Research Insight Open Access

Molecular Basis and Regulatory Network of Sex Determination in Groupers

Liting Wang, Manman Li
Hainan Institute of Biotechnology, Haikou, 570206, Hainan, China
Corresponding authors: manman.li@hitar.org

International Journal of Aquaculture, 2025, Vol.15, No.4 doi: 10.5376/ija.2025.15.0018
Received: 01 Jun., 2025
Accepted: 15 Jul., 2025
Published: 02 Aug., 2025

Preferred citation for this article:
Wang L.T., and Li M.M., 2025, Molecular basis and regulatory network of sex determination in groupers, International Journal of Aquaculture, 15(4): 184-196 (doi: 10.5376/ija.2025.15.0018)

Abstract Grouper is a category of important value among marine economic fish. Due to its unique reproductive characteristics of hermaphrodite and female premature reproductive characteristics, gender can undergo two-way conversion in its life cycle. This phenomenon makes grouper an ideal model for studying the mechanisms of gender determination and differentiation of fish, but also poses challenges to aquaculture breeding. This study reviews the molecular basis and overall regulatory network of grouper gender determination mechanism, including sexual conversion in gender type and life cycle, reproductive gonadal histological characteristics, endocrine regulation of gender differentiation, identification and expression patterns of key gender-related genes, the role of non-coding RNA and epigenetics in gender regulation, the mechanism of action of sex hormones and their signaling pathways, the impact of external environmental factors on gender determination, and the method of constructing a gender regulatory network model in combination with multiomics data. We focused on the functions and interactions of genes that play a key role in the transition process of male and female (such as dmrt1 in male pathway and *Foxl2* in female maintenance, etc.), and explained the role of epigenetic regulators such as miRNA and long-chain non-coding RNA in gender plasticity, as well as the role of pituitary-godal axis hormone signal in inducing gender reversal. Research on grouper gender determination not only helps to deeply understand the plasticity mechanism of gender differentiation in vertebrates, but also provides theoretical basis and practical guidance for controlling gender through molecular means in aquaculture and improving production efficiency.

Keywords Grouper; Gender differentiation; Non-coding RNA; Epigenetics; Sex hormones

1 Introduction

Grouper (*Epinephelus*) is a warm-water reef fish widely distributed in tropical and subtropical waters of the Indian Ocean and the Pacific Ocean. It is an important marine aquaculture economic fish in China and Southeast Asia. Grouper has delicious meat, high nutritional value, strong market demand, and has huge economic value and development prospects. At the same time, there are many types of grouper, and different species have their own characteristics in body color patterns and growth rates. They are important resources for the marine fishery and ornamental aquarium industries (Qu et al., 2021).

Most grouper belong to the hermaphrodite female premature type, that is, the individual first develops and matures into functional females during puberty, and then a gender reversal of transition from female to male can occur later in the life cycle (Shi et al., 2010). In natural groups, juvenile and subadult individuals are mostly females, and only a few individuals can develop into males, and are often induced by the dominant females in the population to become males by social factors when they lose their original males. Grouper gender conversion is usually irreversible and does not redirect back to females once an individual turns from female to male (Evliyaoğlu et al., 2019). However, under artificial induction conditions, there have also been studies that have reported cases of male individuals re-expressing female traits after special treatment. Grouper's unique two-way gender conversion mechanism provides valuable materials for scientific research, but also complicates its breeding and breeding management: the proportion of male individuals in breeding groups is low and the structure of the breeding group is unstable, which brings difficulties to artificial breeding and strain improvement.

Among vertebrates, the gender determination and differentiation mechanisms of fish are the most diverse and plastic. Unlike higher vertebrates, fish are often significantly affected by environmental factors such as temperature, light, and density, in addition to genetic factors. Studying the gender determination mechanism of fish (especially species with gender-transforming ability) is not only of great theoretical significance for understanding the evolution and plasticity of vertebrate gender development, but also has direct application value for aquaculture practice. In recent years, with the development of high-throughput sequencing and omics technology, many progress has been made in the research on grouper gender decisions. Based on reviewing the latest research progress in various aspects, this study attempts to propose a molecular network model for grouper gender regulation and discusses the current main challenges and future research directions.

2 Biological Basis of Grouper Gender Decision and Differentiation

2.1 Gender type and gender transition in life cycle of grouper

The gender types of grouper are mainly hermaphrodites, and most species are typical female premature type and are heteromature. In natural populations, a dominant male usually leads a certain number of females to form a breeding group. If male fish in the group are missing for reasons such as death or removal, females with the highest rank (largest size or highest social status) in the group will be induced to initiate gender reversal and transform into new males within weeks to months (Palma et al., 2019). Experimental observations of oblique grouper showed that in a group of 1 male and 3 females, the dominant female fish can turn from female to male after only about one month. This mechanism of gender change caused by social factors is believed to be closely related to group stability and reproductive success. In addition, there are also differences between "primary male" and "secondary male" in grouper: the so-called primary male refers to individuals who develop directly into males at the time of not going through the female stage (the proportion in grouper is very low), while secondary males are males that are transformed from mature females again.

2.2 Gonadal structure and histological characteristics

The histological characteristics of grouper during gender differentiation and conversion are very typical. Groupers during the juvenile period have undifferentiated primitive dual potential glands that can develop into ovaries or sperm. Under normal circumstances, its gonads first differentiate into the ovary, which is histologically manifested as ovarian cavity formation, oocyte proliferation and growth. When an individual matures into a functional female, ovarian tissue predominates, containing oocytes and helper cells at different developmental stages. It is worth noting that in female mature ovaries, a small number of scattered spermatogonia or primary spermatogonia can often be observed to exist in ovarian tissues, which is more common in protogynous fish such as grouper. These potential male cells do not develop into sedentary structures at the female stage, but proliferate and differentiate rapidly at the onset of sexual reversal. During the process of grouper turning from female to male, the gonad undergoes obvious tissue remodeling: oocytes gradually degenerate and absorb, the ovarian cavity is closed, the semen tubular structure begins to appear and develop and mature, and spermatogonia proliferates and forms semen tissue (Wang et al., 2017; Wu et al., 2017). Transitional gonads (also known as intergonadal) are the intermediate stages of grouper's gender conversion. The microstructure is often manifested as having both ovarian and sperm components - both residual oocytes and developing sperm cells can be seen in the same gonad.

2.3 Overview of hormone regulation during gender differentiation

Sex steroid hormones play a key role in the gender differentiation of grouper. Similar to many fish, the rise and fall of estrogen and androgen levels directly affect the direction of differentiation of the gonads. During female differentiation, high expression of aromatase (Cyp19a1a) in ovarian tissue converts androgens such as testosterone into estrogen, which increases the level of estradiol, thereby promoting the undifferentiated gonads to ovarian development. Experiments have shown that administration of aromatase inhibitors at critical periods of grouper gender decisions will lead to hindered differentiation of female fish and tend to develop towards male direction (Wang et al., 2018), demonstrating that estrogen signaling is indispensable for ovarian differentiation. In addition, gonadotropins also play an upstream regulatory function in gender differentiation. The follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secreted by the pituitary gland regulate the synthesis and secretion of endogenous hormones in the gonads through receptors (FSHR and LHR) acting on the surface of the gonads, thereby affecting the development direction of gonads. During the female-to-male transition from grouper to male, the study observed that the gene expression of pituitary FSH and gonadal FSHR was gradually increased; experimental injection of recombinant FSH protein can successfully induce gender reversal of mature female fish. This finding suggests that the gonadotropin-sex hormone axis is one of the key endocrine pathways in grouper gender conversion.

3 Gender Determines Key Genes and Their Expression Patterns

3.1 Identification and expression of key genes related to male and female

3.1.1 The role of dmrt family genes in male decisions

The *Dmrt1* gene (Doublesex and Mab-3 Related Transcription factor 1) is widely regarded as one of the core decision makers in the gender pathway of vertebrate males, serving as a “testicle determinant” or semen maintenance factor in many species (Augstenová and Ma, 2024). Fish such as grouper do not have the *Sry* gene on the Y chromosome like mammals, but the high expression of *dmrt1* in most fish males suggests its important position in gender decisions. For example, in Nile tilapia, *dmrt1* is one of the most expressed genes in the testicles during the gender differentiation stage; gene editing causes *Dmrt1* function to be depleted, which will lead to severe testicular dysplasia in XY males, degeneration and disappearance of spermatogonia in the semen, support cells mistakenly transform into follicle cells, and abnormal activation of female pathway genes such as *Foxl2* and *cyp19a1a*. Recent grouper transcriptome analysis further confirmed that dmrt1 was significantly upregulated early in the process of gender reversal, consistent with androgen-induced testicular development. In addition to *Dmrt1*, other members of the Dmrt gene family such as *dmrt2* and *dmrt3* have also been reported to be involved in gonadal development in different species, but their effects are mostly indirect or auxiliary (Zhang et al., 2020). Several DMRT family genes have also been identified in the grouper genome, but their functions need further research.

3.1.2 The relationship between *Foxl2* and female maintenance

Foxl2 is one of the important transcription factors that maintain ovarian fate and oocyte survival. In mammals, the sustained expression of *Foxl2* is essential for inhibiting male genes and maintaining the ovarian phenotype; in fish, *Foxl2* is also considered a marker gene for the female pathway (Fan et al., 2019). In most female individuals such as grouper, *Foxl2* is expressed at high levels in ovarian tissue, while in male sprites, which are extremely low in male sprites. *Foxl2* mainly enhances estrogen synthesis by promoting the transcription of the aromatase cyp19a1a, thereby supporting ovarian development. During the gender reversal of grouper, the expression dynamics of *Foxl2* also echo the process of the transition from the ovary to the sperm: *Foxl2* was rapidly downregulated in the early stage of sexual reversal, while male factors such as dmrt1 and amh were upregulated, marking that the "switch" of molecular regulation was switched from female mode to male mode (Lyu et al., 2019). It is worth noting that in recent years, an ancient paralogous gene of *Foxl2, foxl3*, has also been found in fish. Its function is both similar and antagonistic to *Foxl2*. In orange-spot grouper, *Foxl3* was shown to directly inhibit the expression of cyp19a1a, thereby promoting schizophrenia differentiation; while *Foxl2* promoted cyp19a1a to maintain the ovary.

3.1.3 Synergistic mechanism of bidirectional regulation of genes

In the molecular network of grouper gender determination, some key factors do not promote a certain gender in one direction, but work together in an antagonistic pairwise manner to form a steady-state "bistable" switch. This mechanism is widely present in vertebrate gender decisions. For example, *Dmrt1* and *Foxl2* form a mutual inhibitory network between the male pathway and the female pathway: *Dmrt1* not only activates male genes in the testicles, but also prevents the activation of ovarian genes by inhibiting the expression of *Foxl2*; accordingly, in addition to upregulating estrogen synthetase in the ovary, *Foxl2* also inhibits the expression of male-related genes (including *Dmrt1*) and prevents the initiation of male programs. This mutual suppression of each other's "competition" ensures that the gonads differentiate in a single direction. Once a side has the advantage, it will further consolidate itself and inhibit the other party through positive feedback, thereby maintaining the gender stability of the gonads. However, in hermaphrodites such as grouper, the equilibrium can be broken under certain conditions, causing the otherwise stable ovarian state to turn to the genital state. This often involves the interference of external signals on the "two-way regulatory gene" network. For example, when environmental or endocrine changes cause the Foxl2/cyp19a1a pathway to weaken, the dmrt1/sox9 pathway may have relatively upper hand, thereby driving gonads towards male development. In addition to the Dmrt1-Foxl2 pair, there are other similar antagonist groups in fish gender decision-making. For example, the Wnt4/β-catenin signaling pathway tends to support ovarian development, while Amh/Amhr2 signaling promotes schizophrenia differentiation (Figure 1) (Wu et al., 2017); the Cyp19a1a product estradiol maintains ovarian structural integrity, while androgen (11-KT) drives schizophrenia development (Peng et al., 2020). These factors form key nodes in the gender regulatory network through complex regulatory relationships.

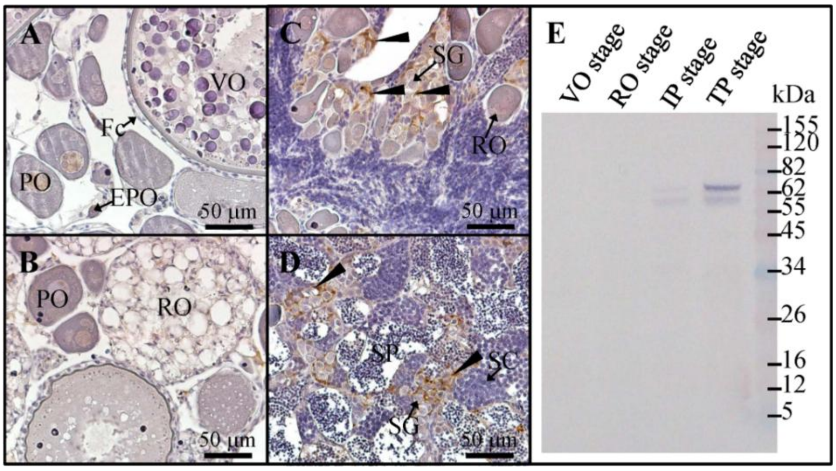

Figure 1 Location of Amh in the testes (Adopted from Wu et al., 2017)

Image caption: (A) Western blotting of grouper testicular and ovarian protein extracts using anti-Amh antiserum and anti-Actin antiserum. The black arrowhead denotes Amh protein. (B and C) Immunohistochemical staining of testes using anti-Amh antiserum. Black arrowheads indicate the positive signal (brown color) of anti-Amh antiserum. (D and E) Immunofluorescent staining of testes using anti-Amh antiserum and anti-Vasa antiserum. Vasa is a germ cell marker. White arrowheads indicate a positive signal (green color) of anti-Amh antiserum. OT, ovarian tissue; RO, regressed oocyte, SC, spermatocyte; SG, spermatogonia, SP, sperm; TT, testicular tissue (Adopted from Wu et al., 2017)

3.2 Differential gene groups revealed by expression profiling analysis

With the help of high-throughput sequencing technology, the analysis of the transcriptomes of grouper's different gender and sexual transition stages can reveal a large number of gender-related differentially expressed gene populations. These genes include transcription factors, hormone synthetases, receptors, signaling pathway molecules, and metabolic and structural proteins (Li, 2024). By comparing the gene expression profiles of female, primary male and secondary male, the researchers found that a considerable portion of gene expression showed significant differences between male and female. Genes related to ovarian function such as *cyp19a1a, Foxl2, bmp15, figla*, etc. are highly expressed in female samples, but are low and even non-expressed in male samples (Peng et al., 2020). Some cell proliferation and differentiation factors also show dynamic changes during sexual reversal. For example, the spatiotemporal expression changes of genes such as *Wnt4, igf1, FST* (ovarian-related) and *cxcl12*, *gsdf* (spermally related) reflect the process of ovarian tissue regression and sperm occurrence. In addition to conventional protein-encoding genes, transcriptome analysis also reveals the potential role of many non-coding RNAs in gender differences: dozens of microRNAs and long-chain non-coding RNAs are significantly differentially expressed between male and female gonads, which may be involved in gender fate decisions by influencing post-transcriptional regulation of key genes. The gender transition process may vary slightly between different grouper individuals, so screening of different genes requires stricter criteria and sufficient repetition. Through time series transcriptome data, a series of gender-related genes can be sorted in order of expression peaks, and their order and causal relationship in gender reversal can be inferred. Studies have conducted multi-stage sampling and sequencing of grouper induced by artificial androgen treatment and found that first responded to some stress and metabolic genes, followed by reproductive axis-related genes (upregulation, ultimately, the gonad direct target gene is expressed in large quantities, thus delineating a top-down timeline of molecular events (Han et al., 2018).

3.3 The correlation between spatiotemporal expression patterns and gender transformation stages

Different spatiotemporal expression patterns of gender-related genes are of great significance to understanding the stages of grouper gender transition. The expression of many key genes in different stages of development and in different tissues is strictly regulated. In the embryonic/littlefish stage, some gender-related genes may show male-female expression differences early, indicating the initial differentiation direction of the gonad; while others only initiate expression and regulate gonadal function when the gonad develops rapidly during puberty. In grouper, since their gender can be changed acquired, it is necessary to focus not only on gene expression in the early stage of gender differentiation (the male-female-determining phase), but also on reprogramming when sexual reversal occurs after maturation. Dmrt1 and cyp19a1a can be regarded as "opposite indicators" of gonad fate: in the mature ovaries of female fish, cyp19a1a is highly expressed while DMrt1 is nearly silent; while in the male fish's semen, DMrt1 maintains a high level while cyp19a1a is suppressed for a long time. At the initiation of sexual reversal, the expression of both increases and decreases: the significantly increased DNA methylation level in the promoter region of the aromatase gene leads to a decrease in its transcription, while the de-repression of the DMRT1 promoter achieves a large amount of transcription. This suggests that the molecular switches of grouper females to males are directly related to the transition of these key gene expression states (Figure 2) (Wang et al., 2018).

For example, *Foxl2* and *amh* genes: *Foxl2* is continuously expressed in female ovaries to maintain follicles, while *amh* is mainly expressed by male sperm support cells to inhibit excessive primitive germ cells; during sexual reversal, *Foxl2* is gradually downregulated and *amh* begins to appear in the newly formed sperm tissue. The expression changes of these genes correspond to the histological process of follicle reduction and spermatogenesis (Lyu et al., 2019). In addition, hormone receptors and signaling pathway components also show phase-specific expression patterns. The gonadotropin receptor fshr is relatively low in the ovaries in the pre-sexual reversal stage, but it is enhanced in residual follicles and newly formed semen tissue at the beginning of the reversal, suggesting that FSH signal is involved in the trigger of reversal; recent brain and pituitary transcriptome analysis of oblique band grouper under natural social reversal conditions showed that after determining a new social status (preparing for sexual transition), the clock genes and melatonin synthesis-related genes in the brain were rapidly upregulated after the dominant female fish were determined to have a new social status (preparing for sexual transition). This may mean that circadian signaling and light sensing are involved in the initiation of socially induced gender-transforming behaviors (Wang et al., 2018). This spatiotemporal expression association of brain-godal axis genes provides clues for us to understand how environmental factors act on the gonad through the central role.

4 The Role of Non-Coding Rna and Epigenetic Mechanisms in Gender Regulation

4.1 Regulatory functions of miRNA and lncRNA in gender decisions

With the development of genomics, it has been recognized that a large number of non-coding RNAs (ncRNAs) play an important role in the regulation of gene expression, including during the process of gender determination and differentiation in fish. In the gonadal tissues of fish such as groupers, abundant microRNAs (miRNAs) and long-chain non-coding RNAs (lncRNAs) were detected, and their expressions often differ between male and female individuals, suggesting that they are involved in the regulation of the gender pathway. Some miRNAs have been found to have sex-biased expression: specifically high in male or female gonads, thereby targeting genes that regulate gender-related. Studies have shown that miR-26a-5p is upregulated during androgen-induced sexual transition, which can inhibit the expression of the aromatase cyp19a1a, leading to a decrease in estrogen synthesis, thereby promoting the maleization process. In a study on sexual reversal of turtles, dozens of miRNAs significantly related to gender differences were identified, among which miR-26a-5p, miR-212-5p, miR-202-5p, etc. play a role in the sexual reversal process and are predicted to target multiple gender-related genes (Zhang et al., 2021). Together with the target genes, these miRNAs form a network that regulates gonad development. Long-chain non-coding RNA (lncRNA) affects gene expression through various mechanisms: some lncRNAs can act as "sponges" of miRNAs, binding and isolating miRNAs to relieve their inhibition of target genes; others can directly bind to transcription factors or chromatin modification complexes to regulate gene transcription. In the study of fish gender differentiation, lncRNA is an emerging field but has shown importance. Reports show that in the gonads of species such as carp and raw fish, many lncRNAs have co-expression patterns with adjacent gender-related genes, suggesting that they may participate in regulation in concert (Yuan et al., 2019).

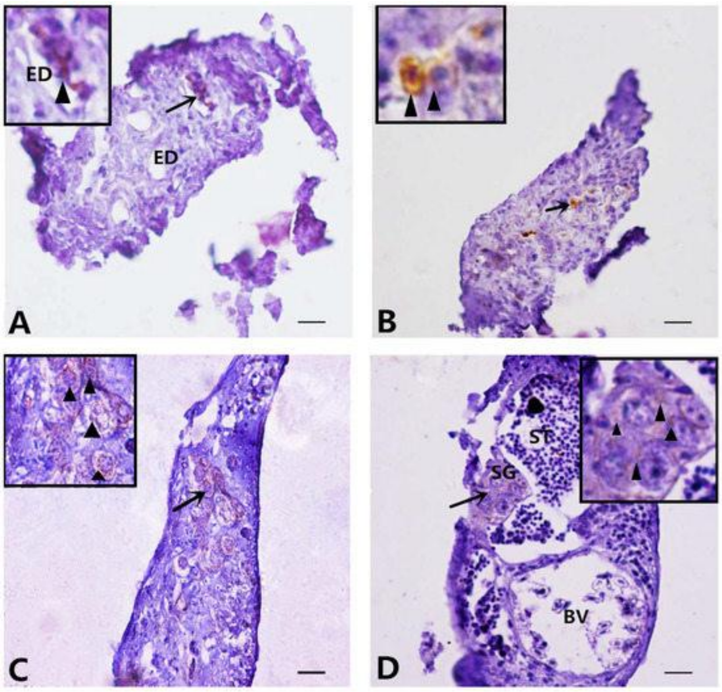

Figure 2 Tracing of Dmrt1-expressing cells during MT-feeding-induced sex reversal. The black arrows show the high magnification of the picture. Scale bars, 25 μm. Dmrt1 is detected in goniae cells around the efferent dust (black arrowheads) of MT-feeding fish at 12 dat (A). Dmrt1 is detected in a few of spermatogonia scattered in the gonads (black arrowheads) at 24 dat (B), and then they appear in gathered spermatogonia (black arrowheads) at 36 dat and 96 dat (C, D) of the MT-feeding fish. ED: efferent dust; BV: blood vessel; SG: spermatogonia; ST: spermatid (Adopted from Wang et al., 2018)

4.2 Dynamic changes in DNA methylation and histone modification in gender transition

Epigenetic regulation is another key aspect of gender determination and transition mechanisms. DNA methylation is one of the most common epigenetic markers, and can enable or silence gene expression for a long time by affecting the methylation state of the promoter region of the gene. In fish gender studies, DNA methylation is often negatively correlated with the expression of gender-related genes. In grouper, the latest study analyzed the methylation status of the aromatase cyp19a1a promoter region of female fish and sexually reversing male fish, and found that the cyp19a1a promoter in female fish ovaries had a low methylation level (large number of CpG sites were not methylated), while in the semen tissue was highly methylated (Peng et al., 2020). As females switch to males, the methylation degree of cyp19a1a promoter gradually increases, consistent with its reduced gene expression. Especially during the sexual reversal induced by artificial MT, after removal of MT, a partial reversal of male cyp19a1a promoter methylation status was restored and reactivated expression, causing certain signs of female reversal in gonad tissue. This result shows that DNA methylation not only participates in the reprogramming of gene expression during grouper gender reversal, but its changes may be reversible under certain conditions, thus conferring certain plasticity to the gender. In Japanese crucian carp, which induced gender reversal in temperature, studies have observed that the promoter region of genes related to schizophrenia is activated with the increase of histone H3K27 demethylation (activation marker), while the ovarian gene obtains H3K27 trimethylation (repression marker) and is silenced (Li et al., 2016). This suggests that during the gender transition of grouper, the histone modification status at key loci will also undergo corresponding changes.

4.3 Research on the association between epigenetic markers and gender plasticity

The high gender plasticity of hermaphrodite fish is based on the fact that their gene expression programs can be reset, and reprogramming of epigenetic markers is considered one of the main ways to achieve this. In mammals, gender is often irreversible once it is decided, which is partly because key genes and chromatin regions involving gender are locked by stable epigenetic modifications, forming a "oneway street". However, in fish such as grouper, there is still considerable reversibility after gender determination, and their ovaries can be transformed into sperm.

This means that its gene regulatory network remains responsive to environmental and endocrine signals in the adult stage, and its epigenetic state is not an unshakable endpoint, but more like a switchable reversible switch. Some scholars have suggested that "episogenetic sensitivity" may be one of the key factors in the ability of hermaphrodite fish to evolve gender conversion. Specifically, in the grouper genome, the epigenetic status of certain important sites associated with gender decisions may be more likely to be remodeled than in the general species (Nakamura et al., 2021). For example, endocrine changes caused by external factors (temperature, social stress, drugs) can trigger changes in DNA methylation or histone modification at these key sites, thereby reversing their expression status and thus reversing the gender function of the gonad (Soyano et al., 2022). New technologies in recent years, such as ATAC-seq, ChIP-seq, etc., can be used to analyze chromatin accessibility and histone marker distribution. If applied to sample comparison of grouper gonad differentiation/reversal, it will inevitably reveal the contribution of epigenetics to gender plasticity in a deeper way.

5 Regulatory Mechanism of Sex Hormones and Their Signaling Pathways

5.1 Estrogen and androgen synthesis and its metabolic genes (such as *cyp19a1a, hsd17b*)

The anabolic genes of sex steroids play a central role in gender regulation, and the aromatases *Cyp19a1a* (P450arom) and 17β-hydroxysteroid dehydrogenase (HSD17 family) are key enzymes responsible for estrogen synthesis and androgen conversion, respectively. cyp19a1a aromatizes androgens such as androgens such as androsene and testosterone into estradiol, and is therefore a gene necessary for ovarian differentiation and maintenance. During the gender determination of grouper, cyp19a1a is expressed at high levels in female gonads to ensure a steady stream of sufficient estrogen production, thereby promoting follicle development and inhibiting the misinitiation of male pathways (Cheng et al., 2020). Studies have confirmed that knocking out cyp19a1a (ovarian aromatase) in zebrafish and tilapia by gene editing will cause partial or even complete sexual reversal in XX females, further demonstrating their function in female differentiation. In grouper, there has been no reports of cyp19a1a knockdown, but evidence through transcriptome and epistemological regulation has shown that cyp19a1a downregulation is one of the early molecular events of sexual reversal. There are many subtypes of 17β-HSD in fish, among which 17β-HSD type 3 is responsible for converting androstenedione to testosterone in male testes; while 17β-HSD type 1 mainly converts estrone to estradiol in the ovary (Liu et al., 2017). Another related gene, *cyp11b* (11β-hydroxylase), catalyzes the conversion of testosterone to 11-ketotestosterone, which is the synthesis step of fish's main androgens. Studies on orange-spot grouper show that *cyp11b* is highly expressed in male semen and sexual reversal individuals, while almost non-expressed in female ovaries. This means that the expression of *cyp11b* can be used as one of the molecular markers of male tissues. In fact, in some early stages of sexual reversal (such as after artificial MT treatment), upregulation of *cyp11b* often precedes complete schizophrenia.

5.2 Effects of changes in hormone levels on gonadal remodeling

The most direct manifestation of grouper gender reversal is the rebalancing of sexual steroid hormone levels: estrogen falls from its peak, and androgen rises significantly. This transition of the endocrine environment plays a decisive role in the remodeling of gonadal tissue. During the process of female fish turning into male fish, the continuous decline in estradiol (E2) levels will cause follicle cells to lose support signals and a large number of oocytes enter atresia and apoptosis, thus making room for sperm hyperplasia (Wu et al., 2020). Meanwhile, androgens, especially 11-ketotestosterone (11-KT), are significantly elevated, which is the main hormone driver that promotes schizobulin formation and spermatogenesis. Some studies have quantified the changes in hormones in artificially induced sexual reversal experiments: after injecting a certain dose of cortisol intraperitoneal cavity, it was found that the serum 11-KT concentration increased significantly within a few weeks. At the same time, oocytes in ovarian tissue began to degenerate, spermatogonia appeared and developed in large quantities, and finally the gonads completed semenization (Soyano et al., 2022). If cortisol treatment is stopped at this time, the 11-KT level will gradually decrease, the developed semen tissue will stop maturing and partially degenerate, and some individuals may even restore part of the ovarian structure, indicating that the sexual reversal results may also be unstable when hormones are insufficient. This suggests that our gonadal remodeling requires sustained androgenic signals to consolidate. At the population level, sex hormone changes are also associated with gender ratio: environmental stress such as high temperature or high density can trigger an increase in cortisol in fish and indirectly lead to an increase in androgen, thus tending toward a higher male production ratio. In Japanese fish such as cuckoo, high temperature-induced male bias has been shown to be closely related to the increase in serum cortisol and 11-KT (Wang et al., 2020). For grouper farming, if you want more fish to turn males as soon as possible (for example, breed whole male groups for breeding), you can consider applying androgens or reducing estrogen levels within the appropriate developmental window to promote sexual reversal. However, the dosage and timing need to be grasped to avoid negative impact on fish growth. In addition to the gonads themselves, changes in hormone levels also have feedback on the central hormone axis: When androgens are elevated, the secretion of hypothalamic GnRH and pituitary gonadotropin may be inhibited to maintain internal homeostasis. This feedback regulation can sometimes affect the thoroughness of sexual reversals, such as excessive androgens may inhibit FSH through negative feedback, causing the gonadal support cells to not be stimulated sufficiently. Therefore, it is often necessary to balance the effects of various hormones during manual operations. If necessary, combined use of gonadotropins and sex steroids can be achieved successfully.

5.3 Functions of hormone receptors and downstream signaling pathways in gender regulation

Sex hormones work by binding to their receptors and triggering downstream gene expression, so the role of hormone receptors and related signaling pathway molecules cannot be ignored. Take estrogen receptor ER and androgen receptor AR as examples: ER includes ERα and ERβ, which are expressed in ovarian cells. After binding to E2, it can regulate a series of ovarian development and maintenance genes; AR is expressed in support cells and germ cells, and after binding to androgen, it can activate spermgenesis-related genes (Cheng et al., 2020). Studies have shown that in socially sexual conversion fish such as blue-headed parrot, high levels of cortisol in the brain of female fish may block sexual conversion by binding to glucocorticoid receptor GR, interfering with the path of estrogen receptor; while when dominant female fish lose community pressure and decrease cortisol, the estrogen receptor signal is relatively enhanced, and the motivation to convert to males is reduced. This suggests that GR-mediated stress signals and ER-mediated female maintenance signals affect gender stability or switching. During the maleization of grouper, GR may also be directly involved in inhibiting ovarian genes: Studies have speculated that the elevated cortisol-GR complex can bind to the glucocorticoid response element (GRE) of the cyp19a1a promoter to recruit inhibitory cofactors, thereby directly reducing the transcription of aromatase genes. This molecular mechanism provides clues to understanding how environmental stress (such as high temperature, high density) affects gender through endocrine pathways. In addition, FSH receptors (FSHR) and LH receptors (LHR) mediate the effects of gonadotropins. FSH mainly promotes follicle growth and estrogen synthesis, so FSHR is highly expressed on ovarian granules cells; while during sexual reversal, FSHR is also expressed in newly formed schizophrenia support cells, indicating that FSH may promote schizophrenia development through these receptors. Some experiments with FSH injections were able to induce female fish to turn male, further demonstrating the importance of FSH-FSHR signaling for reversal initiation. In pattern fish such as bluefish, if Amhr2 is dysfunctional, it will lead to abnormal germ cell number and disordered gonad development. For grouper, the AMH-Amhr2 pathway is also likely to play a role in inhibiting female structure and promoting semen formation-as in experiments with oblique band grouper, through overexpressing the amh gene in juveniles, the production of functional male individuals has been successfully induced (Wu et al., 2020). All these show that hormone receptors and downstream signals are indispensable parts of the gender regulatory network, and their mechanism of action often affects gene regulation.

6 The Impact of External Environmental Factors on Gender Decisions

6.1 The relationship between temperature, density, nutrition and gender bias

External environmental factors are important variables that affect the gender decision and sexual ratio of fish, and even play a decisive role in some species. For hermaphrodite fish such as grouper, environmental factors mainly affect the timing and rate of gender conversion, and the impact on initial gender differentiation is still unclear. But in many bony fish, temperature has been shown to significantly tilt the sex ratio of progeny, which provides a reference for understanding the gender plasticity of fish. One experiment showed that males accounted for about 63% of the offspring of tilapia bred at 27 °C, while males accounted for as high as 97.8% under high temperature treatment at 37 °C. Similarly, juvenile fish with zebrafish genotype of female can all develop into "pseudo-male" individuals after treatment at 37 °C. These high temperature-induced maleization effects are related to the increased cortisol levels in the body and the inhibition of aromatase activity. In addition to temperature, breeding density and social structure are also key factors. Groupers may delay sexual maturation and sexual transition under high-density feeding conditions, as crowded environments usually increase fish body stress levels (elevated cortisol) and alter their behavioral ratings, which may inhibit the formation of dominant individuals. Many hermaphrodite fish are more likely to undergo sexual reversal or earlier in solitary or low-density conditions. This is because social stimulus is reduced and individuals do not need to maintain female identity to integrate into the group structure, so they tend to be more male (Wu and Chang, 2017). The relationship between nutritional status and gender is also worth mentioning. Full nutrition and rapid growth often enables the fish to reach the physique required for sexual maturity, which may promote gender switching (in hermaphrodite fish, large individuals are usually able to play the role of males). Some studies have pointed out that sexual reversal will only occur when the weight of a red spot grouper reaches a certain threshold, otherwise small females tend to maintain females to preferentially increase their body shape. Therefore, in breeding practice, improving the nutrition of the bait and managing stocking density well, so that some individuals can grow rapidly, which is expected to shorten the time required for their sexual reversal, which is beneficial for breeding plans that require male fish to be used as seeds.

6.2 Experiment on induction of gender conversion by exogenous hormone treatment

Artificial application of exogenous hormones is a classic way to change the gender of fish, and has been tried on many species such as grouper. For hermaphrodite fish, exogenous hormones are mainly used to accelerate or synchronize their gender conversion process, so that they can change in the direction expected by humans. The most commonly used method is to feed or inject androgens to induce maleization of female fish. Literature reports that semen tissue can be observed in orange grouper less than one instar after MT feeding for more than 8 weeks, although sexual reversal is temporary at this time (requiring continuous hormone administration). Once the medication is stopped, the unstable semen structure may partially degenerate or even restore ovarian function (Figure 3) (Soyano et al., 2022). On the other hand, the application of estrogen (such as estradiol E2) can be used to inhibit maleization and maintain the female state. In commercial seedling cultivation, there have been attempts to prevent the juvenile fish from maleization by short-term soaking estrogen to obtain a whole female population. However, since grouper is usually female first and then male, the whole female group has little significance for production, but the whole male group has attracted more attention. In addition to sexual steroids, gonadotropins (such as human chorionic gonadotropins hCG, carp luteinizing hormone, etc.) are also used to try to trigger sexual reversal. For example, injection of large amounts of hCG in mature female fish can indirectly increase endogenous androgen levels, and as a result, partial reversal was observed on oblique band grouper (Peng et al., 2020). Furthermore, the application of corticosteroid hormones provides another idea. In practical applications, MT is still the most effective and commonly used inducer. To ensure food safety, hormonal feed is rarely used directly in commercial cultivation, but MT treatment is still a convenient means to establish a whole-male or whole-female experimental group in scientific research. It is also worth noting that different species and developmental stages respond to hormones differently, and the dose and treatment time need to be adjusted. Therefore, for grouper, it should be based on its own physiological optimization plan. Some studies compared the effects of MT oral injection and intramuscular injection on grouper, and found that oral injection is mild but takes longer, the injection starts quickly but the dose is not easy to control and has a risk of trauma.

6.3 Interaction between environmental factors and gene expression

Environmental factors affect gene expression through endocrine pathways, and changes in gene expression will in turn shape the phenotype. This interaction is particularly complex in the process of gender determination. Taking temperature as an example, high temperature not only directly affects the activity and gene expression of aromatase in the gonad, but also inhibits the expression of genes such as *Foxl2* and *cyp19a1a* through cortisol-mediated pathways, thus making gender differentiation biased towards males. In addition, high temperatures may alter the activity of DNA methylases and histone modifier enzymes in the gonad, resulting in changes in the apparent modification status of certain key genes (Wang et al., 2020). Looking at social factors, the changes in group structure are transmitted to the gonads through neuroendocrine signals. When a female fish becomes the dominant group, its brain undergoes neuroendocrine changes: cGnRH surges in the hypothalamus, and pineal AANAT (the enzyme that controls melatonin synthesis) activity is upregulated. Increased melatonin secretion is thought to inhibit female reproductive behavior in female fish, thereby releasing their maleization potential. Meanwhile, the rise of GnRH stimulates pituitary FSH secretion, which affects the expression of cyp19a1a and dmrt1 in the gonadal hormone axis, thereby completing the transmission from social signals to molecular gene expression. In addition, the nutritional state and metabolic environment may affect gonad cell proliferation by regulating the mTOR pathway, thereby changing the expression of certain genes in the gonad. A typical example is that in the early stages of sexual reversal, some heat shock protein genes (HSPs) are upregulated in response to changes in the cellular environment, which can be seen as a direct reflection of gene expression by environmental stress. As the reversal deepens, HSP expression decreases and is replaced by other differentiated genes, which shows that the environmental effect is mainly in the initial initiation stage, and subsequently the self-sustaining conversion process by the gene network (Chen et al., 2022).

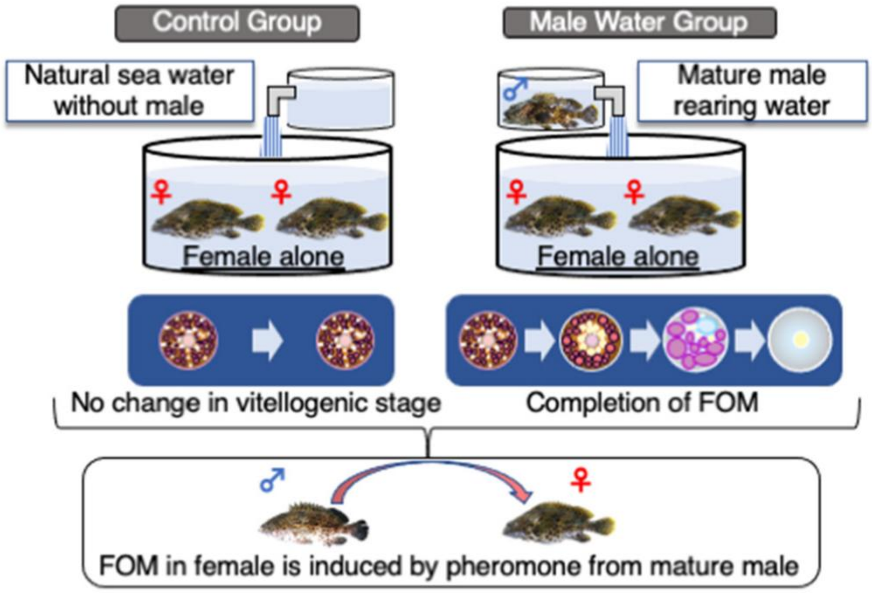

Figure 3 Induction of final maturation in females by pheromones released from mature males (Adopted from Soyano et al., 2022)

7 Construction and Analysis of Gender Regulation Network Model

7.1 Integration and visualization of gene regulation network map

Given that grouper gender decision involves the synergy of multi-level and multi-factors, it is of great significance to establish a comprehensive regulatory network model. Integrating the aforementioned genes, hormones and environmental signals can draw a network map of grouper gender regulation. Through this integration, the decentralized regulatory relationship is presented as an overall network map, and the multi-factor interaction pattern of gender determination can be visually seen (Li and Xu, 2024). Some studies have been trying similar integrations: for example, a team conducted a genome-wide and multiomic comparison of female, primary and secondary grouper groups in oblique bands, and constructed a schematic diagram of the regulatory network of gender-related genes based on the data, highlighting the core position of the brain-pituitary-godal axis. Their results show that the axis connects the upstream environment and the downstream gonads, which plays a total scheduling role, and the key nodes of the molecular network such as Dmrt1, Foxl2, and Cyp19a1a determine the gonadal direction at the end of this axis (Nagarajan et al., 2013; Wu et al., 2023).

7.2 Multi-omics data-driven network modeling method

In order to build a gender regulation network more comprehensively and objectively, integrating multi-omics data is a necessary means. Single transcriptome data reveals associations at the gene expression level, while protein interactions, apparent modifications, and phenotypic information all need to be included to reflect the real biological network (Miao et al., 2021). Specifically, transcriptomics can provide a list of differential genes and expression correlations, proteomics can verify the actual abundance and modification status of key regulatory proteins, and epigenomics (methylation group, histone modification group) reveals which gene regulation may be apparently affected. Hormone level data and physiological indicators are also important variables in modeling. Modern systems biology provides a variety of methods to combine different types of data (Tanvir et al., 2023). For example, network inference algorithms in machine learning can be used to integrate transcription factor-DNA binding data, miRNA target gene data with expression data to reconstruct a directed regulatory network, and then superimpose the effects of hormones and environmental factors on the nodes in it. In the study of grouper, there have been attempts to integrate genome and transcriptome data analysis: Hawe's team assembled high-quality reference genomes, combined with different gender and sexual reversal transcriptome data, drew heat maps and pathway maps of grouper gender-related genes, and used known gender pathway genes to annotate and verify the results (Hawe et al., 2019).

In the future, kinetic modeling methods can be used: fit differential equation models through time series data, simulate the changes in key genes and hormone concentrations over time, so as to infer the regulatory relationship and intensity between each component. This strategy based on multiomics and computational models will help us to quantitatively understand grouper gender regulation networks (Valous et al., 2024).

7.3 Identification and functional verification of key regulatory factors of network nodes

In the gender regulatory network built, there are often some "key nodes" that play a decisive role in network behavior. Identifying these key regulators and verifying their functions is of great significance for a deep understanding of the gender-determining mechanisms. Dmrt1, Foxl2, Cyp19a1a, Amh, etc. are obviously the central nodes of grouper gender network, and their state transitions basically determine the fate of gonadal tissue (Zhang et al., 2017). Therefore, functional verification work for these genes is also underway. For example, knocking out or mutating these genes through gene editing technology and observing the impact on the gender phenotype is the most direct verification method. In addition, transgene overexpression or gene therapy can also be used, such as overexpressing dmrt1 or amh in female fish, to simulate its overactivation to see if it is sufficient to induce sexual reversal (Li et al., 2021). Through the results of these functional experiments, we can determine the causal relationships and necessary conditions in the network model, and then optimize the network structure. In addition to the genetic level, hormone nodes in the regulatory network can also perform functional verification. For example, the administration of hormone receptor antagonists is observed to block the expected gender switch response; or to verify whether the axis is necessary for gender switch through hypothalamic-pituitary damage/transplantation experiments. Further, cross-species verification can also be tried: expressing the important gender genes of grouper in pattern fish to detect whether they can interfere with the gender development of pattern fish, thereby providing evidence for the function of grouper genes from the side. It should be emphasized that there are multiple redundancy and compensation mechanisms in the gender regulation network.

Acknowledgements

During this study, we sincerely thank my colleagues for their literature and the two review experts for their suggestions on the revisions.

Conflict of Interest Disclosure

The authors confirm that the study was conducted without any commercial or financial relationships and could be interpreted as a potential conflict of interest.

References

Augstenová B., and Ma W.J., 2025, Decoding *Dmrt1*: insights into vertebrate sex determination and gonadal sex differentiation, Journal of Evolutionary Biology, 2025: voaf031.
https://doi.org/10.1093/jeb/voaf031

Dong J.J., Li J., Hu J., Sun C.F., Tian Y.Y., Li W.H., Yan N.N., Sun C.X., Sheng X.H., Yang S., Shi Q., and Ye X., 2020, Comparative genomics studies on the dmrt gene family in fish, Frontiers in Genetics, 11 563947.
https://doi.org/10.3389/fgene.2020.563947

Evliyaoğlu E., Eroldoğan O.T., Yılmaz H.A., Genç M.A., Genç E., Duncan N., Aktaş M., and Güroy D., 2019, Artificial sex reversal of white grouper (*Epinephelus aeneus*) utilizing aromatase inhibitor (Fadrozole), Aquaculture Research, 50(5): 1539-1546.
https://doi.org/10.1111/ARE.14030

Fan Z., Zou Y., Liang D., Tan X., Jiao S., Wu Z., Li J., Zhang P., and You F., 2019, Roles of forkhead box protein L2 (foxl2) during gonad differentiation and maintenance in a fish the olive flounder (*Paralichthys olivaceus*), Reproduction Fertility and Development, 31(11): 1742-1752.
https://doi.org/10.1071/RD18233

Han Y., Peng C., Wang L., Guo J., Lu M., Chen J., Liu Y., Li S., Zhao M., Zhang Y., and Lin H., 2018, Female-to-male sex reversal in orange-spotted grouper (*Epinephelus coioides*) caused by overexpressing of Amh in vivo, Biology of Reproduction, 99: 1205-1215.
https://doi.org/10.1093/biolre/ioy157

Hawe J.S., Theis F.J., and Heinig M., 2019, Inferring interaction networks from multi-omics data, Frontiers in Genetics, 10: 535.
https://doi.org/10.3389/fgene.2019.00535

Li J.Y., and Xu Q.B., 2024, Molecular mechanisms of axis development: insights from cilia biology, International Journal of Marine Science, 14(4): 256-265.
https://doi.org/10.5376/ijms.2024.14.0029

Li M.M., 2024, Molecular mechanisms underlying the diversification of aquatic life forms, International Journal of Aquaculture, 14(4): 184-194.
https://doi.org/10.5376/ija.2024.14.0019

Lyu Q.J., Hu J., Yang X.K., Liu X.C., Chen Y.B., Xiao L., Liu Y.L., Wang Q., Chen J.X., Huang M.W., Yu Z.S., Yang H., Shi H., Zhang Y., and Zhao H.H., 2019, Expression profiles of dmrts and foxls during gonadal development and sex reversal induced by 17α-methyltestosterone in the orange-spotted grouper, General and Comparative Endocrinology, 274: 26-36.
https://doi.org/10.1016/j.ygcen.2018.12.014

Miao Z., Humphreys B., McMahon A., and Kim J., 2021, Multi-omics integration in the age of million single-cell data, Nature Reviews Nephrology, 17: 710-724.
https://doi.org/10.1038/s41581-021-00463-x

Nagarajan G., Aruna A., and Chang C., 2013, Neurosteroidogenic enzymes and their regulation in the early brain of the protogynous grouper *Epinephelus coioides* during gonadal sex differentiation, General and Comparative Endocrinology, 181: 271-87.
https://doi.org/10.1016/j.ygcen.2012.10.013

Nakamura M., Nozu R., Nakamura S., Higa M., Bhandari R.K., Kobayashi Y., Horiguchi R., Komatsu T., Kojima Y., Murata R., Soyano K., Ogawa S., Hirai T., Matsubara H., Tokumoto T., Kobayashi T., Kagawa H., Adachi S., Yamauchi K., and Nagahama Y., 2021, Morphological and physiological studies on sex change in tropical fish: sexual plasticity of the ovaries of hermaphroditic and gonochoristic fish, Galaxea Journal of Coral Reef Studies, 24(1): 5-17.
https://doi.org/10.3755/galaxea.g2021_s6r

Palma P., Takemura A., Libunao G.X., Superio J., Jesus-Ayson E.G., Ayson F., Nocillado J., Dennis L., Chan J., Thai T., Ninh N., and Elizur A., 2019, Reproductive development of the threatened giant grouper Epinephelus lanceolatus, Aquaculture, 509: 1-7.
https://doi.org/10.1016/J.AQUACULTURE.2019.05.001

Peng C., Wang Q., Shi H.R., Chen J.X., Li S.S., Zhao H.H., Lin H.R., Yang J.C., and Zhang Y., 2020, Natural sex change in mature protogynous orange-spotted grouper (*Epinephelus coioides*): gonadal restructuring sex hormone shifts and gene profiles, Journal of Fish Biology, 97(3): 785-793.
https://doi.org/10.1111/jfb.14434

Qu M., Cao X., Wang H., Lin L., and Ding S., 2021, Gonadal structure and expression localization of sex-related genes in the hermaphroditic grouper *Epinephelus akaara* (Perciformes: Epinephelidae), Aquaculture, 542: 736902.
https://doi.org/10.1016/J.AQUACULTURE.2021.736902

Shi S.Y., Zhang Z.Y., Li S.S., Liu S.Q., Lu D., Liu M., Meng Z., Cheng C., Liu X., and Lin H., 2010, Molecular identification of the Kiss2/Kiss1ra system and its potential function during 17Alpha-methyltestosterone-induced sex reversal in the orange-spotted grouper epinephelus coioides1, CABI, 83: 63-74.
https://doi.org/10.1095/biolreprod.109.080044

Soyano K., Amagai T., Yamaguchi T., Mushirobira Y., Xu W., Phạm N., and Murata R., 2022, Endocrine regulation of maturation and sex change in groupers, Cells, 11(5): 825.
https://doi.org/10.3390/cells11050825

Tanvir R.B., Islam M.M., Sobhan M., Luo D., and Mondal A.M., 2023, Mogat: an improved multi-omics integration framework using graph attention networks, bioRxiv, 2023: 535195.
https://doi.org/10.1101/2023.04.01.535195

Valous N., Popp F., Zörnig I., Jäger D., and Charoentong P., 2024, Graph machine learning for integrated multi-omics analysis, British Journal of Cancer, 131: 205-211.
https://doi.org/10.1038/s41416-024-02706-7

Wang Q., Huang M., Peng C., Wang X., Xiao L., Wang D., Chen J., Zhao H., Zhang H., Li S., Yang H., Liu Y., Lin H., and Zhang Y., 2018, MT-feeding-induced impermanent sex reversal in the orange-spotted grouper during sex differentiation, International Journal of Molecular Sciences, 19(9): 2828.
https://doi.org/10.3390/ijms19092828

Wang Q., Liu Y., Peng C., Wang X., Xiao L., Wang D., Chen J., Zhang H., Zhao H., Li S., Zhang Y., and Lin H., 2017, Molecular regulation of sex change induced by methyltestosterone -feeding and methyltestosterone -feeding withdrawal in the protogynous orange-spotted grouper, Biology of Reproduction, 97: 324-333.
https://doi.org/10.1093/biolre/iox085

Wang X.X., Ma X., Wei G.B., Ma W.R., Zhang Z., Chen X.P., Gao L., Liu Z.B., Yuan Y., Yi L.Z., Wang J., Tokumoto T., Huang J., Chen D., Zhang J., and Liu J., 2021, The role of DNA methylation reprogramming during sex determination and transition in zebrafish, Genomics Proteomics and Bioinformatics, 19(1): 48-63.
https://doi.org/10.1016/j.gpb.2020.10.004

Wu G.C., and Chang C.F., 2017, Primary males guide the femaleness through the regulation of testicular Dmrt1 and ovarian Cyp19a1a in protandrous black porgy, General and Comparative Endocrinology, 261: 198-202.
https://doi.org/10.1016/j.ygcen.2017.01.033

Wu G.C., Dufour S., and Chang C.F., 2020, Molecular and cellular regulation on sex change in hermaphroditic fish with a special focus on protandrous black porgy *Acanthopagrus schlegelii*, Molecular and Cellular Endocrinology, 520: 111069.
https://doi.org/10.1016/j.mce.2020.111069

Wu G.C., Li H.W., Tey W.G., Lin C.J., and Chang C.F., 2017, Expression profile of amh/Amh during bi-directional sex change in the protogynous orange-spotted grouper *Epinephelus coioides*, PLoS ONE, 12(10): e0185864.
https://doi.org/10.1371/journal.pone.0185864

Wu X., Yang Y., Wang T., Zhong C., Tao Y., Chang L., Meng Z., and Liu X., 2023, Transcriptome profiling of the gonad during sex differentiation in hermaphroditic brown-marbled grouper (*Epinephelus fuscoguttatus*), Aquaculture Reports, 30: 101542.
https://doi.org/10.1016/j.aqrep.2023.101542