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Comparison of ovarian mRNA expression levels in wild and hatchery-produced greater amberjack *Seriola dumerili*

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The greater amberjack *Seriola dumerili* is a promising candidate for aquaculture production. This study compares the ovary transcriptome of greater amberjack sampled in the wild (WILD) with hatchery-produced breeders reared in aquaculture sea cages in the Mediterranean Sea. Among the seven sampled cultured fish, three were classified as reproductively dysfunctional (DysF group), while four showed no signs of reproductive alteration (NormalF group). The DysF fish showed 1,166 differentially expressed genes (DEGs) compared to WILD females, and 755 DEGs compared to the NormalF. According to gene ontology (GO) analysis, DysF females exhibited enrichment of genes belonging to the biological categories classified as Secreted, ECM-receptor interaction, and Focal adhesion. Protein–protein interaction analysis revealed proteins involved in the biological categories of ECM-receptor interaction, Enzyme-linked receptor protein signaling, Wnt signal transduction pathways, and Ovulation cycle. KEGG pathway analysis showed DEGs involved in 111 pathways, including Neuroactive ligand-receptor interaction, Steroid hormone biosynthesis, Cell cycle, Oocyte meiosis, Necroptosis, Ferroptosis, Apoptosis, Autophagy, Progesterone-mediated oocyte maturation, Endocytosis and Phagosome, as well as Hedgehog, Apelin, PPAR, Notch, and GnRH signalling pathways. Additionally, DysF females exhibited factors encoded by upregulated genes associated with hypogonadism and polycystic ovary syndrome in mammals. This study -which is part of a broader research effort examining the transcriptome of the entire reproductive axis in greater amberjack of both sexes-, enhances our comprehension of the mechanisms underlying the appearance of reproductive dysfunctions when fish are reared under aquaculture conditions.

Rearing in captivity is often associated with reproductive dysfunctions of variable degrees in fish[1,2]. In males, reproductive dysfunctions may impact spermatogenesis[3,4], ultimately leading to the production of sperm of low volume and quality[3–6]. In females, commonly observed reproductive dysfunctions include (a) the incapacity of oocytes to enter vitellogenesis (process of oocyte uptake of vitellogenin and accumulation of its derived yolk proteins in the form of yolk granules), (b) the arrest of vitellogenesis before completion, resulting in the loss of vitellogenic follicles via atresia, and (c) the failure of fully vitellogenic oocytes to undergo maturation, consequently leading to the lack of ovulation and spawning[1,2,7].

Confinement-induced stress[3,8–10], lack of suitable environmental conditions for reproduction[11–13] and nutritional deficiencies[14,15] are considered as the main causes of reproductive dysfunctions in aquaculture. The functional alterations of the brain-pituitary-gonad axis associated with these reproductive dysfunctions have not yet been fully elucidated. Low plasma levels of luteinizing hormone (Lh) concomitantly with high Lh pituitary content have been observed in fish failing to undergo oocyte maturation, ovulation and spawning, suggesting a reduced pituitary secretion of this hormone at the completion of vitellogenesis[2,7,16–18]. In fact, the stimulation of

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Lh release through the administration of gonadotropin releasing hormone agonists (GnRHa) is widely used as a therapy to alleviate this reproductive dysfunction in several fish species[1,2,5,7,18–24].

The greater amberjack *Seriola dumerili* has a circumglobal distribution in the temperate zones of the three oceans and adjacent seas, including the Mediterranean Sea[25–27]. A worldwide consumers' appreciation, rapid growth and high market prices[28,29] make the greater amberjack an excellent candidate for domestication and aquaculture production throughout the world, along with other congeneric species[30].

It has been well documented that greater amberjack caught from the wild in the Mediterranean Sea and reared under routine farming conditions (thereafter referred to as "wild-caught" fish) undergo severe reproductive dysfunctions. These same dysfunctions have been documented also in hatchery-produced (F1 generation) greater amberjack in aquaculture facilities in the Mediterranean Sea. In wild-caught fish reared in outdoor tanks, the occurrence of atretic degeneration of vitellogenic follicles was the first documented dysfunction[31,32]. Recently, a few studies on the oogenesis of wild-caught individuals confirmed the limited capacity of this species to spawn spontaneously[15,33]. In fact, wild-caught females had low ovary relative mass (gonado-somatic index, GSI), more than 50% of atresia of vitellogenic follicles during the phase of active gametogenesis, and up to 100% atretic vitellogenic follicles during the spawning season[15,33]. Wild-caught males, on the other hand, had low GSI, small seminiferous tubules, early cessation of the active spermatogenesis phase, and a high rate of germ cell apoptosis[4]. The gametogenesis impairment in both sexes was associated with low plasma concentrations of sex steroids in males[4] and females[15,33,34]. Spermatogenesis impairment was also documented in hatchery-produced cultured greater amberjack. It was characterized by a precocious arrest of spermatogenesis, associated with the dysregulation of a large number of genes involved in various interconnected biological processes, such as steroidogenesis, cell cycle, meiosis, cell assembly, and apoptosis[35,36].

This study reports a comparative analysis of the ovary transcriptomes of wild greater amberjack reproducing in their natural reproductive grounds around the Pelagie Islands (Sicily, Italy) and hatchery-produced females reared in aquaculture sea cages, both sampled during the natural reproductive season in the Mediterranean Sea. This work is part of a broader research effort aimed at describing the effects of rearing in captivity on gene expression along the reproductive axis of greater amberjack, in order to elucidate the causes of reproductive dysfunctions in aquaculture.

Methods

Ethics

For this study, wild fish sampled in nature and hatchery-produced, farmed greater amberjack females were used. Wild fish beyond the size of first maturity[37] were caught by an authorized commercial purse-seine fishing vessel during routine fishing operations. Farmed fish were produced at Argosaronikos Fishfarming S.A. (Salamina Island, Greece) under routine farming conditions. The use of farmed fish in this study was approved by the Greek National Veterinary Services (authorization number: AP 31337). All procedures involving animals were conducted in accordance with the "Guidelines for the treatment of animals in behavioural research and teaching"[38], the "Ethical justification for the use and treatment of fishes in research: an update"[39], and the "Directive 2010/63/EU of the European parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes"[40]. The authors complied with the ARRIVE guidelines.

Sampling

Three wild and seven farmed greater amberjack females were sampled between 18th May and 1st June 2021 during the active gametogenesis period of the wild population in the Mediterranean Sea[15]. Wild individuals were caught around the Pelagie Islands (Sicily, Italy) from a purse-seine fishing vessel and sampled immediately after death. Farmed, hatchery-produced (first generation, F1) fish were produced from eggs obtained in Argosaronikos Fishfarming S.A. in 2017, following spawning induction of wild-caught breeders[23,24] and were reared in sea cages under common aquaculture practices. When the fish reached reproductive maturity (2021), they were fed a commercial broodstock diet (Skretting, Vitalis Prima) 3 to 5 times a week until apparent satiation.

Before sampling, hatchery-produced fish were confined in a small PVC cage connected to the rearing cage. They were tranquilized with about 0.01 ml l^{-1} of clove oil (Roumpoulakis E.P.E., Greece) dissolved in ethanol at a 1:10 ratio. Subsequently, they were gently directed into a PVC stretcher and brought aboard a service vessel, where they were deeply anesthetized with 0.03 ml l^{-1} of clove oil in a 1-m^3 tank. The fish were then euthanized by decapitation, placed in crushed ice and transferred to the farm facility for biometric data collection and tissue sampling. The time interval between fish death and sampling ranged between 30 min and 2 h.

For each fish, biometric data (fork length, FL, nearest cm; body mass, BM, nearest hg; gonad mass, GM, nearest g) were recorded, and the gonadosomatic index was calculated as GSI = 100 GM BM^{-1} (Table 1). Ovaries were excised and preserved as below specified.

Histological analysis of greater amberjack ovaries

For the histological analysis of ovaries, 1-cm thick gonad slices were cut and fixed in Bouin's solution, dehydrated in ethanol, clarified in xylene and embedded in paraffin wax. Five-µm thick sections were then stained with hematoxylin–eosin. The assessment of reproductive state was performed on the basis of the most advanced oocyte stage and the occurrence of post-ovulatory (POFs) and atretic vitellogenic follicles[15,19]. According to[41], atretic vitellogenic follicles were identified on the basis of the fragmentation of the zona radiata, invasion of hypertrophic granulosa cells into the oocyte and progressive yolk reabsorption.

Sampling date	Fish origin and ID	Fork length (cm)	Body mass (kg)	Gonad mass (g)	GSI	Most advanced oocyte stage	Group[a]
31/05/2021	Wild, GNW1	96.0	11.5	450.0	3.9	Mid-late vitellogenesis	WILD
31/05/2021	Wild, GNW2	101.0	12.7	250.0	2.0	Mid-late vitellogenesis	WILD
31/05/2021	Wild, GNW8	100.0	10.8	100.0	0.9	Mid-late vitellogenesis	WILD
18/05/2021	Hatchery-produced, GNC16	79.5	8.5	124.4	1.5	Mid-late vitellogenesis	NormalF
18/05/2021	Hatchery-produced, GNC17	77.0	8.1	133.8	1.6	Mid-late vitellogenesis	NormalF
18/05/2021	Hatchery-produced, GNC18	80.8	8.1	79.4	1.0	Mid-late vitellogenesis	NormalF
18/05/2021	Hatchery-produced, GNC20	77.3	7.3	145.1	2.0	Mid-late vitellogenesis	NormalF
01/06/2021	Hatchery-produced, GNC23	91.0	11.8	150.3	1.3	Atresia of the most advanced vitellogenic follicles	DysF
01/06/2021	Hatchery-produced, GNC25	89.0	10.7	145.0	1.4	Atresia of the most advanced vitellogenic follicles	DysF
01/06/2021	Hatchery-produced, GNC26	89.0	10.0	149.8	1.5	Atresia of the most advanced vitellogenic follicles	DysF

Table 1. Greater amberjack sampling date, origin, biometric data, gonado-somatic index (GSI), most advanced oocyte state and designated group. *DysF* reproductively dysfunctional farmed fish, *NormalF* non-dysfunctional farmed fish, *WILD* wild fish with normal spermatogenic activity. [a]For comparative transcriptome analysis, fish were grouped as described in the "Results" section.

RNA extraction and sequencing

For RNA-seq, small ovary samples were stored in RNA later® (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.), transported to the laboratory within one week, and frozen at -80 °C. Total RNA extraction was performed on 2.5 mg ovary samples, lysed and homogenised with TissueLyser II (Qiagen, Germany) setting 2' and 20 Hz frequency, by RNeasy® Plus Micro kit (Qiagen, Germany) following the manufacturer's protocol. The quantity and quality of extracted total RNA were checked by Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, U.S.) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, U.S.), respectively. High-quality RNA samples (RIN range 7–8) were then used to prepare the mRNA libraries by SureSelect Strand Specific RNA Library Preparation kit (Agilent Technologies, Santa Clara, California, U.S.). In particular, poly-A selection and directional mRNA libraries were carried out using 1 µg of total RNA. Finally, paired-end sequencing (2 × 75 bases) was performed on the Illumina NextSeq platform (Illumina Inc., San Diego, California, U.S.).

RNAseq data analysis

Raw sequencing data in FASTQ format were quality-checked using the FastQC program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and adaptor sequences as well as low quality regions (phred cut-off < 25) were trimmed using fastp (version 0.20.0) (with parameters: -detect_adapter_for_pe -x -q 25 -n 1 -l 50 -y -w 8)[42]. Cleaned reads were aligned onto the *Seriola dumerili* reference genome (Sdu_1.0, assembly accession GCF_002260705, https://www.ncbi.nlm.nih.gov/assembly/GCF_002260705.1) using STAR (version 020,201)[43] with default parameters. Read counts per gene were performed by featureCounts (version 1.6.0)[44] and differential gene expression analysis was carried out using DESeq2[45]. Only genes with an adjusted P value ≤ 0.05, $|\log_2(FC)| > 1.5$ and $|\log_2(FC)| < -1.5$ were used for downstream analyses.

DAVID (Database for Annotation, Visualization, and Integrated Discovery database https://david.ncifcrf.gov/tools.jsp)[46] and ShinyGO (http://bioinformatics.sdstate.edu/go)[47] were used to perform the functional annotation of differently expressed genes (DEGs), and the gene ontology (GO) enrichment analysis. By applying a false discovery rate (FDR) < 0.05, these analyses were able to identify specific categories (biological processes, molecular functions, cellular components and pathways), potentially involved in reproductive dysfunctions. A protein–protein interaction (PPI) network based on DEGs associated with each comparison was built using STRING (https://string-db.org/). DEGs were mapped to Kyoto encyclopedia of genes and genomes (KEGG) pathways using KEGG Mapper—Search (https://www.genome.jp/kegg/mapper/search.html)[48].

All queries launched on DAVID, ShinyGO and STRING were restricted to taxon ID 41447 (*Seriola dumerili*).

Results

Evaluation of reproductive state

The three wild fish had mid-to-late vitellogenesis as the most developed oocyte stage (maximum oocyte diameter ≈ 450 µm) (Table 1; Fig. 1a). This stage of oogenesis was the expected stage at the time of sampling and served as the normal reference group for the RNAseq comparative study (WILD group). Among the seven hatchery-produced greater amberjack females, four had mid-to-late vitellogenic oocytes (maximum oocyte diameter ≈

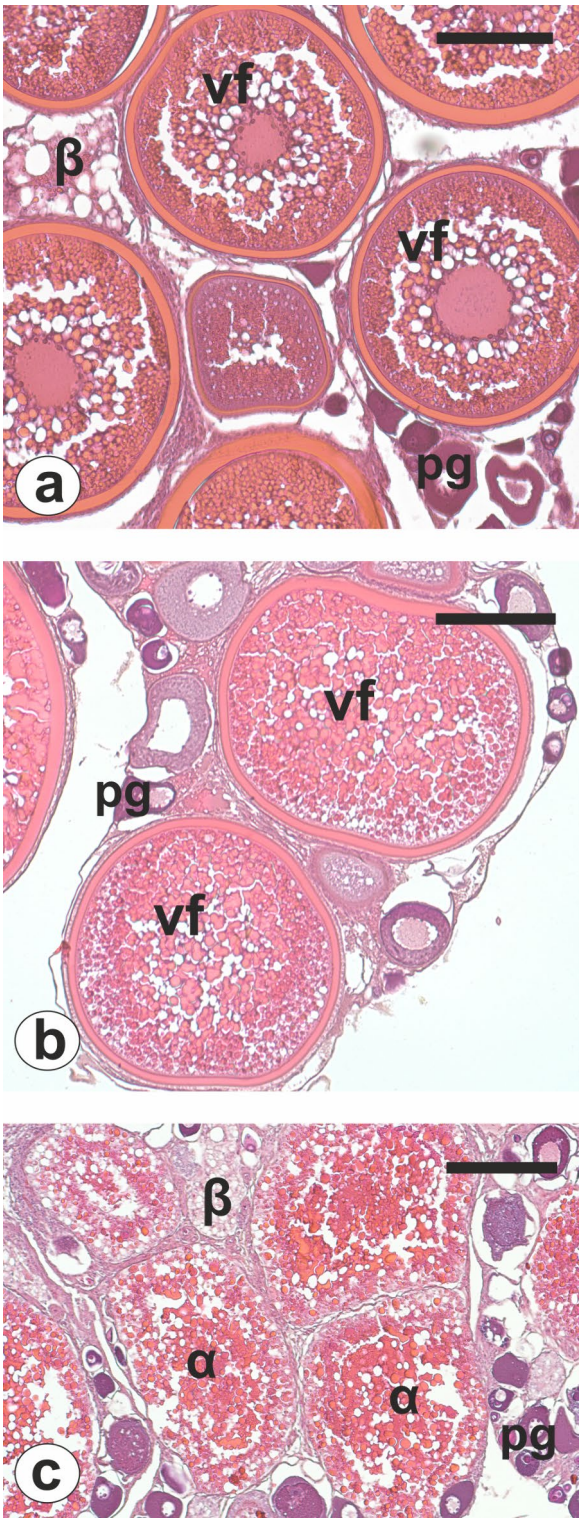

Figure 1. Micrographs of ovary sections from wild and hatchery-produced greater amberjack. (**a,b**) Ovaries from wild and hatchery-produced fish, respectively, showing mid-late vitellogenesis as the most advanced oocyte stage and minor atresia of vitellogenic follicles. (**c**) Ovary from a hatchery-produced fish showing major atresia of the most advanced vitellogenic follicles (100% atresia). H-E staining. Magnification bars = 150 µm in (**a,b**), 200 µm in (**c**). *pg* primary growth ovarian follicles, *α* alpha atretic follicle, *β* beta atretic follicle, *vf* vitellogenic follicle.

410 µm) as the most advanced oocytes stage and displayed mild (< 50%) atresia of vitellogenic follicles (Table 1; Fig. 1b). These fish were considered as fish undergoing normal oogenesis and were designated as "non-dysfunctional farmed" fish (NormalF) for ovary RNAseq analysis. The other three individuals were affected by severe (> 50%) atresia of the most advanced vitellogenic follicles and were designated as "dysfunctional farmed fish" (DysF) for ovarian RNAseq analysis. The atretic vitellogenic follicles were in the *alpha* or *beta* stage of atresia. *Alpha* atretic follicles were characterized by large vitellogenic follicles showing a breakdown of the nuclear envelope, *zona radiata* disappearance, coalescence of yolk granules (Fig. 1c). Atretic follicles in the *beta* stage were smaller than *alpha* atretic follicles and were devoid of yolk granules (Fig. 1a, c).

RNAseq and differential gene expression analysis

The ovary comparative transcriptome analysis among the three groups of fish in different reproductive conditions (WILD, NormalF and DysF) produced an average of 28 million paired-end reads per sample. After an appropriate cleaning procedure, high-quality reads were aligned to the *Seriola dumerili* reference genome. About 90% of the cleaned reads were uniquely mapped to the reference genome. Reads generated in this study are freely available through the SRA (Short Read Archive) database under the BioProject accession number PRJNA1056146.

The transcriptome analysis identified 21,678 genes, of which 19,621 were shared among the three groups, and 351, 359, and 176 genes were specifically expressed in the WILD, NormalF, and DysF groups, respectively (Fig. 2a, Supplementary Table S1). The principal component analysis (PCA) of the 1000 most variable genes showed a clear separation of samples belonging to the DysF group, from the WILD and NormalF groups (Fig. 2b).

Differential gene expression analysis showed 1166 dysregulated genes in the comparison DysF vs. WILD, of which 1072 were downregulated and 94 upregulated; 755 dysregulated genes in the comparison DysF vs. NormalF, of which 687 were downregulated and 68 upregulated; and only 20 dysregulated genes in the comparison NormalF vs. WILD, of which 11 were downregulated and nine upregulated (Supplementary Table S2). Among the observed DEGs, five were shared between the DysF vs. WILD and NormalF vs. WILD comparisons, seven were shared between the DysF vs. NormalF and NormalF vs. WILD comparisons. Additionally, 621 genes were shared between the DysF vs. NormalF and DysF vs. WILD comparisons (Fig. 3; Supplementary Table S3).

GO enrichment analysis

Biological categories related to gene ontology (GO) enrichment analysis performed on DEGs of each comparison were showed in Table 2. DysF fish exhibited statistically significant enrichment of genes, compared with non-dysfunctional groups (NormalF and/or WILD) in various biological categories (Supplementary Table S4). Specifically, the DysF group showed enriched genes associated with cellular components, including the GO terms Secreted (i.e., vascular endothelial growth factors; insulin like growth factor binding protein 4; inhibin subunit alpha; neuron-derived neurotrophic factor) and Extracellular matrix (i.e., nidogen-1-like; tenascin-1-like; collagen type VI alpha 3 chain; adamts genes). Additionally, DysF females showed significantly enriched genes with molecular functions related to the GO terms Receptors (i.e., AXL receptor tyrosine kinase; eph receptor A3; androgen receptor-like; low density lipoprotein receptor-related protein 5; platelet-derived growth factor receptor; sphingosine-1-phosphate receptor 2; smoothened, frizzled class receptor). Furthermore, KEGG pathways significantly enriched by DEGs in the DysF group included ECM-receptor interaction (i.e., integrin, alpha 1; collagen type VI alpha 3 chain; dystroglycan 1; laminin, alpha 4), Focal adhesion (i.e., integrin, alpha 1; collagen, type IV, alpha 5; platelet derived growth factor d) and Regulation of actin cytoskeleton (i.e., integrin alpha 1; fibroblast growth factor 5; fibroblast growth factor receptor 2, moesin-like).

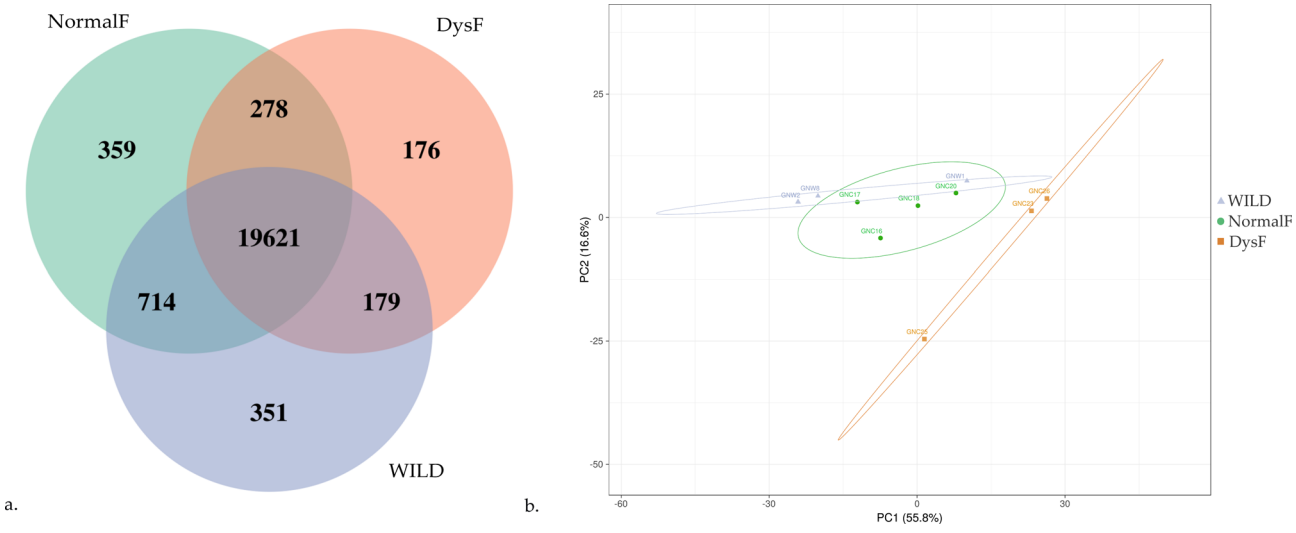

Figure 2. Venn diagram (**a**) and PCA (**b**) of shared and unique genes related to ovary samples of wild (WILD), dysfunctional (DysF) and normal (NormalF) hatchery-produced greater amberjack.

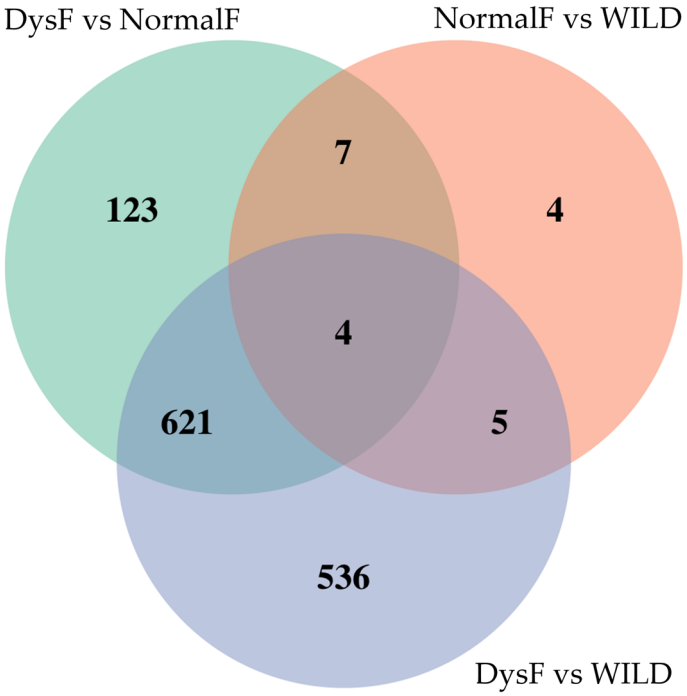

Figure 3. Venn diagram of shared and unique DEGs in the DysF vs. NormalF, DysF vs. WILD and NormalF vs. WILD comparisons. *DysF* dysfunctional farmed group, *NormalF* non-dysfunctional farmed group, *WILD* wild group.

	Category	Term	Count	FDR
DysF vs. NormalF	UP_KW_CELLULAR_COMPONENT	KW-0964~secreted	21	1.59E−04
	UP_KW_CELLULAR_COMPONENT	KW-0272~extracellular matrix	8	1.24E−03
	UP_KW_MOLECULAR_FUNCTION	KW-0675~receptor	20	3.41E−03
	KEGG_PATHWAY	sdu04512: ECM-receptor interaction	17	4.60E−08
	KEGG_PATHWAY	sdu04510: focal adhesion	21	5.42E−05
	KEGG_PATHWAY	sdu04810: regulation of actin cytoskeleton	18	6.15E−03
DysF vs. WILD	UP_KW_CELLULAR_COMPONENT	KW-0964~secreted	29	8.63E−06
	UP_KW_CELLULAR_COMPONENT	KW-0272~extracellular matrix	10	4.65E−04
	KEGG_PATHWAY	sdu04512: ECM-receptor interaction	21	8.61E−09
	KEGG_PATHWAY	sdu04510: focal adhesion	31	1.29E−07

Table 2. Gene ontology enrichment analysis of DEGs from wild and hatchery-produced greater amberjack ovaries. Count indicates DEGs belonging to specific category. FDR (false discovery rate) is statistically significant ($P < 0.05$), corrected for multiple testing within each category using the Benjamini–Hochberg procedure, describing the level of significance of the enrichment. Term denomination corresponds to that provided by DAVID annotation tool. No enriched category or term were identified in NormalF vs. WILD comparison. *DysF* dysfunctional farmed group, *NormalF* normal farmed group, *WILD* wild group.

Although no enriched biological category emerged, a few dysregulated genes involved in inflammation (*ptx3*), angiogenesis (*xdh*, *ptx3*), neurogenesis/dendritic spine morphogenesis (*ephb3*, *ngf*), and neurological/behavioural disorders (*ngf*, citron Rho-interacting kinase-like-*LOC111231034*) were found in the NormalF vs. WILD comparison.

Relationship between enriched pathways and PPi network analysis

The relationship among the top 20 enriched pathways obtained by ShinyGO are shown in Fig. 4. All enriched KEGG pathways were interconnected except for "cell surface receptor signalling" in DysF vs. NormalF and "ECM-receptor interaction" in both comparisons.

A network based on Protein–Protein interaction (PPi) was generated to evaluate functional relations between DEGs for each comparison (Figs. 5 and 6; Supplementary Table S5). Three main protein-interaction groups emerged both in DysF vs. NormalF and DysF vs. WILD comparisons. Many of these proteins are involved in

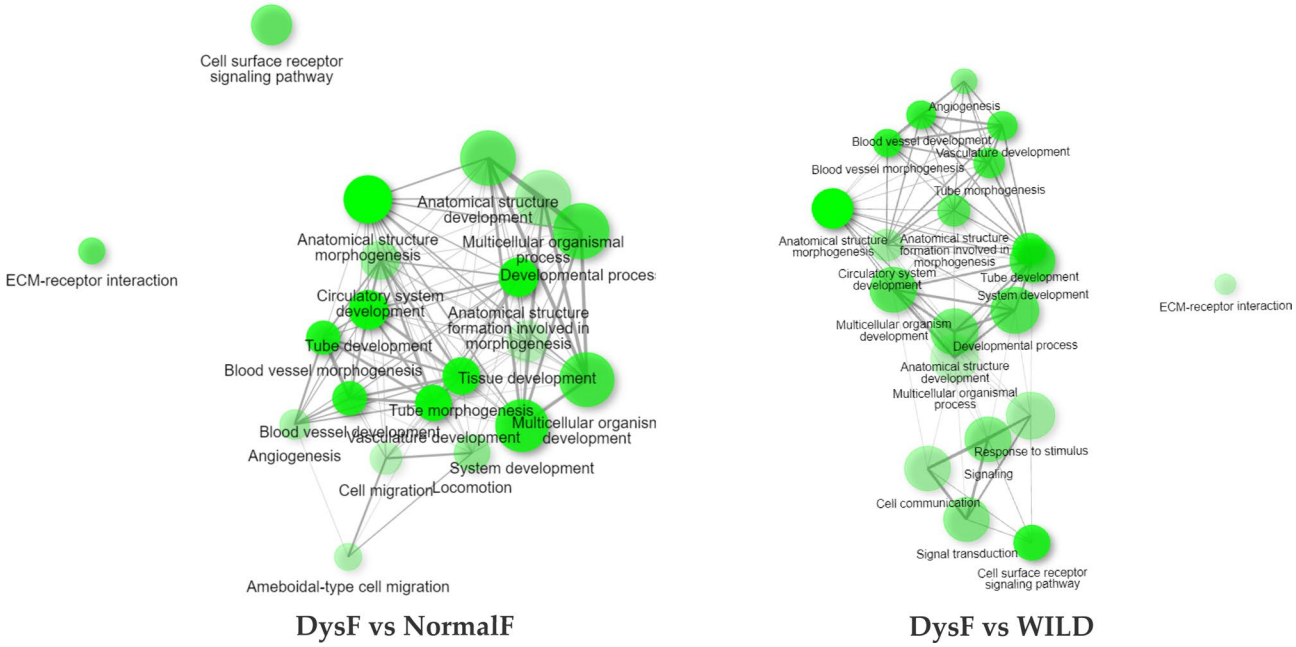

Figure 4. Relationship between enriched pathways in ovary samples from the three analysed groups. Pathways (nodes) are connected if they share 20% or more genes. Darker notes are more significantly enriched gene sets. Larger nodes represent larger gene sets.

biological categories associated with extracellular matrix (ECM-receptor interaction), signaling (Enzyme-linked receptor protein signaling), and transduction (Wnt signal transduction) pathways. Moreover, several proteins associated with the Ovulation cycle category (AMH, AXL, Inhibin subunit beta Aa, Lutropin-choriogonadotropic hormone receptor, and ADAMSTs) were identified. No protein interaction groups emerged from the comparison of NormalF vs. WILD.

KEGG analysis

The KEGG analysis showed DEGs involved in 111 pathways (Supplementary Table S6). Among these pathways, those likely associated with the reproductive function such as oogenesis or follicular atresia include: neuroactive ligand-receptor interaction, Steroid hormone biosynthesis, Cell cycle, Oocyte meiosis, Necroptosis, Ferroptosis, Apoptosis, Autophagy, Progesterone-mediated oocyte maturation, Endocytosis, and Phagosome, as well as Hedgehog, Apelin, PPAR, Notch, GnRH, p53, and mTOR signalling pathways. Moreover, in the DysF vs. NormalF comparison, several dysregulated gene-encoding factors associated with mammal hypogonadism, such as neuronal guanine nucleotide exchange factor and semaphorin-3F-like or polycystic ovary syndrome (PCOS), such as thymocyte selection-associated high mobility group box, chemokine (C–X–C motif) ligand 14, protein-lysine 6-oxidase-like and small glutamine rich tetratricopeptide repeat co-chaperone beta, were identified. In the same comparison, several dysregulated genes encoding factors involved in immune system functioning and inflammation, such meprin A subunit beta-like, galectin-2-like; cysteinyl leukotriene receptor 2-like and C-X-C motif chemokine 10-like, were found.

Discussion

The integration of histological and RNA-seq data from wild and hatchery-produced female greater amberjack provided novel insights into the molecular mechanisms underlying the observed oogenesis impairment in hatchery-produced fish. Histological evaluation of the ovaries revealed that three out of seven hatchery-produced greater amberjack females exhibited a reproductive dysfunction similar to that observed in individuals caught from the wild as juveniles and subsequently reared in captivity until reaching reproductive maturity[15,33]. Although the number of fish used in this study was limited due to the scarcity of hatchery-produced greater amberjack breeders and the challenges of collecting samples from wild breeders on commercial fishing vessels, transcriptome analyses revealed statistically significant differences in gene expression between fish showing reproductive dysfunction and those with no histological evidence of such dysfunction. Among the 19,621 genes expressed in all the three groups, approximately 4 to 6% of them were differentially expressed between the two groups, with the majority of dysregulated genes showing a downregulation in dysfunctional fish.

GO enrichment analysis

GO enrichment analysis in reproductively dysfunctional fish revealed enrichment of genes associated with secretion, extracellular matrix protein synthesis, extracellular matrix-receptor interaction, and focal adhesion. Additionally, genes encoding for growth factors such as vascular endothelial growth factors and several receptors, including the low-density lipoprotein receptor, were identified. Dysregulation of a few genes involved in

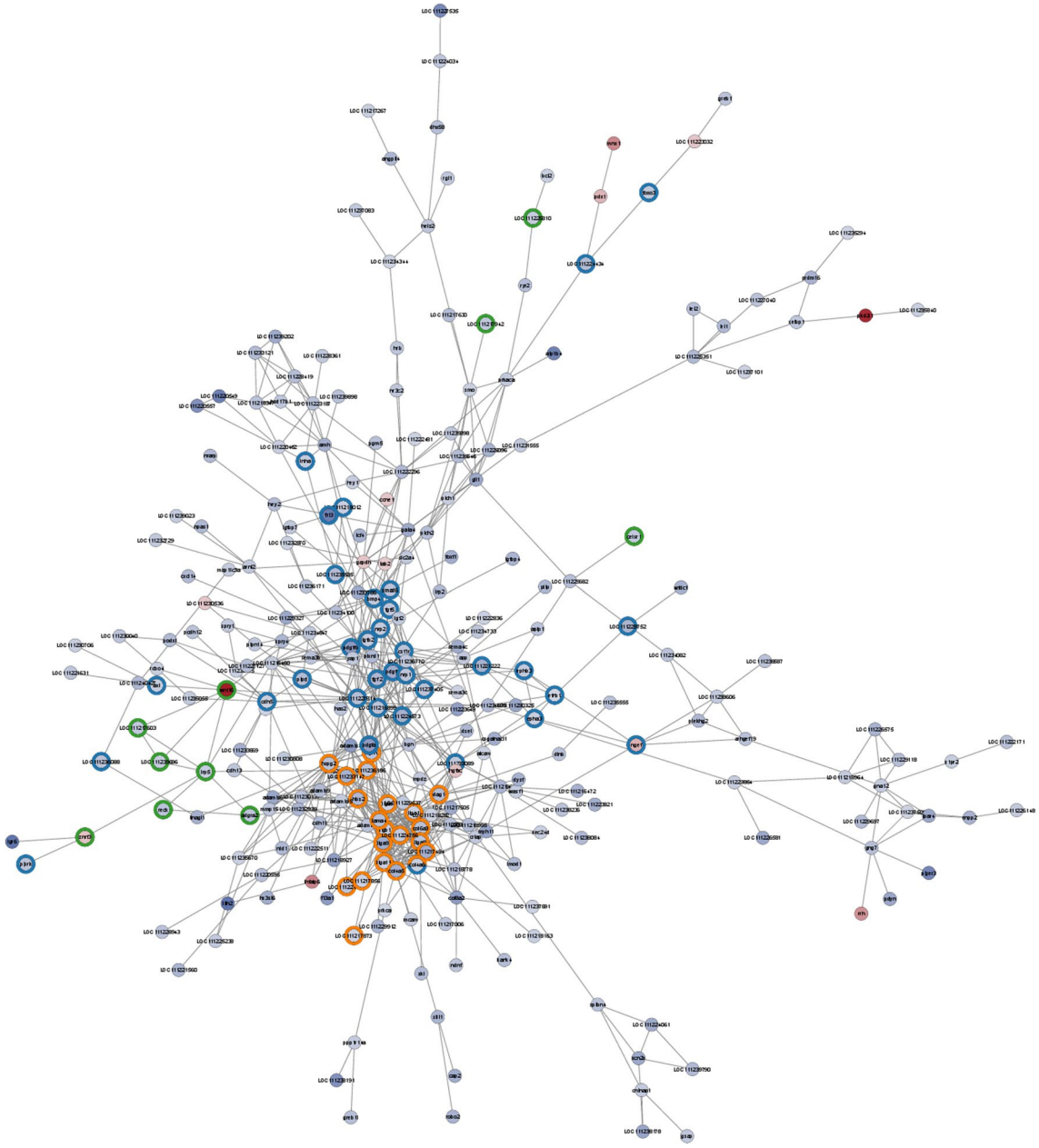

Figure 5. Protein–protein interaction (PPi) networks in DysF vs. NormalF. Networks were built using a confidence protein interaction (score = 0.5). Node background indicates gene upregulation (red, $\log_2FC > 1.5$) or downregulation (blue, $\log_2FC < -1.5$). Node contour indicates biological categories: ECM-receptor interaction (orange), Enzyme-linked receptor protein signaling pathway (blue), Wnt signal transduction pathway (green).

morphogenesis, inflammation, and neurological/behavioral disorders suggests that fish in the NormalF group experienced limited captivity-related stress, which did not affect reproductive function. Indeed, no enriched biological categories, KEGG pathways or PPi networks were identified when comparing NormalF to WILD fish. These findings contrast with those observed in males in a previous study, where molecular evidence of reproductive dysfunction was detected even in individuals with no histological evidence of gametogenesis alterations[35]. However, it cannot be ruled out that hatchery-produced, farmed fish initially classified as normal, may later develop a reproductive dysfunction. Indeed, all fish from the same origin used as breeders, showed no response to spawning-induction treatments, suggesting potential dysfunctionality across all hatchery-produced broodstocks[49].

Recently, a dysregulation of genes involved in extracellular matrix has been found in the testis of reproductively dysfunctional greater amberjack males[35]. Changes in the expression of genes encoding extracellular matrix proteins have also been observed during the testicular maturation in rainbow trout *Onchorhynchus mykiss*, suggesting their involvement in the reorganization of seminiferous tubules during the testicular cycle[48]. In humans, factors within the extracellular matrix have been reported to create the complex environment necessary for the interaction between ovarian follicles and their surroundings, ensuring follicle growth and development[50].

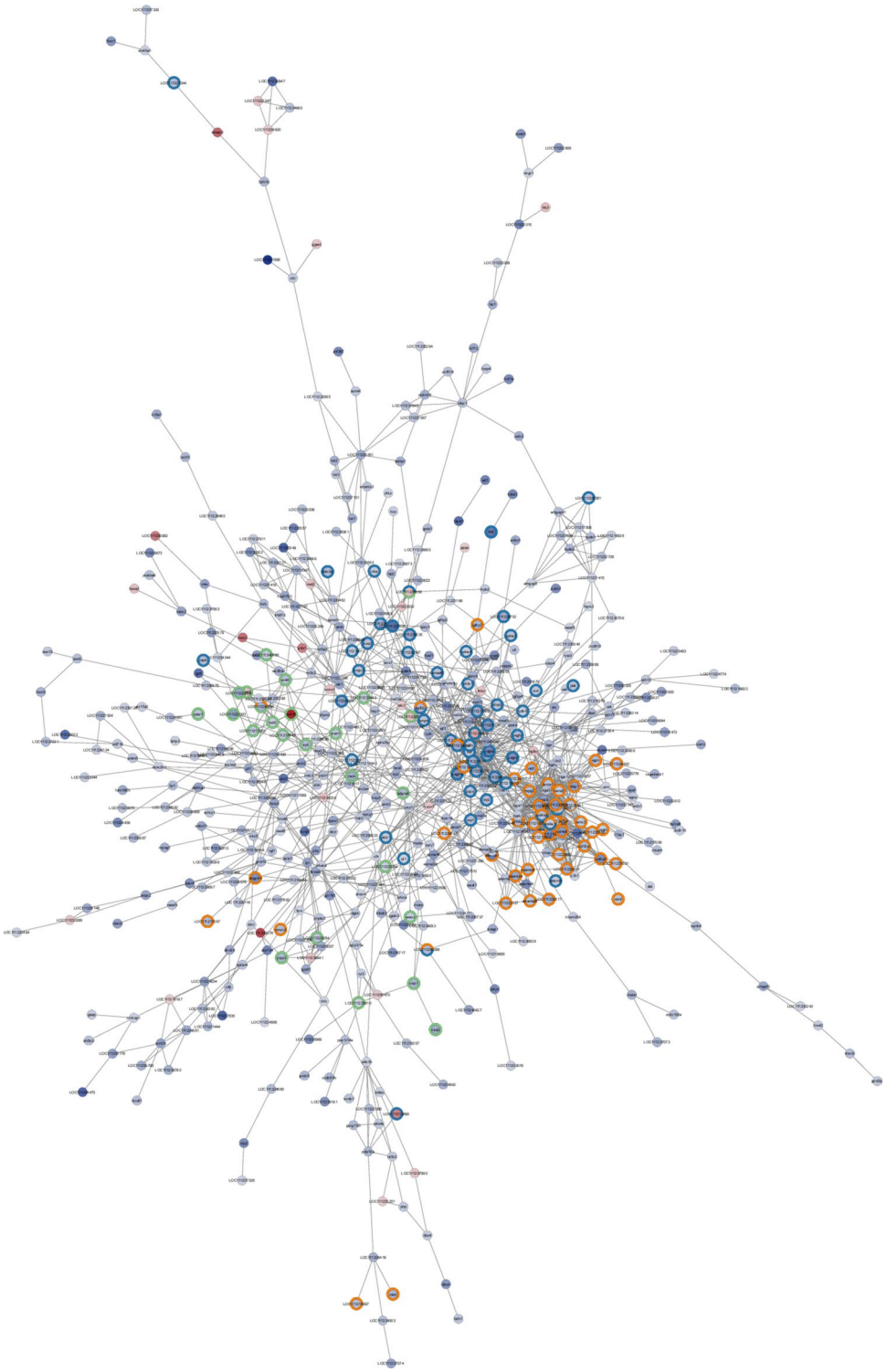

Figure 6. Protein–protein interaction (PPi) networks in DysF vs. WILD. Networks were built using a confidence protein interaction (score = 0.5). Node background indicates gene upregulation (red, $\log_2$FC > 1.5) or downregulation (blue, $\log_2$FC < 1.5). Node contour indicates biological categories: ECM-receptor interaction (orange); Enzyme-linked receptor protein signaling pathway (blue); Wnt signal transduction pathway (green).

Additionally, downregulation of extracellular matrix and cell adhesion molecules has been identified in the RNA expression profile of ovaries from patients affected by PCOS, an endocrinopathic condition characterized by oligo/anovulation, hyperandrogenism and polycystic ovaries[51]. In the present study, the ovarian follicle

degeneration characterizing the reproductive dysfunction was associated with the downregulation of several genes involved in cell-to-cell and cell-to-matrix interactions, such as genes encoding for collagens, integrins and laminins.

The observed downregulation of genes encoding for growth factors, including vascular endothelial growth factors that promote angiogenesis[52], is coherent with the observed extensive atresia of vitellogenic follicles in the DysF group. Angiogenesis is a complex process regulated by a balance of pro- and anti-angiogenetic factors, and disruption of this process may result in ovarian dysfunction[53]. In mammals, active angiogenesis is associated with gonad development and folliculogenesis, and follicles that fail to acquire satisfactory vascular support usually undergo atresia[54]. In prepubertal zebrafish *Danio rerio*, during the transition from primary to secondary oocyte growth, biological processes related to angiogenesis, including blood vessel morphogenesis and lymph vessel formation are enriched in up-regulated genes. This suggests that formation of blood vessels is necessary to ensure sufficient supply of nutrients, hormones and oxygen to support the rapid growth of follicles in fish[55].

Different lipoprotein receptors have been found to bind vitellogenin[34,56,57]. The downregulation of lpr5 observed in the present study may reduce the ability to uptake vitellogenin and complete vitellogenesis. In greater amberjack, significantly lower expression of lipoprotein receptor (*vtgr* and *lrp13*) gene has been observed in wild-caught, captive-reared fish compared to fish sampled in nature[34]. This reduced expression was associated with a reduced number of vitellogenic oocytes and, consequently, lower fecundity. Despite good egg quality observed in wild-caught greater amberjack treated with GnRHa[22–24], fecundity was significantly lower compared to fish in the wild[41].

Relationship between enriched pathways and PPi network analysis

The outputs of the ShinyGO analysis, alongside the GO enrichment analysis, clearly indicated that the observed gene dysregulation in dysfunctional greater amberjack involves many interconnected pathways related to ovarian morphogenesis and development. In the DysF group, many interconnected proteins associated with the biological categories Enzyme-linked receptor protein signalling pathway and Wnt signal transduction pathway (both involved in cell proliferation, differentiation, development, inflammatory response and apoptosis), ECM-receptor interaction and Ovulation cycle, were encoded by downregulated genes. Among the interconnected proteins involved in Ovulation cycle, AMH, AXL, Inhibin Subunit Beta A, Lutropin-choriogonadotropic hormone receptor and ADAMSTs play recognized roles in folliculogenesis and follicular atresia. Anti-Müllerian hormone (AMH) is known as a critical testicular signal driving the development of the male reproductive tract in vertebrates[58]. In humans, it is synthesized and secreted by granulosa cells of small ovarian follicles and is actively involved in folliculogenesis[59]. Recent evidence suggests that AMH may also indirectly affect folliculogenesis by modulating GnRH neuron activity, which controls pituitary gonadotropin secretion[59]. Moreover, during mouse embryonic development, AMH and its receptors are expressed by migratory GnRH neurons, suggesting AMH signaling involvement in congenital hypogonadotropic hypogonadism pathogenesis[60]. AXL, a member of the Tyro3-Axl-Mer (TAM) receptor tyrosine kinase subfamily, transduces signals from the extracellular matrix into the cytoplasm, influencing cellular functions such as growth, cell survival, cell proliferation, migration and differentiation, and has been implicated in human reproductive diseases including some forms of hypogonadotropic hypogonadism[61,62]. The inhibin subunit beta A (INHBA) gene encodes a member of the transforming growth factor-beta (TGF-β) superfamily and is proteolytically processed to generate a subunit of the dimeric activin and inhibin protein complexes. These complexes activate and inhibit, respectively, follicle stimulating hormone (Fsh) secretion from the pituitary gland[63]. Increased expression of the β-subunit of Fsh (Fshβ) was found in both young and adult inhba-deficient zebrafish; however, the adult females exhibited ovarian follicles arrested at the full-grown stage without oocyte maturation and ovulation taking place[64].

The lutropin-choriogonadotropic hormone receptor, a member of the G protein coupled receptor family, mediates Lh activity in the ovary. In mammals, it is upregulated by Fsh and chorionic gonadotropin and it is downregulated by decreased receptor gene transcription and/or increased mRNA degradation following Lh stimulation (post-transcriptional downregulation)[65,66]. In the present study, downregulation of this gene in the dysfunctional fish suggests insufficient pituitary gonadotropin secretion, typical of captivity-induced reproductive dysfunction[2].

Disintegrin and metalloproteinase enzymes belonging to the ADAMTS family are involved in tissue morphogenesis, tissue remodeling, inflammation and vascular biology, and participate in follicle rupture and ovulation processes in mammals[67]. Dysregulation or functional changes in ADAMTS proteases have been linked to reproductive disorders, such as PCOS and premature ovarian failure[68].

KEGG analysis

Consistent with the widespread gene dysregulation, reproductive dysfunction involved many KEGG pathways. Several genes encoding cell receptors involved in the Neuroactive ligand-receptor interaction (a signaling pathway directly related to neuro function[69]), such as lutropin-choriogonadotropic hormone receptor, thyroid hormone receptor beta isoform X1, prostaglandin E2 receptor EP3 subtype, and *d* prodynorphin, were downregulated in dysfunctional fish. In rodents, prodynorphin, a preproprotein proteolytically processed to form opioid peptides that bind the kappa-type of opioid receptor[70], is expressed in granulosa and luteal cells under gonadotropin regulation[71]. Neurons in the central nucleus of the Amygdala (CeA)[72] and hypothalamic neurons[73] expressing prodynorphin, kisspeptin, and neurokink B (Kdyn neurons) have been shown to regulate GnRH pulsatility. Inactivation of prodynorphin gene from multiple sources, both intrinsic and extrinsic to the CeA, induces anxiety-like behavior in mice[72]. It has been hypothesized that the altered GnRH pulsatility in women affected by PCOS may result from a reduced hypothalamic opioid inhibitory activity[73].

In dysfunctional fish in the present study, downregulation of several genes involved in GnRH and steroid hormone biosynthesis pathways was observed. In the latter pathway, the following genes were found to be downregulated: steroid 17-alpha-hydroxylase (*cyp17A1*), cholesterol side-chain cleavage enzyme (*cyp11a1*), and cytochrome P450 family 19 subfamily A member 1 (*cyp19a1*; aromatase). The *cyp17A1* gene encodes for the steroidogenic enzymes that produce progestins, mineralocorticoids, glucocorticoids, androgens and estrogens[74]. The *cyp11a1* encodes for a cytochrome P450 monooxygenase that catalyzes the side-chain hydroxylation and cleavage of cholesterol to pregnenolone, the precursor of most steroid hormones[75]. The *cyp11a1* gene produces a cytochrome P450 monooxygenase that catalyzes the aromatization of androstenedione, testosterone, and 16a-hydroxyandrostenedione[76].

The failure of oocytes to undergo maturation after completion of vitellogenesis observed in cultured fish is often due to a reduced pituitary release of gonadotropins, particularly Lh, which triggers oocyte maturation via 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) synthesis[1,2,7]. Low plasma Lh levels have also been found in wild-caught, captive-reared fish belonging to the same stock as the parents of the fish analysed in the present study[77].

Therefore, the observed dysregulation in cultured greater amberjack of genes involved in steroid synthesis, cell cycle, meiosis and progesterone-mediated oocyte maturation pathways may have originated from insufficient Lh plasma levels, in the present study. Furthermore, the widespread depression of steroid hormone synthesis, associated with the downregulation of androgen receptor-like genes in DysF fish, may have been exacerbated by the atretic loss of vitellogenic oocytes and the associated death of follicular and thecal cells. These cells are involved in testosterone synthesis and its conversion to 17-β estradiol.[78].

The alteration of *PPAR* and *Apelin* signaling pathways may also have played a role in the oogenesis impairment of DysF greater amberjack. In mammals, adipokines, including apelins, are present in different ovarian cells and can affect steroidogenesis and ovulation through the activation of the nuclear transcription factors PPARS, which are involved in ovarian development and follicular atresia. Their alteration may induce infertility[79,80]. Although the gene expression data and histological evidence of extensive atresia of vitellogenic follicles, indicate that the observed reproductive dysfunction affected the ability of vitellogenic oocytes to proceed towards maturation, the alteration of Wnt, Notch, and Hedgehog signalling pathways, strongly suggest that the oogenesis impairment may also affect early oogenesis stages. These three pathways represent highly conserved mechanisms that contribute to stem cell maintenance and proliferation capacity[81–84]. Apoptosis play an important role if the regulation of fish spermatogenesis[85] and a high density of apoptotic spermatogonia was observed in wild-caught Atlantic bluefin tuna *Thynnus thynnus*[3] and greater amberjack males confined in captivity[4], suggesting that the anti-apoptotic pathways protecting stem germ cells from apoptosis might not work properly under prolonged captivity-induced stress.

The dysregulation of genes encoding immune system and inflammation factors observed in the DysF vs. NormalF comparison, along with the alterations in Endocytosis and Phagosome pathways, aligns with the reported involvement of the immune system in the inflammatory process associated with follicular atresia[41]. Moreover, the dysregulation of Endocytosis and Phagosome pathways may be linked to the reduced ability of the ovaries in dysfunctional fish to internalize egg yolk precursors by micropinocytosis, resulting from the loss of vitellogenic oocytes through atresia.

The presence of upregulated genes associated with Necroptosis and Apoptosis pathways in dysfunctional fish may also be associated with follicular atresia. Oocyte atresia in teleost fish involves at least three different processes: autophagy (self-digestion of oocyte and follicular cell components), heterophagy (phagocytosis of egg components by granulosa cells that act as macrophages) and follicular cell death by apoptosis[41]. No information is available regarding the involvement of necroptosis in teleost fish atresia; in mammals, however, recent studies demonstrated that oxidative stress triggers necroptosis in granulosa cells and oocytes[86].

Conclusions

In the present study, we report that 50% of hatchery-produced greater amberjack females were affected by extensive atresia of ovarian follicles in vitellogenesis. This dysfunction involved dysregulation of genes associated with many biological categories and showed molecular traits common to the PCOS and congenital hypogonadism, both conditions associated with infertility in mammals. Ongoing analyses aim to characterize gene expression dysregulation in the pituitary and brain of reproductively dysfunctional greater amberjack, with the goal of enhancing our understanding of reproductive dysfunction along the entire reproductive axis, in fish reared in captivity. This knowledge may, hopefully, lead to the development of better protocols for the reproductive control in a species of emerging interest for the aquaculture industry.

Data availability

Reads generated in this study are freely available through the SRA (Short Read Archive) database under the BioProject accession number PRJNA1056146 https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1056146. All the other data produced and/or analyzed during the current study are included in this article in Supplementary Tables.

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Competing interests
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Additional information
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