Differential pituitary mRNA expression in wild and hatchery-produced greater amberjack *Seriola dumerili* exhibiting normal and impaired spermatogenesis

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Abstract MAX 250 WORDS

The greater amberjack *Seriola dumerili* is a promising aquaculture species in the Mediterranean Sea. However, first generation (F1) hatchery-produced individuals exhibit incomplete gametogenesis when reared in tanks and absence of spontaneous spawning when reared in cages. As part of a broader investigation into the impacts of captivity, we provide a comparison of the pituitary transcriptome between wild and F1 male greater amberjack sapled during spermatogenesis. Wild males (WILD group, $n = 4$) displayed normal testicular morphological development with abundant luminal spermatozoa. Among F1 males, some had normal testicular development (NormalF, $n = 4$), while others had dysfunctional testicular development (DysF, $n = 2$). Transcriptomic analysis revealed 301 differentially expressed genes (DEGs) in the DysF vs. WILD comparison and 456 DEGs in the NormalF vs. WILD comparison, while no DEGs were detected between DysF and NormalF groups. Protein–protein interaction (PPI) analysis identified broad regulatory networks involving 246 proteins across eleven functional categories in DysF vs. WILD, and 382 proteins across 10 categories in NormalF vs. WILD. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed DEGs enriched in 62 (DysF vs. WILD) and 84 (NormalF vs. WILD) pathways. Notably, several dysregulated pathways common to all F1 males are associated with reproductive functions, as well as nucleotide metabolism, calcium signaling, MAPK and mTOR signaling, neuroactive ligand–receptor interaction, Gonadotropin Releasing Hormone (GnRH) and ErbB signaling pathways. Among the DEGs, the identified genes were linked to GnRH neuron migration, panhypopituitarism and hypogonadotropic hypogonadism. These findings suggest that reproductive dysfunctions in F1 male greater amberjack may involve a broad suppression of pituitary activity, potentially mediated by dysregulation of the opioid, endocannabinoid and dopaminergic systems.

1. Introduction

The domestication process of any potential fish species begins with the confinement of wild individuals in captivity and their rearing in optimal environmental and nutritional conditions to allow the proper function of their reproductive axis, the maturation of their gonads and the spawning of viable gametes (Mylonas et al., 2010). However, when confined in captivity, wild fish are often affected by reproductive dysfunctions, due to captivity induced stress or the exposure to inappropriate environmental conditions, unknown to the broodstock managers (Mylonas et al., 2010). Reproductive dysfunction in many cultured fishes can be alleviated through the use of exogenous reproductive hormones, modifications of environmental conditions or a combination of both (Duncan et al., 2012, 2013; Mylonas et al., 2010, 2016).

Significant research efforts have been ongoing in the eastern Mediterranean Sea to domesticate the greater amberjack *Seriola dumerili* (Risso, 1810), a promising aquaculture candidate due to its wide distribution in temperate waters around the world (Bauchot, 1987; Cervigon, 1993; Smith, 1997). Efforts to establish greater amberjack reproductive control begun with monitoring the reproductive function of wild-caught, captive-reared juveniles reared to reproductive maturity, and the description of the effects of confinement on gametogenesis (Zupa et al., 2017a, b; Pousis et al., 2018, 2019). These studies demonstrated that wild-caught greater amberjack were reproductively dysfunctional, characterized by low sex-steroid plasma levels and limited gonadal development, precocious cessation of spermatogenesis and a high rate of germ cell apoptosis in males (Zupa et al., 2017a), and extensive atresia of vitellogenic follicles in females (Zupa et al., 2017b; Pousis et al. 2018, 2019). Through improvements in rearing conditions, nutrition and feeding, broodstock management, and the administration of exogenous reproductive hormone protocols, successful control of reproduction has been achieved (Fakriadis et al., 2019, 2020a, b, 2024; Fakriadis and Mylonas, 2021). As a result, high quality fertilised eggs have been obtained over the last decade, leading to the establishment of commercial protocols for larval rearing and on-growing (Pérez et

al., 2020; Loufi et al., 2024), and eventually giving rise to the first generation (F1) of greater amberjack breeders produced in the eastern Mediterranean.

These hatchery-produced greater amberjack reached sexual maturity (puberty) in 5 years, as confirmed by the presence of fully vitellogenic follicles in females and the production of viable sperm in males (Lancerotto et al., 2024). However, these reproductively mature, hatchery-produced fish exhibited reproductive dysfunctions that were even more severe than the ones described above for their wild-caught parents, characterized by incomplete gametogenesis when fish were reared in tanks or the absence of spontaneous spawning when they were reared in cages. These dysfunctions have been addressed recently (Lancerotto et al., 2025), and reproductive control was achieved through the administration of specifically designed single-chain recombinant Follicle stimulating hormone (Fsh) and Luteinizing hormone (Lh). In contrast, treatment with a Gonadotropin-Releasing Hormone (GnRH) agonist—previously shown to be effective in wild-caught broodstock (Fakriadis et al., 2020a, b)— did not yield positive results when applied to these hatchery-produced broodstock (Lancerotto et al., 2024). The above results were not unexpected, since the existence of a significantly lower reproductive success of individuals born in captivity has been reported in many fish species (Pankhurst and Fitzgibbon, 2006; Mañanos et al., 2009; Jerez, 2018; Papadaki et al., 2018), as well as in other vertebrates reared for commercial, conservation or research purposes (Farquharson et al., 2018).

Recently, a research program based on the comparative study of the transcriptome of the reproductive axis of aquacultured greater amberjack was launched (NewtechAqua, 2023), in order to improve our understanding of the molecular mechanisms underlying their reproductive dysfunctions. Within this research program, gonads, pituitaries and brains from wild (fishery-caught) and F1 hatchery-produced greater amberjack were sampled during the gametogenesis phase of the reproductive cycle. In both sexes, the transcriptome analysis of the gonads of F1 reproductively dysfunctional greater amberjack documented the dysregulation of a large number of genes involved in many interconnected biological processes (Lavecchia et al., 2023). In males,

alterations of the biological processes of *steroidogenesis*, *cell cycle*, *meiosis*, *cell assembly*, and *apoptosis* were reported. In females, the alterations involved the biological processes *ECM-receptor interaction*, *Enzyme-linked receptor protein signaling* and *Wnt signal transduction* pathways and *ovulation cycle*, as well as *111* Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Lavecchia et al., 2023). Additionally, reproductively dysfunctional females exhibited an upregulation of genes encoding factors that in mammals are associated with hypogonadism and polycystic ovary syndrome.

Being a continuation of the above-mentioned research efforts on the reproductive axis of greater amberjack, the present study presents a comparison of the pituitary transcriptome between wild and hatchery-produced (F1) male greater amberjack sampled during the period of spermatogenesis.

2. Methods

2.1. Ethics

For this study, wild and F1 hatchery-produced male greater amberjack were used. Adult wild fish commercially caught from an authorized purse-seine fishing vessel during routine fishing operations, were purchased and sampled onboard immediately after death. Hatchery-produced fish were obtained from Argosaronikos Fish Farm S.A. (Salamina Island, Greece). The use of hatchery-produced fish in the present study was approved by the Greek National Veterinary Services (AP 31337). All procedures involving animals were conducted in accordance to the "Guidelines for the treatment of animals in behavioural research and teaching" (Anonymous, 1998), the Ethical justification for the use and treatment of fishes in research: an update (Metcalfe and Craig, 2011) 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" (European Union, 2010). The authors complied with the ARRIVE guidelines.

2.2. Sampling

The pituitaries analyzed in the present study were taken from the same fish used in Lavecchia et al.'s (2023), in which details of fish origin, farming condition, sacrifice and sampling were reported. Briefly, for the present study, pituitaries from ten adult males (four wild and six hatchery-produced) were collected during the active gametogenesis period of the reproductive season 2021. Wild individuals were fished around the Pelagie Islands (Sicily, Italy) and sampled immediately after death; hatchery-produced fish belonged to a stock maintained under common aquaculture practices by Argosaronikos Fish Farm S.A. (Salamina Island, Greece). Before sampling, captive-reared fish were tranquillised with about 0.01 ml L^{-1} of clove oil (Roumpoulakis E.P.E., Greece), 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 and euthanized by decapitation.

For each fish, biometric data (fork length, FL, nearest cm; body mass, BM, nearest hg; gonad mass, GM, nearest g) were recorded and gonadosomatic index (GSI = 100 GM BM^{-1}) estimated (Table 1), and the pituitary was excised after careful craniotomy, and preserved as below specified. The identification of reproductively dysfunctional individuals was based on the histological analysis of the testes (Lavecchia et al., 2023). In particular, fish were classified as undergoing normal spermatogenesis if they showed all stages of spermatogenesis, seminiferous tubules with large lumen and abundant luminal spermatozoa (Fig. 1a), whereas they were classified as reproductively dysfunctional if they showed arrested spermatogenesis and small amount of luminal spermatozoa (Fig. 1b). For the analysis of pituitary RNA expression, the sampled fish were then grouped as follows: wild fish (**WILD**, reference group; N = 4); hatchery-produced fish showing a gonad histological appearance similar to wild fish (normal farmed group, **NormalF**; N = 4); hatchery-produced fish showing reproductive dysfunction (**DysF**; N = 2) (Table 1).

2.3. RNA extraction and sequencing

For RNA-seq, whole pituitaries 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 whole pituitaries, lysed and homogenized with TissueLyser II (Qiagen, Germany) setting 2' and 20 Hz frequency, by Rneasy® Plus Minikit (Qiagen, Germany) following the manufacturer's protocol. The quantity and quality of extracted Total RNA were checked for quantity and quality respectively 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 (2x75 bases) was performed on the Illumina NextSeq 500 platform (Illumina Inc., San Diego, California, U.S.).

2.4. RNAseq data analysis

Sequencing raw 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 cutoff < 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) (Chen et al., 2018). 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 020201) (Dobin et al., 2013) with default parameters. Read counts per gene were performed by featureCounts (version 1.6.0) (Liao et al., 2014) and differential gene expression analysis was carried out using DESeq2 (Love et al., 2014). Only genes with an adjusted P value ≤ 0.05, $|\log2(FC)| > 1.5$ and $|\log2(FC)| < -1.5$ were used for downstream analyses.

DAVID (Database for Annotation, Visualization, and Integrated Discovery database https://david.ncifcrf.gov/tools.jsp) (Sherman et al., 2021) was used to perform the functional annotation of Differently Expressed Genes (DEGs) and the 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 KEGG pathways using KEGG Mapper – Search (https://www.genome.jp/kegg/mapper/search.html) (Kanehisa and Sato, 2020). All queries launched on DAVID and STRING were restricted to taxon ID 41447 (*Seriola dumerili*).

3. Results

3.1. RNAseq and gene expression

The pituitary comparative transcriptome analysis among the three groups of male greater amberjack in different reproductive conditions (WILD, NormalF and DysF) produced an average of 16 million paired-end reads per sample. After an appropriate cleaning procedure, high quality reads were aligned to the *Seriola dumerili* reference genome. About 95% of cleaned reads were uniquely mapped to the reference genome.

The transcriptome analysis identified 18176 genes, of which 16234 were common to the three groups, and 889, 90, and 8 genes were specifically expressed in WILD, NormalF and DysF respectively (Fig. 2; Supplementary Table 1). The principal component analysis (PCA) of the 1,000 most variable genes, showed a clear separation of samples belonging to the two hatchery-produced groups from the WILD group (Fig. 3). Differential gene expression analysis identified 301 and 376 DEGs in DysF vs. WILD and NormalF vs. WILD comparisons, respectively (Table 2; Supplementary Tables 2a and 2b). No differentially expressed genes were found in DysF vs. NormalF comparison. Among the DEGs, 166 were common to the comparisons DysF vs. WILD

and NormalF vs. WILD, whereas 135 and 210 were exclusively associated to DysF vs. WILD and NormalF vs. WILD, respectively (Fig. 4).

3.2. GO enrichment analysis

Biological categories related to gene ontology enrichment analysis performed on DEGs of each comparison are reported in Table 3 and Supplementary Table 3. In DysF vs. WILD, a statistically significant enrichment of *purine metabolism* and *nucleotide metabolism* pathways was identified; in NormalF vs. WILD, a statistically significant enrichment of *antiviral defense* biological process was identified.

3.3. Protein-Protein interaction network analysis

The PPi analysis relative to the DysF vs. WILD comparison evidenced a wide network (Fig. 5) including 246 proteins belonging to eleven biological categories (Table 4 and Supplementary Table 4).

The PPi analysis relative to the NormalF vs. WILD comparison, evidenced a wide network (Fig. 6) including 382 proteins belonging to ten biological categories (Table 5 and Supplementary Table 5).

3.4. KEGG analysis

The KEGG analysis showed DEGs involved in 62 and 84 pathways in the comparison DysF vs. WILD and NormalF vs. WILD, respectively (Supplementary Tables 6a and 6b).

Among the dysregulated KEGG pathways common to the two hatchery-produced fish groups, those likely associated with the reproductive function involving at least two DEGs and common to the two comparisons were: Purine metabolism, Protein processing in endoplasmic reticulum, Nucleotide metabolism, Regulation of actin cytoskeleton, Biosynthesis of cofactors, Oxidative phosphorylation, Cell adhesion molecules, Focal adhesion, Endocytosis, Calcium signaling

pathway, MAPK signaling pathway, mTOR signaling pathway, Neuroactive ligand-receptor interaction, GnRH signaling pathway, ErbB signaling pathway, Metabolic pathways, Cytokine-cytokine receptor interaction.

3.5. Dysregulated genes potentially involved in the reproductive dysfunction

An in-depth screening of all differentially expressed genes (DEGs) between DysF and WILD revealed a number of dysregulated genes functionally associated with neuronal activity, nervous system development and nervous diseases (e.g., Neuron-derived neurotrophic factor, like; Proprotein convertase subtilisin/kexin type 2; Proenkephalin a; Gamma-aminobutyric acid type A receptor beta4 subunit; Ephrin RBD domain-containing protein; Apolipoprotein A-Ib; Seizure related 6 homolog (mouse)-like 2), hormone synthesis and secretion (e.g., Growth hormone 1; Luteinizing hormone, beta polypeptide; Growth hormone releasing hormone receptor b; Hydroxysteroid (11-beta) dehydrogenase 1-like a; Somatolactin alpha; Dehydrogenase/reductase (SDR family) member 7Cb), extracellular matrix degradation and cell adhesion (e.g., Matrix metallopeptidase 9; Fibronectin type-III domain-containing protein; Norrin cystine knot growth factor NDP), inflammation (e.g., Interleukin-8; TNF_2 domain-containing protein; Chemokine (C-X-C motif) ligand 12a), ion channels (e.g., Calcium voltage-gated channel auxiliary subunit alpha2delta 3; Potassium voltage-gated channel, KQT-like subfamily, member 1.1; Voltage-dependent calcium channel gamma-7 subunit; Calcium channel, voltage-dependent, R type, alpha 1E subunit b).

The screening of all differentially expressed genes between NormalF and WILD revealed a dysregulation of genes involved in the pituitary function (e.g., Insulin-like growth factor 1; Growth hormone 1; Luteinizing hormone, beta polypeptide; Gamma-aminobutyric acid (GABA) A receptor, alpha 1; Arrestin, beta 1; NMDA receptor synaptonuclear signaling and neuronal migration factor a; Proenkephalin a; Muscarinic acetylcholine receptor; Cholinergic receptor, nicotinic, beta 5b). Moreover, many strongly dysregulated genes were functionally related to

neuronal activity, nervous system development and nervous diseases (e.g., C-Type Lectin Domain Family 3 Member A; Arrestin, beta 1; Apolipoprotein A-Ib; G_PROTEIN_RECEP_F1_2 domain-containing protein; Somatostatin Receptor 2a; Whirlin b; Gamma-synuclein; Somatolactin alpha; Neuropilin (NRP) and tolloid (TLL)-like 1; Neuron-derived neurotrophic factor, like; Glutamate receptor, ionotropic, kainate 1a; Glycine receptor, alpha 4a; Sonic Hedgehog Protein); inflammation and immunity (e.g., Interleukin-8; Radical S-adenosyl methionine domain containing 2; Complement component 1; q subcomponent-like 3b; Ig-like domain-containing protein; MX dynamin like GTPase 1; DEXH (Asp-Glu-X-His) box polypeptide 58; Regulator of G protein signaling 9 binding protein; Follistatin-like 3**),** ion channels (e.g., Potassium voltage-gated channel, KQT-like subfamily, member 1.1; Transient receptor potential cation channel, subfamily M, member 3; Sodium channel protein; Voltage-dependent T-type calcium channel subunit alpha).

4. Discussion

The present study revealed that around 18,000 genes were expressed in the pituitary of greater amberjack adult males sampled during the reproductive season. A high number of dysregulated genes were found in F1 fish, with NormalF showing a higher number of dysregulated genes (≈2.1%) than DysF (≈1.7%). ……..more about this peculiarity

The GO enrichment analysis demonstrated a significant enrichment of genes in DysF associated with KEGG pathways related to basic cellular functions, such as nucleotide metabolism. The dysregulation of nucleotide metabolism might have played a crucial role in the mechanism underlying the reproductive dysfunction, because several hypophysiotropic hormones act via G-protein coupled receptors to stimulate pituitary hormone synthesis and secretion (Ravindra and Aronstam, 1990). In particular, GnRH, the hypothalamic hormone responsible for Fsh and Lh release from the pituitary, causes the activation of heterotrimeric G-proteins with

consequent effects on cyclic AMP production, as well as on the soluble and particulate guanylyl cyclases that generate cGMP (Perrett and McArdle, 2013). Therefore, ……..

The enrichment of genes related to antiviral defence (comparison NormalF vs WILD) suggests that a viral infection could be affecting all

greater amberjack; however, no macroscopic sign of disease was observed during the sampling operations (A.C., R.Z. C.P., C.C.M., personal observations). Whatever the cause of the enrichment of the antiviral defence process was, the activation of genes involved in the response to viruses, such as Interferon induced with helicase C domain 1, induces signalling cascades leading to the production of type 1 interferon (IFN-α e IFN-β) and other pro-inflammatory cytokines (Bernal-Bermúdez et al., 2023) that may have further affected the reproductive health state of the fish.

The PPi analysis confirmed that the immune system functioning was affected by the farming condition, along with several other biological categories, including secretion, signaling, receptor and ion transport. The KEGG analysis revealed a large number of dysregulated pathways in both hatchery-produced groups, with a greater number observed in NormalF, consistent with the higher number of dysregulated genes identified in this group. The wider dysregulation of genes and pathways observed in fish with apparently normal spermatogenesis confirms our previous hypothesis that these individuals were actually in an early phase of testicular dysfunction (Lavecchia et al., 2023), and suggests the existence of a temporal shift between the occurrence of gene dysregulation at the pituitary level and its effects on testis gene expression and spermatogenesis. Among the dysregulated pathways revealed by the KEGG analysis, PPAR, MAPK, MTOR, ERBb and GnRH signaling pathways are all involved in pituitary hormones synthesis and secretion. The PPAR has been reported to regulate gonadotrope cell proliferation and to be involved in Fshβ, Lhβ, and GnRH-R transcription (Takeda et al., 2007). In pituitary gonadotrope cells, MAPK signaling pathway is activated by GnRH through its receptor GnRH-R (Stamatiades and Kaiser, 2018). The MTOR pathway is involved in the IGF-1-mediated GH secretion (Di Pasquale et al., 2018). The ERBb signaling pathway is involved in GH, Lh and Fsh secretion through mechanisms involving MAPK/ERK and PI3K/Akt pathways (Cooper et al., 2011). The GnRH signaling pathway is the main mechanism that regulates, though the activation of the MAPK pathway, Fsh and Lh release (Stamatiades and Kaiser, 2018); however, in teleost

fish it has been recently demonstrated that GnRH stimulates mainly Lh release, whereas Fsh release is stimulated by cholecystokinin (Hollander-Cohen et al., 2024; Uju et al., 2025).

Among the many dysregulated genes in DysF, Neuron-derived neurotrophic factor and Ephrin RBD domain-containing protein are expressed along the GnRH neuron migratory route and may enhance GnRH neuron migration (Wierman et al., 2011; Messina et al., 2020). In mammals, Neuron-derived neurotrophic factor is implicated in hypogonadotropic hypogonadism, a congenital disease associated with infertility and characterized by delayed or absent puberty and low gonadotropin and sex steroid levels (Messina et al., 2020). The gene Proprotein convertase subtilisin/kexin type 2 (*pcsk2*) encodes a member of the subtilisin-like proprotein convertase family involved in the proteolytic activation of polypeptide hormones and neuropeptides precursors (https://www.genecards.org/cgi-bin/carddisp.pl?gene=PCSK2&keywords=pcsk2), and *Pcsk2*-null mice showed dysregulation of peptides that play a crucial role in the endocrine control, such as somatostatin and neuropeptide Y (Gagnon et al., 2011). Proenkephalin a, a strongly upregulated gene in dysfunctional fish, encodes a preproprotein proteolytically processed to generate multiple protein products, including the pentapeptide opioids Met-enkephalin and Leu-enkephalin, which bind to mu- and delta-opioid receptors to modulate the perception of pain (https://www.genecards.org/cgi-bin/carddisp.pl?gene=PENK). Enkephalins are known inhibitors of GnRH neurons (Roa, 2013) and their increase due to stress exposure is reported to block ovulation in mammals (Jin et al., 1988). In teleost fish, enkephalins play an inhibitory role in the tonic inhibitory control of the reproductive axis acting at both the hypothalamic and pituitary levels and modulating, through μ and δ opioid receptors, GnRH and gonadotropin release (Zohar et., 2010; Shinde et al., 2024). Gamma-aminobutyric acid (GABA) A receptor, alpha 1, which was downregulated in DysF, is involved in the neural circuits that control reproduction. GABA linking to its receptors has an inhibitory effect on neurotransmission in the central nervous system, promotes the release of GnRH and enhances the responsiveness of pituitary cells to GnRH (Di Giorgio et al., 2019; Trudeau et al., 2000). Growth hormone 1 and growth hormone releasing

hormone receptor b, play important roles in steroidogenesis and gametogenesis (Ponce et al., 2018; Tesarik et al., 2021) and disturbances of GH kinetics has been reported in women with polycystic ovary syndrome (Kaltsas et al., 1999), a mammalian disorder showing common molecular traits to the ovarian dysfunction described in female greater amberjack previously (Lavecchia et al., 2024). The downregulation of Lhβ in DysF may also have played a role in the spermatogenesis impairment, although reproductive dysfunction in hatchery-produced fish is generally associated with insufficient pituitary gonadotropin release rather than inhibition of gonadotropin synthesis (Zohar and Mylonas, 2001; Mylonas et al., 2010; Rosenfeld et al., 2012). Finally, dysregulation of Somatolactin alpha, a fish specific pituitary hormone strongly upregulated in both hatchery-produced groups, may have contributed to the reproductive dysfunction as this gene seems to be associated with sexual maturation (Peyon et al., 2013), and its plasma concentration has been found to increase at the time of oocyte maturation/spermiation and spawning in coho salmon *Oncorhynchus kisutch* (Kaneko and Hirano, 1993).

Among dysregulated genes in NormalF, the strongly downregulated Insulin-like growth factor 1 (IGF-1) plays a crucial role in the control of pituitary cells, modulating Lη, Fση, GH e PRL synthesis through intracellular signaling pathways such as PI3K–AKT–mTOR (Skarra and Thackray, 2015). In humans, low serum levels of IGF-1 have been associated with panhypopituitarism, a severe condition characterized by a complete or partial deficiency of pituitary hormones. This deficiency can result from various causes and may lead to adrenal insufficiency, hypothyroidism, hypogonadism and growth hormone deficiency (Castillo et al., 2019). Beta-arrestin, a protein encoded by a strongly downregulated gene in NormalF, attenuates G protein-mediated signaling by competing with the G protein for receptor interaction, leading to receptor desensitization, and acts also as a multifunctional signal transducer by serving as an adapter to connect the activated receptors with diverse signaling pathways within the cell (Yang et al., 2022). Numerous receptor systems use this mechanism for G protein-independent signaling, including opioid, cannabinoid, apelin, GH secretagogue, and D2-like dopamine receptors (Yang et

al., 2022). In particular, beta-arrestin is essential for turning off dopaminergic signaling and redirecting it toward secondary signaling pathways (Del'guidice et al., 2011). NMDA receptor synaptonuclear signaling and neuronal migration (Kramer and Wray, 2000), and Hedgehog protein (Balordi and Fishell, 2007) are implicated in neuronal migration and nervous system development. In particular, the gene for NMDA receptor synaptonuclear signaling and neuronal migration factor a is implicated in the migration of GnRH neurons, and has been associated with hypogonadotropic hypogonadism (Quaynor et al., 2015). Hedgehog signaling pathway plays pivotal roles in embryonic development and its dysregulation during pituitary development results in malformation of the gland and affects the interaction between GnRH and pituitary (Bian et al., 2023). The dysregulation of acetylcholine receptors, such as Muscarinic acetylcholine receptor and Cholinergic receptor, nicotinic, beta 5b may have played a role in the reproductive dysfunction. In fact, acetylcholine has also been established as an autocrine and a paracrine factor in the pituitary gland and may be implicated in the inhibition of the activity of pituitary gonadotrophs (Zemkova et al., 2013).

The present study suggests the existence of a complex determinism underlying the spermatogenesis of hatchery produced F1 greater amberjack. A wide pituitary gene dysregulation occurred in individuals that still were undergoing normal spermatogenesis and preceded the onset of the histologically-evident spermatogenesis impairment. The mechanism of the reproductive dysfunction involves a multiplicity of genes involved in the control of pituitary activity, and includes a general inhibition of pituitary activities through the dysregulation of opioid, endocannabinoid and dopamine systems, all of them acting through metabotropic G-coupled receptors (Turu and Hunyady, 2010; Beaulieu et al., 2015; Che and Roth, 2023). In reproductively dysfunctional greater amberjack, the alteration of these three systems might have caused a general depression of pituitary activity, a condition associated with severe endocrine diseases reported in mammals, such as panhypopituitarism (Carrière et al., 2004) and hypogonadotropic hypogonadism (Kulvinder et al., 2016). The latter pathology has been correlated with ontogenetic anomalies

caused by dysregulation of genes involved in neuronal cell migration, including GnRH neurons (Kulvinder et al., 2016). In men affected by hypogonadotropic hypogonadism, GnRH administration may restore normal levels of pituitary and gonadal hormones, allowing for testicular growth and spermatogenesis (Martin et al., 1990).

The present data also suggest that co-administration of GnRHa with dopamine antagonists, such as pimozide, shown to be effective in inducing spawning in hatchery-produced fish exhibiting dopaminergic inhibition (Zohar and Mylonas, 2001), could be tested to induce maturation and spermiation in hatchery produced greater amberjack. Moreover, the effectiveness of mu opiod receptor antagonists such as naloxone or naltrexone, reported to attenuate stress-induced suppression of Lh secretion in mammals (Retana-Márquez et al., 2009) and fish (Ganesh and Chabbi, 2013), might be explored. Finally, the present study raises the possibility that a dysregulation of genes involved in neuronal development and GnRH neuron migration might have resulted in a congenital and irreversible pituitary insufficiency, although this would necessitate further studies on the ontogenesis of the nervous and endocrine systems of hatchery-produced greater amberjack.

In conclusion, the present study described the wide pituitary gene dysregulation occurring in greater amberjack hatchery produced individuals in the Mediterranean, in an effort of expanding the aquaculture production thorough domestication of new fish species. The observed dysregulation resulted in the alteration of many biological processes and pathways and showed molecular traits common to the hypogonadotropic hypogonadism described in mammals. As alternative to expensive therapies based on recombinant Fsh and Lh, the use of dopamine or mu opioid receptor antagonists in co-administration with GnRHa might be explored to alleviate the effects of the dysregulation of the opioid, endocannabinoid and dopamine systems.

CRediT authorship contribution statement

Anna Lavecchia: Formal analysis, Investigation, Data curation, Visualization, Writing - Original Draft. **Caterina De Virgilio:** Formal analysis, Investigation, Data curation, Writing - Original Draft. **Caterina Manzari:** Investigation, Data curation, Writing - Original Draft. **Lo Giudice Claudio:** Investigation, Data curation. **Chrysovalentinos Pousis:** Investigation, Data curation, Writing - Original Draft. **Rosa Zupa:** Investigation, Visualization, Writing - Original Draft. **Constantinos C. Mylonas:** Conceptualization, Funding acquisition, Writing - Original Draft. **Ernesto Picardi:** Methodology, Data curation. **Gianluca Ventriglia:** Investigation, Visualization, Writing - Original Draft. **Graziano Pesole:** Conceptualization, Funding acquisition, Resources, Writing - Review & Editing. **Aldo Corriero:** Conceptualization, Funding acquisition, Resources, Writing - Original Draft.

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Declaration of Competing Interest

All the authors declare no competing interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version.

Data availability

Reads generated in this study are freely available through the SRA (Short Read Archive) database under the BioProject accession number PRJNA1055966. All the other data produced and/or analyzed during the current study are included in this article and in Supplementary Tables.

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Table 1 Sampling date, origin, biometric data, gonadosomatic index (GSI), reproductive state and designated group of male greater amberjack.

Sampling date	Fish origin	Fork length (cm)	Fish ID	Body mass (kg)	Gonad mass (g)	GSI	Reproductive state*	Group**
31/05/2021	Wild	95	GNW3	9.3	50	0.5	Advanced spermatogenesis	WILD
31/05/2021	Wild	101	GNW5	13.0	300	2.3	Advanced spermatogenesis	WILD
31/05/2021	Wild	92	GNW6	9.2	100	1.1	Advanced spermatogenesis	WILD
31/05/2021	Wild	93	GNW7	8.8	60	0.7	Advanced spermatogenesis	WILD
01/06/2021	Hatchery-produced	81	GNC21	7.9	54	0.7	Advanced spermatogenesis	NormalF
01/06/2021	Hatchery-produced	75	GNC29	6.1	86	1.4	Advanced spermatogenesis	NormalF
01/06/2021	Hatchery-produced	73	GNC30	8.2	71	0.9	Advanced spermatogenesis	NormalF
01/06/2021	Hatchery-produced	80	GNC31	7.7	69	0.9	Advanced spermatogenesis	NormalF
01/06/2021	Hatchery-produced	76	GNC22	6.8	27	0.4	Arrested spermatogenesis (spent)	DysF
01/06/2021	Hatchery-produced	91	GNC24	12.4	49	0.4	Arrested spermatogenesis (spent)	DysF

GSI, gonado-somatic index. *The reproductive state was assessed as described in the Material and Methods section. **For comparative transcriptome analysis, fish were grouped according to their origin and reproductive state as described in the Results section. DysF, reproductively dysfunctional farmed fish; NornalF, non-dysfunctional farmed fish; WILD, wild fish with normal spermatogenic activity.

Table 2. Number of upregulated and downregulated genes in the three groups of greater amberjack males.

	TOTAL GENES	DEGs	UP	DOWN
DysF vs. WILD	18,086	301	141	160
NormalF vs. WILD	18,168	376	163	213
DysF vs. NormalF	17,287	-	-	-

Table 3. Gene ontology enrichment analysis of DEGs from pituitaries of wild and hatchery-produced greater amberjack males.

	Category	Term	Count	FDR
DysF vs. WILD	KEGG_PATHWAY	sdu00230:Purine metabolism	8	2.87E-02
	KEGG_PATHWAY	sdu01232:Nucleotide metabolism	6	4.87E-02
NormalF vs. WILD	UP_KW_BIOLOGICAL_PROCESS	KW-0051~Antiviral defense	3	4.67E-02

Table 4. PPi interaction network biological categories DysF vs. WILD

# Genes	Category	Description	FDR value
76	UniProt Keywords	Signal	4.47E-11
52	GO Cellular Component	Extracellular region	2.68E-06
37	UniProt Keywords	Disulfide bond	4.23E-05
19	GO Molecular Function	Signaling receptor regulator activity	0.0016
11	KEGG Pathways	Cytokine-cytokine receptor interaction	0.0031
14	TISSUES	Cardiovascular system	0.0161
11	UniProt Keywords	Secreted	0.0287
5	TISSUES	Right atrium	0.0322
4	UniProt Keywords	Glycolysis	0.0346
13	UniProt Keywords	Ion transport	0.0429
4	KEGG Pathways	Intestinal immune network for IgA production	0.0476

Table 5. PPi interaction network biological categories NormalF vs. Wild

# Genes	Category	Description	FDR value
11	STRING Clusters	Mixed, incl. Negative regulation of viral genome replication, and Receptor-transporting protein	9.19E-10
84	UniProt Keywords	Signal	1.55E-09
54	GO CellularComponent	Extracellular region	0.00051
14	UniProt Keywords	Secreted	0.0048
36	UniProt Keywords	Disulfide bond	0.008
108	GO Biological Process	Multicellular organismal process	0.0197
9	GO Biological Process	Axoneme assembly	0.0197
10	GO Biological Process	Defense response to virus	0.0197
45	GO Cellular Component	Plasma membrane bounded cell projection	0.0228
11	Reactome Pathways	Interferon Signaling	0.0498

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Table 5. PPi interaction network biological categories NormalF vs. Wild

Figure capitons

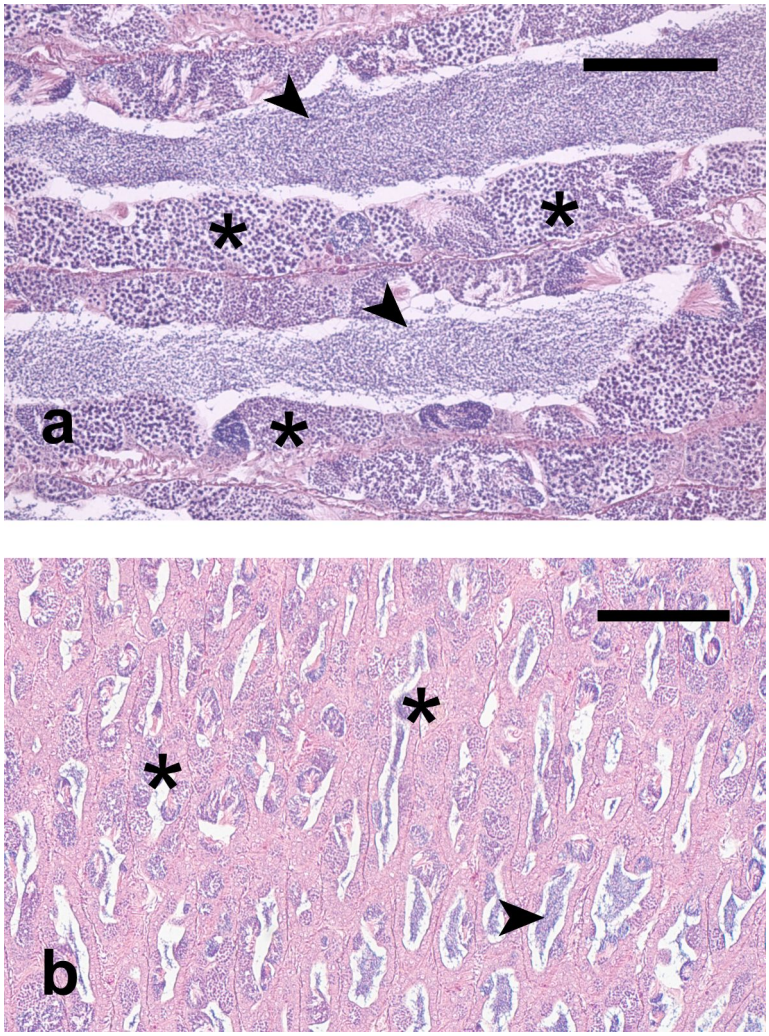

Fig. 1. Micrographs of histological section from greater amberjack testes. a) Hatchery-produced specimen in active spermatogenesis with histological appearance similar to wild fish (NormalF group). b) Hatchery-produced specimens showing arrested spermatogenesis (DysF group), with small seminiferous tubules together with residual spermatocysts and luminal spermatozoa. Arrowhead indicates luminal spermatozoa; asterisk indicates spermatocysts. Hematoxylin-eosin staining. Bars = 100 μm in (a), 200 μm in (b). Modified from Lavecchia et al. (2023).

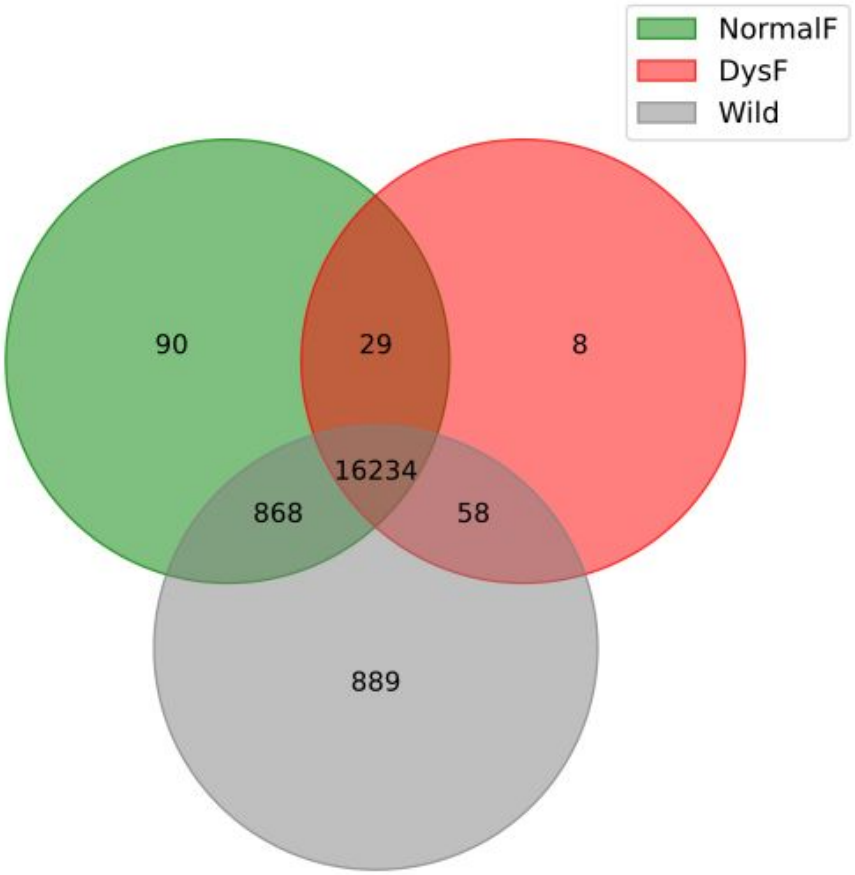

Fig. 2. VENN diagram of shared and unique genes related to pituitary samples of wild (WILD) and hatchery-produced greater amberjack, dysfunctional (DysF) and non-dysfunctional (NormalF).

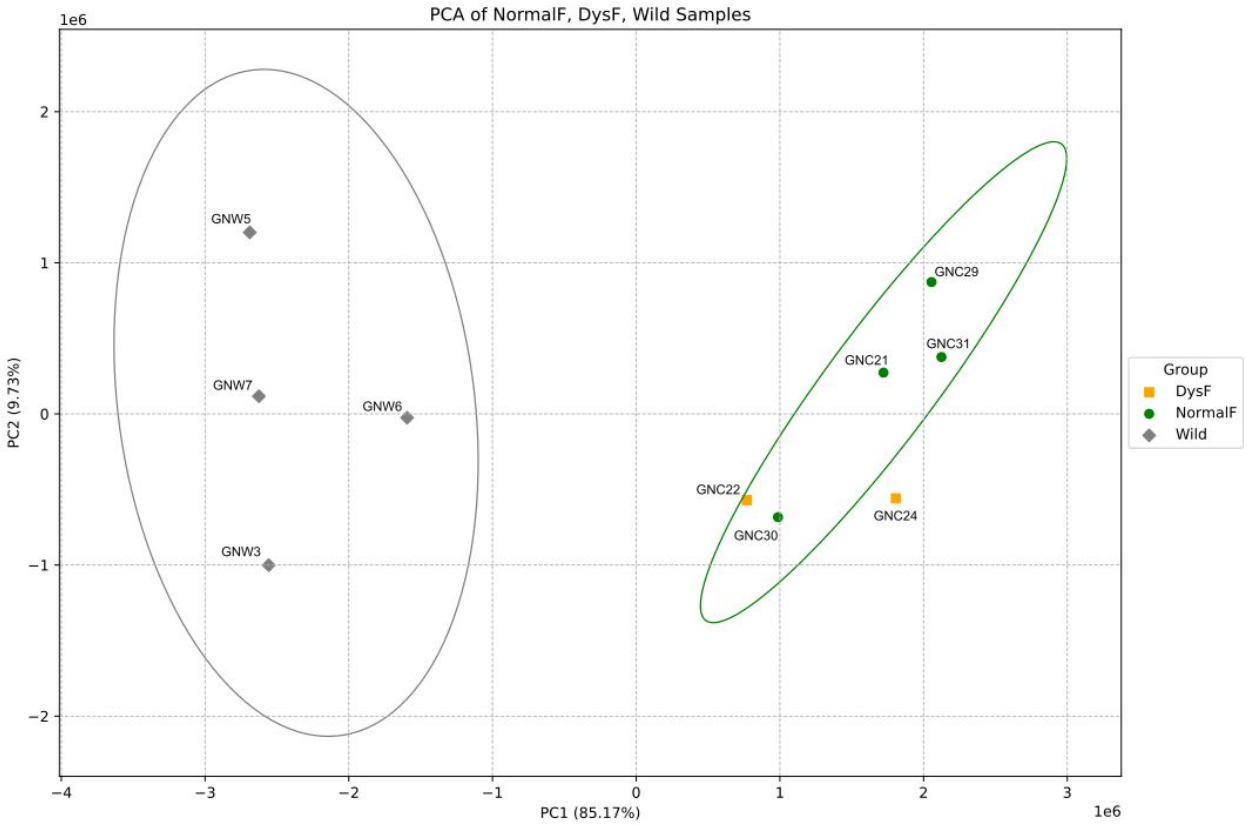

Fig. 3. Principal component analysis (PCA) of shared and unique genes related to pituitary samples of wild (WILD) and hatchery-produced greater amberjack, dysfunctional (DysF) and non-dysfunctional (NormalF).

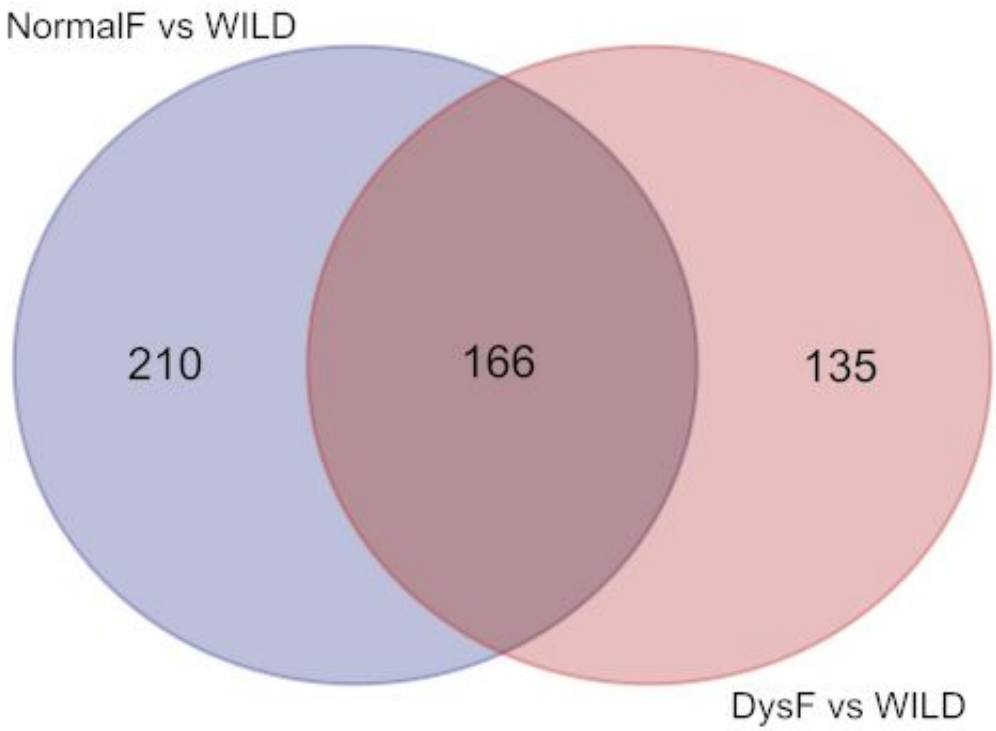

Fig. 4. VENN diagram showing DEGs unique and shared among the two comparisons of pituitaries from male greater amberjack.

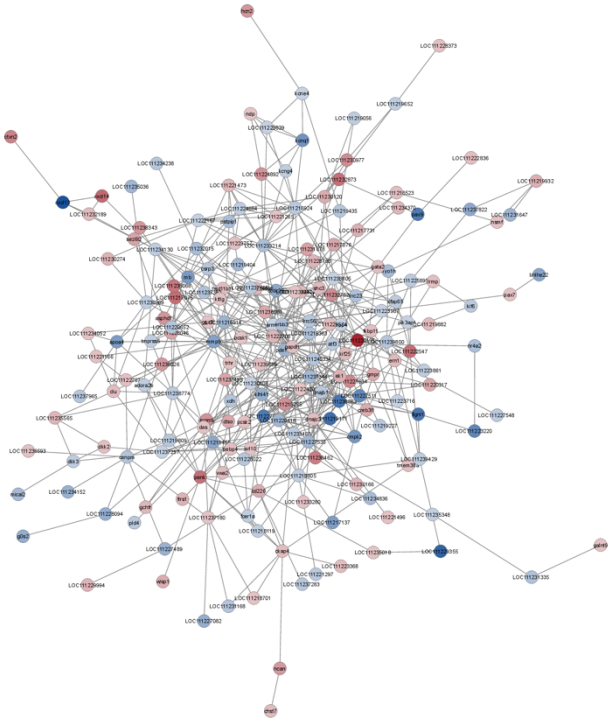

Fig. 5. Protein-Protein interaction (PPi) networks in pituitaries from DysF vs. WILD male greater amberjack. Networks were built using a confidence protein interaction (score = 0.2). Node background indicates gene upregulation (red, log2FC >1.5) or downregulation (blue, log2FC <-1.5).

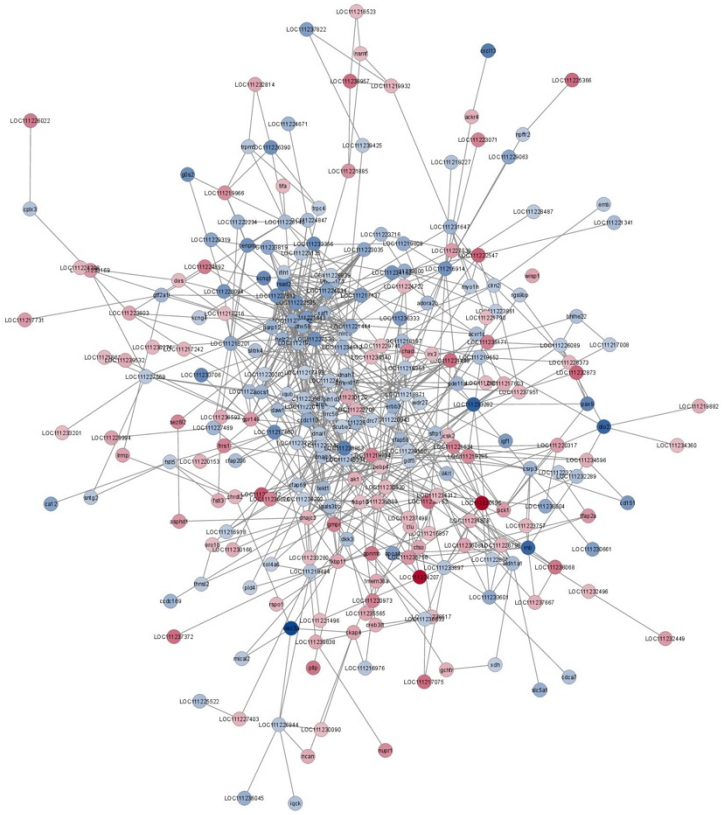

Fig. 6. Protein-Protein interaction (PPi) networks in pituitaries from NormalF vs. WILD male greater amberjack. Networks were built using a confidence protein interaction (score = 0.2). Node background indicates gene upregulation (red, log2FC >1.5) or downregulation (blue, log2FC <-1.5).