Comparison of Gonadal Transcriptomes Uncovers Reproduction-Related Genes with Sexually Dimorphic Expression Patterns in *Diodon hystrix*

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1. Introduction

Spot-fin porcupine fish (*Diodon hystrix*) belonging to the family Diodontidae (Teleostei, Tetraodontiformes) is mainly distributed in the lagoons and coral reefs of tropical sea-
Although *D. hystrix* is widely considered to have tetrodotoxin [3], this fish species is noted for its delicious meat and nutritious skin that is rich in collagen, especially in the Pacific Islands region and southern Hainan Island, China [4,5]. According to the assessment of nutrient value, *D. hystrix* has a high content of amino acids and the rate of essential amino acids is accorded with the FAO/WHO standard. Moreover, the fat contents in skin and muscle tissues are found to be negligible, contributing to a low energy value [6]. *D. hystrix* with such distinguished features is increasingly becoming a highly popular food fish with considerable economic value, the commercial demand is rising rapidly in south China and the market price continues to climb [5,6].

The outstanding growth performance and ease of cultivation make *D. hystrix* a very promising species for commercial aquaculture [6]. As a gonochoristic fish species, however, porcupine fish gonad maturates earlier in males than females under either natural or aquaculture conditions. It is, therefore, difficult, to obtain fertilized eggs using conventional techniques of artificial propagation. With the bloom of porcupine fish industry, artificial breeding unfortunately remains a significant barrier for the successful aquaculture of *D. hystrix*. The commodity fish still has to be captured from the sea. Due to a weak resilience of the natural population of *D. hystrix*, its wild stock is now gravely threatened and the fish resource has been declining greatly in recent years as a result of critical hazards, such as a sharp increase of fish catch and continued destruction of natural habitat [7]. Thus, the large-scale aquaculture of *D. hystrix* is absolutely in great and urgent need of artificial breeding and reproduction management.

The reproduction process, typically including gonad differentiation, development, maturation and gametogenesis, is more complex in fish than in other vertebrate species and is under the influence of genetic and environmental factors [8,9]. Years of practical experience have shown that a thorough knowledge of the mechanisms involved in reproductive regulation could strongly promote the development of efficient management of reproduction, which is a necessary prerequisite for the breeding of aquaculture species. To date, however, the only studies in *D. hystrix* focus mainly on systematics and zoogeography [2,4], characterization of mitochondrial genome and phylogenetic analysis [5], molecular marker development [7], parasitic diseases [10,11], tetrodotoxic poisoning mechanism [3], as well as the analysis of nutrient compositions that can provide reference data for the formulation design of special diets [6]. The research efforts on reproductive biology are severely limited and the underlying mechanism of reproduction remains poorly understood. These research gaps seriously impede the progress of artificial breeding techniques for *D. hystrix*. To provide useful guidance to the future practice of reproductive manipulation, it is essential to pay serious attention to delineating the regulatory mechanisms of the reproductive process. In particular, the efforts to functionally explore the key genes associated with sex differentiation, gonad development and maturation, and gametogenesis are indispensable.

Compared with other commercial aquaculture species, the genetic information available for *D. hystrix* is rather limited. Thus far, reproduction-related genes have been rarely reported in *D. hystrix* and a fundamental understanding of their expression profiles is still lacking. Before elucidating the specific roles of these genes, there is an immediate need to enrich the genomic background knowledge. With improved efficiency, next-generation sequencing (NGS) based transcriptome sequencing is an efficient gene expression profiling technology that is superior in generating a great amount of transcript sequences and mRNA expression data rapidly and cost-effectively, especially for non-model species [12,13]. It has been frequently employed in functional gene identification and gene-expression regulation analysis in aquaculture fish species [14–16] to provide a general representation of the genes that are expressed in specific tissues [17]. The expression patterns of reproduction-related genes generally exhibit significant differences between the sexes both in the developing and developed gonads. By comparative analysis of gonad transcriptomes, many candidate genes and pathways involved in sexual or reproductive regulation and gonad maturation have been identified in silver sillago (*Sillago sihama*) [18], spotted knifefish (*Oplegnathus punctatus*) [19], olive flounder (*Paralichthys olivaceus*) [20], yellow catfish (*Pel-
teobagrus fulvidraco [21], Amur catfish (Silurus asotus) [22], and Amur sturgeon (Acipenser schrenckii) [23]. These studies provide helpful insights into reproduction-related genes and enable the discovery of new gene candidates.

In this study, Illumina-based gonadal RNA sequencing, Trinity de novo assembly and annotation were firstly conducted in *D. hystrix*. Furthermore, comparative transcriptomics was applied to reveal the expression patterns of sex-biased genes and the differences in the expression of the genes which potentially are involved in the regulation of reproduction were analyzed and discussed. This study mainly aimed i) to further enrich the available genetic and genomic data for a deeper understanding of gene expression and functional gene mining, and ii) to identify as many genes putatively related to gonad differentiation, development, maturation, and gametogenesis as possible for future research into the molecular mechanisms of reproduction in *D. hystrix*.

2. Materials and Methods

2.1. Ethics Statement

Animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Animal Research and Ethics Committee of Guangdong Ocean University (NIH Pub. No. 85–23, revised 1996). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Sample Collection and Preparation

For transcriptome sequencing, six adult *D. hystrix* (three males and three females) were obtained from the South China Sea (18°11′2.73″ N, 109°18′32.66″ E) on 12 May 2020 (see Table S1 for the specific information of the fish samples). Live fish were sacrificed by decapitation following anesthetization with a 300 mg/L tricaine methanesulfonate (MS222, Sigma, Saint Louis, MO, USA) immersion bath. After dissection, the determination of fish gender was performed by morphological observation of gonads. The gonad tissues were excised from *D. hystrix* individuals within 1 min from sacrifice, immediately quick-frozen in liquid nitrogen, and then stored at −80 °C until RNA extraction.

2.3. Illumina RNA Sequencing

Six gonad samples (three replicates each sex) were used for the preparation of transcriptome sequencing libraries. The RNA-Seq process was performed as described previously [24]. In brief, total RNA was isolated from female and male *D. hystrix* gonad tissues using a Trizol reagent kit (Life Technologies, Carlsbad, CA, USA). The isolated RNA was quantified by a Nanodrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and its integrity was confirmed by agarose gel electrophoresis and Agilent 2100 BioAnalyzer System (Agilent Technologies, Santa Clara, CA, USA). After purifying mRNA with an Oligo-dT Beads Kit (Qiagen, Hilden, Germany), cDNA libraries were constructed using a TruSeq® Stranded mRNA Sample Preparation kit following the manufacturer’s protocol. RNA sequencing of the libraries was performed using the Illumina HiSeq™ 2000 platform (Illumina, Inc., San Diego, CA, USA) that generates paired-end (PE) reads of 125 bp length.

2.4. De Novo Assembly

By means of SOAPnuke (version 1.5.0) [25], the raw reads were pruned using the software’s quality control with the parameters “-i 10 -q 0.5 -n 0.05 -p 1 -i”. In this step, clean data were generated by removing adapter sequences, reads containing ploy-N sequences and low-quality reads from the raw data. Then, the clean data were de novo assembled by Trinity RNA-Seq Assembler (version r20140717, http://trinityrnaseq.sourceforge.net (accessed on 15 June 2015)) with default parameters [26]. The shorter redundant final linear transcripts were eliminated using CD-HIT-EST when the sequences were totally covered by other transcripts with 100% identity, and the longest ones were defined as unigenes [24].
2.5. Annotation and Classification

Annotation was conducted by aligning sequence data against public databases using BLAST 2.2.26+ software (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 20 April 2016)) with an E-value threshold of 1e-5. The unigenes were subjected to the sequence homology searches against the National Center for Biotechnology Information (NCBI) non-redundant (Nr), Protein family (Pfam), Clusters of Orthologous Groups of proteins (KOG/COG/eggNOG), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Further analysis was performed to obtain the Gene Ontology (GO) functions using the Blast2GO package [27]. The classification of GO terms was visualized using WEGO statistical software [28]. Additionally, KOBAS v2.0 (http://kobas.cbi.pku.edu.cn/home.do (accessed on 24 July 2015)) was employed to analyze the KEGG pathway annotation data and to obtain the pathway categories [29].

2.6. Differential Expression Analysis and Functional Enrichment

By means of the expected number of fragments per kb per million reads (FPKM) method, gene expression levels were calculated using RSEM software (version 1.2.15) [30]. The DESeq2 package was used to identify differentially expressed genes (DEGs) between ovaries and testes [31]. FDR value $\leq 0.01$ and $|\log_2 \text{(Fold Change)}| \geq 1$ were used as the threshold for significantly differential expression. Additionally, GO and KEGG functional enrichment analyses were performed to determine the DEGs that were significantly enriched in GO terms and KEGG pathways at Bonferroni-corrected $p$-value $\leq 0.05$ compared with the whole-transcriptome background. GO enrichment analysis of DEGs was implemented by the topGO package’s (version 2.28.0) Kolmogorov–Smirnov test [32]. Finally, KOBAS v2.0 was used to test the statistical enrichment of DEGs in KEGG pathways [33].

2.7. Validation of DEGs by Real-Time Quantitative PCR (RT-qPCR)

A total of 23 DEGs putatively associated with reproduction were chosen to validate the results of RNA-seq by RT-qPCR analysis. The primer pairs are listed in Supplementary Table S2. Total RNA was isolated from gonad samples using TRIzol according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). The RNA was then subjected to reverse transcription using a RevertAid first-strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania). RT-qPCR was performed on an ABI 7500 qPCR system (Life Technologies Inc., Carlsbad, CA, USA) using SYBR Green Real Time PCR Master Mix (TaKaRa Biotechnology, Dalian, China). The reference gene $\beta$-actin was used as an internal control to determine the relative expression. Three independent biological replicates and two technique repeats were performed for each gene. The relative gene expression levels were calculated using $2^{-\Delta\Delta C_t}$ method. Analysis of Variance (ANOVA) was performed by SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Values with $p < 0.05$ were considered significant.

3. Results

3.1. Overview of Sequencing and Assembly Results

RNA-Seq of the six libraries produced a total of 156.58 million raw reads (46.85 Gb sequencing data) with a mean of 26.10 million, ranging from 21.49 to 40.74 million per sample (Table 1). Approximately 151.89 (97.00%) million clean reads with a mean Q30 of 94.32% were filtered from the raw data (Table 1). The total size of the clean data generated from each library reached more than 6.0 Gb. The statistics of sequencing saturation distribution and gene coverage showed that the sequencing coverage was sufficient to quantitatively analyze the gene expression profiles (Figure S1). All raw sequencing data were submitted to the Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra/ (accessed on 25 October 2020)) of the NCBI database under BioProject accession number PRJNA674446.
Table 1. Summary statistics of the gonadal RNA-Seq data for *D. hystrix*.

| Sample     | Raw Reads   | Clean Reads  | Clean Bases (bp) | GC Content (%) | % ≥ Q20 | % ≥ Q30 |
|------------|-------------|--------------|------------------|----------------|--------|--------|
| Ovary 1    | 23,164,033  | 22,312,200   | 6,686,832,318    | 48.66          | 98.06  | 94.32  |
| Ovary 2    | 22,001,854  | 21,207,110   | 6,354,910,044    | 48.92          | 97.53  | 93.24  |
| Ovary 3    | 21,490,133  | 20,679,487   | 6,198,085,778    | 48.86          | 97.47  | 93.14  |
| Testis 1   | 23,845,599  | 23,454,828   | 7,010,039,950    | 48.74          | 98.32  | 95.25  |
| Testis 2   | 40,744,744  | 39,306,614   | 11,739,627,328   | 48.52          | 98.31  | 95.16  |
| Testis 3   | 25,338,572  | 24,928,306   | 7,457,433,494    | 48.57          | 98.17  | 94.83  |
| Mean       | 26,097,489  | 25,314,758   | 7,574,488,149    | 48.71          | 97.98  | 94.32  |
| Total      | 156,584,935 | 151,888,545  | 45,446,928,892   |                |        |        |

All clean data were then imported to the Trinity package for de novo assembly using the default parameters. The high-quality clean reads were assembled into 139,628 transcripts with a N50 length of 3118 bp (Table 2). Further redundancy elimination resulted in a total of 57,077 unigenes with an average length of 1300 bp. In terms of sequence length distribution, 33,675 (59.00%) unigenes were >500 bp in length and 19,620 (34.37%) unigenes were >1000 bp in length (Table 2). These results demonstrated the high quality of assembly.

Table 2. Summary statistics of the *D. hystrix* gonadal transcriptome assembly.

| Length Range | Transcript | Unigene |
|--------------|------------|---------|
| 300–500 bp   | 30,973 (22.18%) | 23,402 (41.00%) |
| 500–1000 bp  | 30,900 (22.13%) | 14,055 (24.62%) |
| 1000–2000 bp | 31,636 (22.66%) | 8495 (14.88%) |
| >2000 bp     | 46,119 (33.03%) | 11,125 (19.49%) |
| Total number | 139,628    | 57,077  |
| Total length (bp) | 261,271,796 | 74,197,236 |
| N50 length (bp)     | 3118       | 2560    |
| Mean length (bp)     | 1871.2     | 1299.9  |

3.2. Unigenes Annotation

Functional annotation was carried out by aligning the 57,077 unigenes to the protein sequences of public databases. A total of 24,574 (43.05%) unigenes were successfully annotated in at least one of the queried databases (Table 3). Of which, 23,114 (40.50%) and 13,488 (23.63%) unigenes had homologous sequences in the Nr and Swiss-prot protein databases, respectively. Meanwhile, 12,434 (21.78%), 15,157 (26.56%), and 13,699 (24.00%) unigenes could be annotated and classified in the GO, KOG, and KEGG databases, respectively (Table 3). Specifically, 12,434 unigenes annotated in the GO database were divided into three main subcategories and assigned into 56 2nd GO terms (Figure S2), and 15,157 unigenes were successfully clustered into 25 KOG subcategories (Figure S3). Furthermore, 13,699 (24.28%) unigenes were grouped into six main KEGG categories that include 287 secondary pathways (Figure S4).

3.3. Differential Expression Analysis

The levels of gene expression were normalized using the FPKM values. The distribution of gene expression levels was shown as a series of box plots (Figure 1A). About half of the genes were found to be expressed at extremely low levels (0.1 ≤ FPKMs ≤ 1) or not expressed at all (0 ≤ FPKMs < 0.1) in the gonads, and only a small proportion was considered to be highly expressed (FPKMs ≥ 60). To ensure if all gonad samples were reliable, the gene expression patterns of testes and ovaries were visualized via a heatmap using the Pearson correlation coefficient as distance measure (Figure 1B). The correlation analysis clearly indicated that the samples were classified into two major groups, representing testis and ovary, respectively. In addition, the result of principal component analysis...
(PCA) showed that three testis samples (blue circles) formed a cluster and three ovary samples (red circles) formed another distinct cluster (Figure S5).

Table 3. Statistics of the D. hystrix gonadal transcriptome annotation.

| Database       | Annotated Number | Percentage | 300 ≤ Length < 1000 bp | Length ≥ 1000 bp |
|----------------|------------------|------------|------------------------|-----------------|
| COG            | 6281             | 11.00%     | 1522                   | 4759            |
| GO             | 12,434           | 21.78%     | 3570                   | 8864            |
| KEGG           | 13,699           | 24.00%     | 3705                   | 9994            |
| KOG            | 15,157           | 26.56%     | 4048                   | 11,109          |
| Pfam           | 17,519           | 30.69%     | 4206                   | 13,313          |
| Swiss-prot     | 13,488           | 23.63%     | 2933                   | 10,555          |
| eggNOG         | 22,671           | 39.72%     | 7438                   | 15,233          |
| Nr             | 23,114           | 40.50%     | 7469                   | 15,645          |
| All            | 24,574           | 43.05%     | 8824                   | 15,750          |

By comparison of the unigene expression levels in gonadal transcriptomes, a total of 11,487 unigenes were detected to be differentially expressed between the ovaries and testes (FDR ≤ 0.01, |Log2 (fold change)| ≥ 1) (Table S3). Among these DEGs, 6888 testis-biased (downregulated) and 4599 ovary-biased (upregulated) transcripts with significant differences in expression levels between the sexes were obtained (Figure 2A). The further analysis indicated that 46 and 1163 DEGs were specifically expressed (0 ≤ FPKMs ≤ 0.1) in the ovaries and testes, respectively. The remaining 10,278 DEGs were expressed in both gonads (Figure 2B).

3.4. Enrichment Analysis of DEGs

GO functional analysis was performed and the obtained DEGs were finally assigned to 52 2nd level GO terms. Of these functional terms, ‘cellular process’, ‘binding’, ‘single-organism process’, and ‘metabolic process’ annotated the most DEGs (Figure 3). More important, many GO terms associated with reproduction were identified, such as ‘reproductive process’ (GO:0022414), ‘gamete generation’ (GO:0007276), ‘steroid hormone mediated signaling pathway’ (GO:0043401), and ‘oocyte differentiation’ (GO:0009994) (Table S4). GO enrichment analysis showed that 224, 62, and 107 GO terms were significantly enriched in categories ‘biological process’, ‘cellular component’, and ‘molecular function’, respectively.
respectively ($p < 0.05$) (Table S4). The top three most significant GO terms involved in ‘biological processes’ were ‘DNA repair’ (GO:0006281), ‘tRNA processing’ (GO:0008033), and ‘lymph vessel development’ (GO:0001945). The top three most significant terms involved in ‘molecular functions’ included ‘RNA methyltransferase activity’ (GO:0008173), ‘DNA binding’ (GO:0003677), and ‘chemorepellent activity’ (GO:0045499). The top three ‘cellular components’ GO terms were ‘catalytic complex’ (GO:1902494), ‘ribosome’ (GO:0005840), and ‘cytoplasm’ (GO:0005737).

**Figure 2.** Differential gene expression between the ovaries and testes of *D. hystrix*. (A) Volcano plot of the differences in gene expression. Red dots: upregulated, represent ovary-biased genes; Green dots: downregulated, represent testis-biased genes. (B) Venn diagram showing the distribution of testis-specific and ovary-specific DEGs.

**Figure 3.** Functional annotation of DEGs based on GO categorization. The horizontal axis indicates the GO functions, and the vertical axis represents the number of genes with GO function.
KEGG enrichment analysis was also carried out with the DEGs to uncover their functional characteristics. In total, 5598 DEGs were mapped to 198 KEGG pathways, of which ‘endocytosis’ (ko04144), ‘MAPK signaling pathway’ (ko04010), ‘focal adhesion’ (ko04510), and ‘regulation of actin cytoskeleton’ (ko04810) annotated the most genes (Table S5). Meanwhile, 20 KEGG pathways were significantly enriched (\(q\)-value < 0.05). The DEGs upregulated in ovaries and testes were involved in 17 and 12 significant pathways, respectively (Figure 4). Moreover, the enrichment analysis showed that clearly different pathways were enriched between the ovary- and testis-biased DEGs. The ovary-biased DEGs were significantly enriched in pathways such as ‘ribosome biogenesis in eukaryotes’, ‘DNA replication’, ‘pyrimidine metabolism’, ‘cell cycle’, and ‘spliceosome’ (Figure 4A), suggesting the importance of nucleic acid synthesis, protein homeostasis, and cell proliferation and differentiation for ovarian function. Whereas the testis-biased DEGs were highly enriched in pathways such as ‘focal adhesion’, ‘cytokine-cytokine receptor interaction’, ‘cell adhesion molecules (CAMs)’, ‘ECM (extracellular matrix)-receptor interaction’, and ‘MAPK signaling pathway’ (Figure 4B). Such noticeable differences imply the variation in the gene regulatory landscape between the *D. hystrix* ovary and testis.

![Figure 4. Significantly enriched KEGG pathways of DEGs. (A) Pathway enrichment analysis of DEGs upregulated in testes. (B) Pathway enrichment analysis of DEGs upregulated in ovaries. The pathways and rich factor are shown in the vertical and the horizontal axis, respectively. The size of the point indicates the number of genes, and the color indicates the q value.](image)
Figure 4. Significantly enriched KEGG pathways of DEGs. (A) Pathway enrichment analysis of DEGs upregulated in testes. (B) Pathway enrichment analysis of DEGs upregulated in ovaries. The pathways and rich factor are shown in the vertical and the horizontal axis, respectively. The size of the point indicates the number of genes, and the color indicates the \( q \) value.

3.5. Sex-Biased Genes of Interest Related to Reproduction Regulation

A number of GO terms and KEGG pathways known to be associated with gonadal development and maintenance, gametogenesis, oocyte maturation, and sperm motility were found in the present study, such as ‘reproductive process’ (GO:0022414), ‘gonad development’ (GO:0008406), ‘sexual reproduction’ (GO:0019953), ‘germ cell development’ (GO:0007281), ‘gamete generation’ (GO:0007276), ‘ spermatogenesis’ (GO:0007283), ‘ovarian steroidogenesis’ (ko04913), ‘steroid hormone biosynthesis’ (ko00140), ‘Wnt signaling pathway’ (ko04310), and ‘MAPK signaling pathway’ (ko04010) (Table S6). There were 221 and 618 genes grouped into reproduction-related GO terms and KEGG pathways, respectively (Table S6). Based on the resources of annotation and enrichment analyses, 67 DEGs were obtained and these genes were determined to be involved in gonadal differentiation and development, gamete generation and maturation in vertebrates were identified, including gonadal soma-derived factor 1 (gsdf1), SRY-box transcription factor 9 (sox9), anti-Müllerian hormone (amh), doublesex- and mab-3-related transcription factor 1 (dmrt1), Cytochrome P450 aromatase (cyp19a1a), Kelch-like protein 10 (klhl10), zona pellucida sperm-binding protein 1 (zp1), and so on (Table 4). Among these DEGs, 45 were detected to be testis-biased, and 22 were ovary-biased. For example, the expressions of amh and gsdf1 unigenes were upregulated in male gonads, whereas the expressions of zygote arrest protein 1 (zar1) and zona pellucida sperm-binding protein 3 (zp3) were found to be considerably higher in the ovaries (Table 4). In addition, we found that seven genes showed gonad-specific expression patterns. Of which, one gene (cyp19a1a) showed specific expression in ovaries; And the remaining six genes, such as dmrt1, steroid 11-beta-hydroxylase (cyp11b1), transcription factor SOX6 (sox6), and forkhead box protein J3 (foxj3) showed male-specific expression patterns.
Table 4. Searching for differential expression genes (DEGs) putatively involved in reproduction from the gonad transcriptome of *D. hystrix*.

| Unigene ID         | Gene Annotation                                      | Log2 Fold Change (Ovary/Testis) | FDR      |
|-------------------|------------------------------------------------------|--------------------------------|----------|
| c84183.graph_c0   | Relatively higher expression in testis               | −3.63                          | 1.23E-06 |
| c81599.graph_c0   | Gonadotropin-releasing hormone II receptor           | −8.64                          | 5.85E-07 |
| c87605.graph_c0   | Doublesex- and mab-3-related transcription factor 1  | −8.34                          | 4.17E-51 |
| c69078.graph_c0   | Anti-Mullerian hormone                               | −5.03                          | 1.14E-11 |
| c89331.graph_c1   | Anti-Mullerian hormone type-2 receptor              | −5.19                          | 1.37E-20 |
| c90445.graph_c0   | Forkhead box L3                                       | −7.13                          | 7.21E-29 |
| c83812.graph_c1   | Forkhead box protein J3                               | −9.57                          | 1.21E-08 |
| c80643.graph_c0   | Forkhead box protein D3                               | −8.62                          | 7.63E-07 |
| c90293.graph_c0   | Wilms tumor protein 1-interacting protein            | −2.73                          | 6.19E-14 |
| c86435.graph_c1   | Wilms tumor protein homolog                          | −3.11                          | 4.20E-07 |
| c87662.graph_c6   | Wilms tumor protein                                  | −2.75                          | 1.14E-06 |
| c74886.graph_c0   | Gonadal soma derived factor 1                        | −8.11                          | 8.58E-34 |
| c67874.graph_c1   | Steroidogenic acute regulatory protein               | −5.62                          | 1.05E-26 |
| c88056.graph_c0   | 17-beta-hydroxysteroid dehydrogenase 14              | −3.92                          | 1.12E-17 |
| c74550.graph_c0   | 3-oxo-5-beta-steroid 4-dehydrogenase                 | −2.82                          | 1.41E-03 |
| c56757.graph_c0   | 3 beta-hydroxy-steroid dehydrogenase type 1          | −3.75                          | 9.81E-10 |
| c79716.graph_c0   | Steroid 11-beta-hydroxylase STAR-related lipid transfer protein 13 | −8.40                          | 1.94E-11 |
| c83337.graph_c1   | STAR-related lipid transfer protein 9                | −5.63                          | 2.00E-19 |
| c89692.graph_c0   | STAR-related lipid transfer protein 9                | −1.59                          | 2.13E-05 |
| c80342.graph_c0   | Steroidogenic acute regulatory protein               | −5.22                          | 1.05E-26 |
| c89905.graph_c0   | Estrogen receptor b2                                  | −2.41                          | 2.43E-09 |
| c84046.graph_c0   | Estrogen receptor b1                                  | −2.86                          | 1.55E-10 |
| c82367.graph_c0   | Estrogen receptor b1                                  | −3.06                          | 5.18E-13 |
| c89150.graph_c2   | Progesterone receptor                                 | −1.34                          | 6.89E-04 |
| c86613.graph_c1   | Kelch-like protein 10                                 | −9.33                          | 3.60E-31 |
| c85739.graph_c0   | Spermatogenesis-associated protein 5                 | −1.95                          | 1.19E-04 |
| c87439.graph_c0   | Spermatogenesis-associated protein 20                | −2.16                          | 4.59E-04 |
| c86517.graph_c0   | Spermatogenesis-associated protein 7                 | −4.85                          | 4.25E-09 |
| c83923.graph_c0   | Spermatogenesis-associated protein 17                | −6.61                          | 1.43E-13 |
| c84036.graph_c0   | Spermatogenesis-associated protein 6                 | −3.16                          | 5.66E-10 |
| c81488.graph_c0   | Sperm surface protein Sp17                           | −1.77                          | 2.49E-03 |
| c82755.graph_c0   | Sperm acrosome membrane-associated protein 6          | −3.09                          | 2.95E-05 |
| c42266.graph_c0   | Spermine oxidase                                     | −5.52                          | 7.33E-09 |
| c75610.graph_c0   | Ropporin-1-like protein                              | −12.40                         | 3.92E-39 |
| c86581.graph_c5   | Splicing factor 1                                     | −2.21                          | 8.72E-04 |
Table 4. Cont.

| Unigene ID     | Gene Annotation                                        | Log₂ Fold Change (Ovary/Testis) | FDR ¹  |
|----------------|--------------------------------------------------------|---------------------------------|--------|
| c87182.graph_c0 | Fibroblast growth factor receptor-like 1               | −6.42                           | 4.76E-36 |
| c86547.graph_c1 | Fibroblast growth factor 11                           | −7.51                           | 2.39E-10 |
| c73147.graph_c0 | Fibroblast growth factor 7                             | −3.32                           | 5.76E-05 |
| c77489.graph_c0 | Fibroblast growth factor-binding protein 1             | −3.12                           | 2.17E-05 |
| c81519.graph_c1 | Fibroblast growth factor 10                            | −6.61                           | 1.31E-11 |
| c84573.graph_c2 | Fibroblast growth factor-binding protein 3             | −2.05                           | 7.40E-03 |
| c89383.graph_c0 | Fibroblast growth factor receptor 2                    | −5.21                           | 1.75E-13 |
| c63200.graph_c0 | ATP synthase F0 subunit 6                              | −2.86                           | 6.13E-33 |
| c78426.graph_c0 | Cholesterol side-chain cleavage enzyme                 | −2.44                           | 7.91E-05 |
| c73686.graph_c0 | Gonadotropin subunit beta-2                            | 4.47                            | 1.46E-28 |
| c80357.graph_c1 | Catenin beta-1                                         | 1.79                            | 3.63E-07 |
| c85139.graph_c8 | Catenin alpha-2                                        | 2.64                            | 7.20E-23 |
| c80092.graph_c0 | Protein fem-1 homolog C                                | 2.88                            | 8.07E-13 |
| c85792.graph_c0 | Protein fem-1 homolog B                                | 2.26                            | 7.47E-10 |
| c83767.graph_c0 | Zygote arrest protein 1                                | 5.76                            | 2.60E-14 |
| c82641.graph_c1 | 3-oxo-5-alpha-steroid 4-dehydrogenase 1                | 1.40                            | 1.13E-03 |
| c88743.graph_c5 | Steroid hormone receptor ERR2                          | 2.34                            | 4.22E-12 |
| c87163.graph_c3 | 3-keto-steroid reductase                               | 1.90                            | 2.24E-05 |
| c73009.graph_c0 | Inactive hydroxysteroid dehydrogenase-like protein 1   | 1.11                            | 3.55E-04 |
| c85481.graph_c3 | Steroid hormone receptor ERR1                          | 1.47                            | 8.53E-04 |
| c73497.graph_c0 | Hydroxysteroid dehydrogenase-like protein 2            | 1.14                            | 3.41E-04 |
| c72251.graph_c0 | Cytochrome P450 aromatase                              | 3.86                            | 8.36E-08 |
| c87866.graph_c0 | STAR-related lipid transfer protein 13                 | 2.63                            | 1.17E-10 |
| c83997.graph_c0 | STAR-related lipid transfer protein 7                  | 1.63                            | 4.78E-07 |
| c82351.graph_c1 | Membrane-associated progesterone receptor component 1  | 1.01                            | 2.73E-03 |
| c83997.graph_c2 | Membrane-associated progesterone receptor component 2  | 2.87                            | 6.61E-09 |
| c83333.graph_c2 | Progesterone-induced-blocking factor 1                 | 1.66                            | 4.04E-08 |
| c42601.graph_c0 | Zona pellucida sperm-binding protein 3                 | 6.29                            | 2.60E-07 |
| c85704.graph_c1 | Zona pellucida sperm-binding protein 1                 | 3.72                            | 2.40E-29 |
| c87366.graph_c0 | Vasa                                                 | 1.33                            | 1.15E-04 |
| c78173.graph_c1 | Forkhead box protein H1                                | 4.80                            | 6.46E-41 |

¹ False discovery rate.

3.6. RT-qPCR Confirmation of DEGs

A total of 13 testis-upregulated and 10 ovary-upregulated DEGs were chosen and subjected to the statistical verification of expression profiles using RT-qPCR analysis. The
relative expressions of these representative genes were shown in Figure 5. In general, the RT-qPCR results were found to be consistent with those of RNA-seq analysis (Figure 5). DEGs such as *amh*, *sox9*, *dmrt1*, and ropporin-1-like protein (*ropp1l*) were testis-biased (Figure 5A), whereas unigenes such as homologs of *zar1*, membrane-associated progesterone receptor component 1 (*pgrmc1*), and *vasa* were ovary-biased (Figure 5B). Meanwhile, a correlation analysis was conducted and the consistent tendencies of expression levels between the RNA-Seq data and RT-qPCR results ($R^2 = 0.8476$) confirmed the reliability and accuracy of gene expression levels quantified by transcriptomic analysis (Figure 5C).

![Figure 5](image)

Figure 5. Verification of expression profiles of 13 testis-biased (A) and 10 ovary-biased genes (B) using RT-qPCR. (C) Correlation analysis of the RNA-Seq data and RT-qPCR results.

4. Discussion

Gonadal development from undifferentiated to differentiated stages and maturation is the most important determinant for the success of reproduction in fish. This highly complex biological process involves a set of functional genes that can promote the gonadal
differentiation into either an ovary or a testis, and then cause a fish individual to exhibit a male or female phenotype [34]. To date, however, the molecular mechanisms underlying gonadal development have totally been unrevealed in D. hystrix. As an effective way to uncover the gene regulatory networks of gonad development and its dimorphism, transcriptome sequencing and comparative analysis between male and female gonads were employed to identify sex-related genes and to reveal their potential roles by combining differential expression data with biological pathways.

4.1. Sex-Biased Genes Involved in Steroids Synthetic Pathway

Sex steroid hormones, primarily including androgen and estrogen, influence the phenotypic sex by acting as key regulators for gonadal differentiation, development and sex maintenance in fish species [34,35]. In teleosts, the major androgen and estrogen essential to ovarian and testicular development are 11-ketotestosterone (11-KT) and 17β-estradiol (E2), respectively [36]. The syntheses of these sex steroid hormones need a series of genes encoding steroid-metabolizing enzymes. In particular, cyp19a1a, cyp11b2 encoding steroid 11-beta-hydroxylase, hsd11b1 coding for 11 beta-hydroxysteroid dehydrogenase, cyp11a1 coding for cholesterol side-chain cleavage enzyme, and hsd17bs encoding 17β-hydroxysteroid dehydrogenases with 17-ketosteroid reducing activity are considered to be the most essential.

It has been demonstrated that hsd11b1 and cyp11b2 (encodes the key enzyme that converts testosterone to 11-KT) are involved in the pivotal steps in the synthesis of 11-KT in testis, whereas cyp19a1a gene product that catalyzes the conversion of androgens to estrogens is essential for the E2 synthesis in the ovary [37]. Previous reports have indicated that cyp11b2 expression levels were comparatively higher in the testes of some teleost fishes [38,39], implicating a regulatory role for cyp11b2 gene in testicular development. In this study, cyp11b2 gene was expressed at significantly higher levels in D. hystrix testes, while cyp19a1a was found to be upregulated in ovaries compared to testes, exhibiting a similar expression pattern to other fish species [34,37,40,41]. The findings suggested that these genes play potential roles in the development of gonads and may participate in D. hystrix reproduction. Interestingly, the sexual dimorphism of serum E2 and 11-KT levels has already been observed in fish species; the levels of E2 and 11-KT are relatively higher in females and males, respectively [34,41]. In Oreochromis niloticus, both serum E2 levels and cyp19a1a expression were comparatively higher in females than in males [41]. In Oryzias latipes, mutation of cyp19a1a gene led to a marked decrease in the gonadal E2 level in female (XX) individuals [42]. Collectively, higher serum E2 levels may be attributed to the elevated ovarian cyp19a1a expression.

Steroidogenic acute regulatory protein (StAR) participates in the rate-limiting step of steroid biosynthesis by transporting cholesterol to the inner mitochondrial membrane where the cholesterol side-chain cleavage enzyme converts this substrate to pregnenolone [34,35]. In teleosts, the star gene was found to be highly expressed during spermatogenesis, oocyte maturation and ovulation [43,44]. Our study showed that the expression levels of star and cyp11a1 in the testes of D. hystrix were significantly higher than those in the ovaries. The current results showed good agreement with the experimental results in Scatophagus argus [34] and P. olivaceus [45], suggesting that the abundances of star and cyp11a1 may partially affect the rate of steroid synthesis in teleost fish. In fish gonads, sex steroids synthesis-related genes are modulated by the hypothalamic–pituitary–gonadal (HPG) axis, one of the key steps is cAMP-mediated stimulation of star expression [34].

In O. niloticus, star mRNA levels in testes were greatly enhanced by injection of human chorionic gonadotropin (hCG) [46]. Moreover, recombinant follicle stimulating hormone (FSH)/luteinizing hormone (LH) administration increased the star and hsd11b1 expressions, E2/11-KT levels, and finally promoted the ovary and testicular development in S. argus [47]. Thus, understanding the expression and endocrine regulation of steroidogenic genes would greatly help us establish effective methods for controlling reproduction in D. hystrix aquaculture, such as multiple gonadotropin-releasing hormone agonist (GnRHa)
injections or implants that are commonly utilized for the artificial induction of oocyte maturation/ovulation and spermination in fish.

4.2. Candidate Genes Related to Gonad Differentiation and Development

The molecular mechanisms involved in sex determination and gonad differentiation are variable among phyla. Although the top upstream regulators in the sex determination pathway are less conserved, the downstream genes are more conserved. With rare exceptions, almost all currently identified sex-determining genes belong to one of the three protein families (Dmrt, TGF-β and its signaling pathway, and Sox) [21].

Here, regulatory genes that appear to be involved in male gonad differentiation were identified from the D. hystrix gonad transcriptomes. In particular, detection of highly expressed dmrt1 as a male-biased gene would be of great interest. Dmrt1 belonging to the Dmrt gene family generally functions as a conserved transcription factor in the sexual regulatory cascade. Dmrt1 and its paralogs have been claimed as master sex-determining genes in some animal species [21,34], playing essential parts in the differentiation of testis and maintenance of male-specified germ cells [48]. Also, it has been understood that dmrt1 works as an essential factor in gonadal development and gametogenesis in fishes [20,49]. Dmrt genes stimulate male-specific differentiation but repress female-specific differentiation [21]. In O. latipes, the mutation of autosomal dmrt1 was found to be responsible for a male-to-female sex reversal [50]. Comparably, dmrt1-mutated testes exhibited serious testicular development defects and gradual loss of germ cells in zebrafish [51]. In this research, dmrt1 genes were determined to be specifically expressed in the testis; ovarian dmrt1 expression could not be detected by transcriptome analysis and RT-qPCR. The trend of dmrt1 expression was quite similar to those in other fishes such as O. niloticus, and Megalobrama amblycephala [40,52], suggesting that dmrt1 gene is a key player in the testis development in D. hystrix.

A series of sex-determining genes encoding transforming growth factor-β (TGF-β) signal components (e.g., Gsdfβ, amhy, Amhr2, Gdf6Y) have been identified in fish, suggesting that the TGF-β pathway is involved in gonad differentiation. Gonadal soma-derived factor (Gsdf), a TGF-β superfamily member, is found to be expressed specifically in fish gonads, predominately in the Sertoli cells and neighboring spermatogonia of testes [53]. As a direct downstream gene of dmrt1, Jiang et al. found that gsdf gene transcription was regulated by dmrt1 [53]. Recently, the authors further demonstrated that dmrt1 could induce the expression of gsdf with the participation of splicing factor 1 (SF-1, also known as Nr5a1, an important activator of steroidogenic enzymes, including aromatase) [54].

Previous studies have shown that gsdf plays a key role in testicular differentiation in fish, and it is speculated that gsdf acts by suppressing the activator of cyp19a1a and inhibiting estrogen synthesis [53]. Mutation of gsdf in medaka and O. niloticus initiated male-to-female sex reversal [53,55], while overexpression of this gene induced testis differentiation in female O. niloticus [56]. A study involving Oncorhynchus mykiss showed that gsdf may act in the regulation of spermatogenesis by stimulating the proliferation of spermatogonia [57]. In teleost, it was reported that gsdf was expressed at a higher level in the testicular somatic cells compared with ovarian tissues [58]. Sf-1 was significantly upregulated during and after testicular differentiation in black porgy [59]. Similar trends of gsdf and sf-1 expressions were also observed in this study. Therefore, we could deduce that gsdf has a conserved function in the testis differentiation of D. hystrix.

Anti-Müllerian hormone (Amh) encoded by amh has also been identified as a member of the TGF-β family in fish species [18]. Amh suppresses the development of the Müllerian ducts and functions as a key regulator for differentiation of the Sertoli and granulosa cells, germ cell proliferation and steroidogenesis in Leydig cells in gonad development [34]. Lin et al. [51] found that amh mutation resulted in a female-biased sex ratio in zebrafish; the unrestrained germ cell proliferation in male amh mutants led to hypertrophic testes. In XY medaka, Amh type II receptor (amhr2) mutation could promote the sex reversal and amhr2 mutants mostly exhibited the signs of germ cell over-proliferation [60]. Our data
showed that the expressions of *amh* and *amhr2* genes were upregulated in the testes but weakly expressed in the ovaries, implicating the significance of Amh/Amhr2 pathway in the modulation of testicular differentiation and germ cell proliferation in *D. hystrix*.

Several members of the Sox (SRY-related HMG box) gene family has also been found to regulate the differentiation of gonads in fish; typical examples include *sox9*, *sox8*, *sox5*, and *sox3* [18,61]. Here, the abundances of the two transcriptional factors *sox9* and *sox6* were detected in our transcriptome data and they were identified as male-biased genes. Classic studies have clearly demonstrated that *sox9* plays vital roles in the testicular development of male gonad as an important sex-determination gene [35]. *Sox9* was found to be expressed in the testes of rainbow trout [62], and channel catfish [63]. Its crucial role in sex determination of teleost fish has also been confirmed by genetic approaches [21]. Genomic studies have revealed that the *sox9* gene in teleosts has undergone duplication and there are two copies (*sox9a* and *sox9b*) [34,61]. In both male and female medaka, *sox9b* was shown to be pivotal for the survival of germ cells [64]. Certain regulatory genes in male fish may regulate the expression of *sox9b* mRNA in teleost fish. A recent study demonstrated that the Nile tilapia *dmrt1* gene positively regulated the transcription of *sox9b* by directly binding to a specific promoter cis-regulatory element [61]. Moreover, *sox6* gene was reported to be specifically expressed in the testis and involved in the later stages of spermatogenesis in teleost fishes [65]. In this study, both RNA-seq and RT-qPCR analysis demonstrated that *sox9* and *sox6* mRNA levels were much higher in the male gonads. Such expression patterns were similar to those observed in *O. latipes* [66], *S. Sillago* [18], and *S. argus* [34], suggesting a highly conserved function of *sox9* gene in teleosts.

As to the identification of genes that participate in the differentiation of gonads, it is necessary to explore their roles in both undifferentiated and differentiated gonad samples. Importantly, detailed information about the gonad differentiation-related genes should be collected and explored in more developmental stages, resembling what has been performed in other fish species [34]. Hence, the expression profiles of the sex-biased genes identified in this study should be carefully analyzed in the future. In this study, the sex-specific expression of *dmrt1* implicates it as the most promising candidate sex-determining gene in *D. hystrix*. In order to further confirm the precise role of *dmrt1*, molecular genetic studies are firstly required to characterize the transcriptional regulation of putative downstream genes (e.g., *gsdf*, and *sf-1*). Meanwhile, the DMRT gene cluster will be cloned, and then gene structures will be comparatively analyzed, to identify any possible sex-linked polymorphic locus. With the foundation of above basic research, specific gene knockout and rescue experiments would be required to the functional confirmation of *dmrt1* gene in sex determination.

### 4.3. Sex-Biased Genes Involved in Gametogenesis and Gamete Maturation

In fish aquaculture, the eventual aim of artificially induced breeding is to facilitate the development of gonads and then obtain mature gametes [26]. In this study, the expression of many genes associated with oogenesis, oocytes maturation, spermatogenesis and sperm motility was presented, such as *vasa*, *zygote arrest 1* (*zar1*), *zona pellucida sperm-binding proteins* (*zps*), *spermatogenesis-associated proteins* (*spatas*), and *spermine oxidase* (*smox*). Vasa, specifically localized in germline cells, has been well characterized as a crucial player in germ cell formation in larval fish [35]. A previous study has pointed out that *vasa* gene is expressed in primordial germ cells during the first formation of the gonadal anlagen [67]. In Pacific bluefin tuna (*Thunnus orientalis*), *vasa* exhibited a high level of expression in spermatogonia during the first formation of the gonadal anlagen [67]. In Pacific bluefin tuna (*Thunnus orientalis*), *vasa* exhibited a high level of expression in spermatogonia during the first formation of the gonadal anlagen [67].
different species [35]. The functional details of *vasa* in gonadal differentiation in *D. hystrix* need to be clarified in future studies.

In vertebrates, zar1 acts as an oocyte-specific maternal effect gene with an evolutionarily conserved function in the fertilization process [34,35]. The zona pellucida (ZP) encoded by *zp* genes is a glycoproteinaceous matrix surrounding the oocyte and has an important part in species-specific binding of sperm [18]. In zebrafish, the *zp* mRNA levels were enhanced notably during oogenesis, particularly at the previtellogenic stage [71]. It has been shown that *zp1* participates in the generation of oocyte envelope, and *zp3* acts as a major class of female-specific factors in the reproductive process [72]. In the present study, *zp1* and *zp3* were more highly expressed in the ovaries than in the testes, suggesting that these oocyte-specific genes appear to be involved in ovarian folliculogenesis in *D. hystrix*. Regarding male reproduction, sperm acrosome membrane-associated protein 6 (spaca6) can mediate the fusion between ovum and spermatozoa as a sperm membrane component [73]. It has been demonstrated that spatas and sperm surface protein Sp17 (sp17) provide vital functions for spermatogenesis and sperm motility [74]. Here, significantly higher expressions of genes spaca6, spata5, spata6, spata7, smox and sp17 were observed in the male gonad; the expression difference may be associated with the spermatogenesis in *D. hystrix*. Absolutely, these identified gonocyte-specific genes would be helpful for investigating the control mechanisms during oogenesis and spermatogenesis in *D. hystrix*. In order to guarantee the performance of reproductive management of this fish species under aquaculture conditions, more importantly, further efforts to establish an effective technique for inducing gamete maturation are highly encouraged.

5. Conclusions

This is the first study on the gonad transcriptome of *D. hystrix* and a number of 57,077 unigenes were assembled. A comparison of ovarian and testicular transcriptomes detected a set of DEGs supposedly involved in gonadal development and gametogenesis. The conserved expression profiles of the well-known reproduction-related genes imply their similar roles in the gonad differentiation and development of *D. hystrix*. Our findings offer a precious data source for further investigation into the regulatory mechanisms and molecular characteristics of the reproductive process in *D. hystrix*.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/ani11041042/s1, Table S1: The specific information of the fish samples, Table S2: PCR primer sequences used for RT-qPCR, Table S3: Differentially expressed genes identified between ovaries and testes, Table S4: GO enrichment analysis of DEGs, Table S5: KEGG enrichment analysis of DEGs, Table S6: GO terms and KEGG pathways known to be associated with reproduction, Figure S1: Sequencing saturation distribution analysis of gonadal RNA-Seq data, Figure S2: GO classification of the assembled unigenes, Figure S3: KOG classification of the assembled unigenes, Figure S4: KEGG classification of the assembled unigenes, Figure S5: Clustering of testis and ovary samples based on their gonadal transcriptome profiles by principal component analysis (PCA).

**Author Contributions:** Data curation, W.Y., Y.W., Z.L. and G.L.; Formal analysis, W.Y., S.L., and H.H.; Funding acquisition, H.H. and H.C.; Investigation, W.Y., Y.W., Z.L. and X.Y.; Methodology, W.Y. and H.C.; Project administration, H.H.; Resources, H.H. and H.C.; Supervision, H.H. and H.C.; Writing—original draft, W.Y. and H.C.; Writing—review and editing, G.L. and W.Y. All authors have read and agreed to the published version of the manuscript.
Funding: This research was funded by grants from the Key R & D Project of Hainan Province (grant number ZDYF2018225), Major Science and Technology Plan Projects of Hainan Province (grant number ZDKJ2016009), National Science Foundation of Guangdong Province (grant number 2019A1515010958), College of Hainan Tropical Marine Project Leader and Doctoral Research Grant 2016 (grant number RHDXB201612), The Open Project of Key Laboratory of Utilization and Protection of Tropical Marine Biological Resources (Hainan Tropical Ocean University, Ministry of Education (grant number UCTMB20201), Young Creative Talents Project of Guangdong Province Universities and Colleges (grant number 2017GkQNCX092), Guangdong Basic and Applied Basic Research Foundation (grant number 2019A1515110290), and China Postdoctoral Science Foundation (grant number 2020M682832).

Institutional Review Board Statement: Animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Animal Research and Ethics Committee of Guangdong Ocean University (NIH Pub. No. 85–23, revised 1996).

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to the agreement with funding bodies.

Conflicts of Interest: The authors declare no conflict of interest.

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