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The Genetics and Epigenetics of Sex Change in Fish

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Abstract

Fish show extraordinary sexual plasticity, changing sex naturally as part of their life cycle or reversing sex because of environmental stressors. This plasticity shows that sexual fate is not an irreversible process but the result of an ongoing tug-of-war for supremacy between male and female signaling networks. The behavioral, gonadal, and morphological changes involved in this process are well described, yet the molecular events that underpin those changes remain poorly understood. Epigenetic modifications emerge as a critical link between environmental stimuli, the onset of sex change, and subsequent maintenance of sexual phenotype. Here we synthesize current knowledge of sex change, focusing on the genetic and epigenetic processes that are likely involved in the initiation and regulation of sex change. We anticipate that better understanding of sex change in fish will shed new light on sex determination and development in vertebrates and on how environmental perturbations affect sexual fate.

INTRODUCTION

Sexual Plasticity of Fish

Sex change: reproductive strategy in which individuals mature as one sex but change sex some time later as a usual part of their life cycle

Phenotypic plasticity allows an organism to respond to changes in the environment by adopting different phenotypes (1). Phenotypic plasticity is pervasive in nature, but how the genome and the environment interact to trigger phenotypic transitions from a common genomic template is still not fully understood. Phenotypic plasticity is found in many taxa (2), but one of the most fascinating examples is the sexual plasticity of fish, in which we observe remarkable malleability in both gonadal development and sexual fate (3) (**Figure 1**). This malleability is most extreme in sequential hermaphrodites, individuals that change sex during adulthood as a usual part of their life cycle (4–6). Sequential hermaphroditism has been reported in 27 taxonomic families spanning nine orders (7), and three sex-changing strategies are observed: female-to-male (protogynous), male-to-female (protandrous), and sequentially bidirectional sex change (8).

In fish, the sexual fate of an individual may be determined chromosomally, environmentally (e.g., temperature, pH, population, density), or most commonly through a combination of the two (9). Environmental factors can frequently override genetic factors to redirect sexual fate (10),

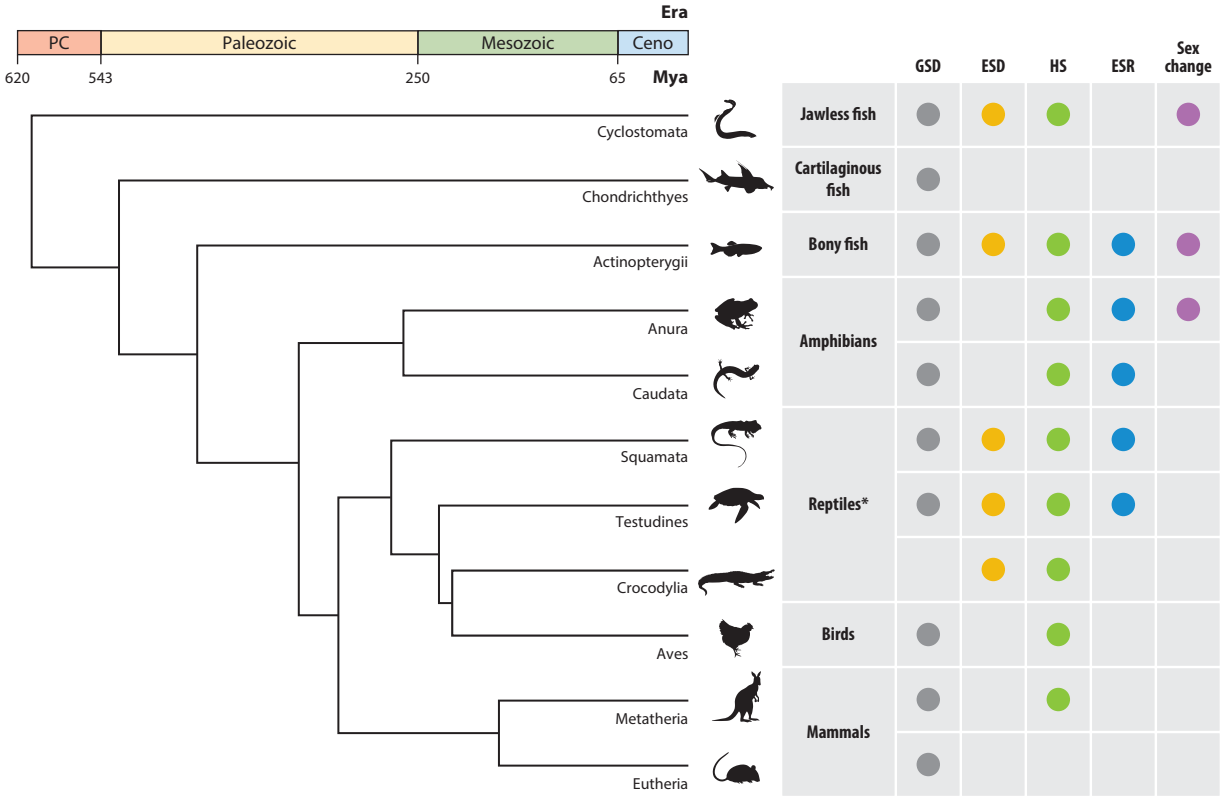


Figure 1

Sex determination and sex change in vertebrates. GSD and ESD coexist in several vertebrate clades for different species. HS during critical time windows affects sex determination in most vertebrate clades except for eutherians, and there is a lack of evidence in chondrichthyans. Fish exhibit remarkable sexual plasticity and may undergo ESR as a result of changes in the external conditions or even complete sex change during adulthood. Reptiles*, nonavian reptiles. Divergence times used to construct the tree were obtained from the TimeTree database (134). Abbreviations: Ceno, Cenozoic; ESD, environmental sex determination; ESR, environmental sex reversal; GSD, genetic sex determination; HS, hormone sensitivity; PC, Precambrian.

Table 1 Evidence of epigenetic modifications in sex-changing and environmental sex-reversal gonochoristic fish

Species	Reproductive strategy	Epigenetic mechanism and genes examined	DNA methylation/histone acetylation patterns across sexes	References
Acanthopagrus schlegelii (black porgy)	Protandrous	DNA methylation of *cyp19a1a*	↑ in testis versus ovary	14
Cynoglossus semilaevis (half-smooth tongue sole)	Gonochoristic—ZW GSD subject to TIM	DNA methylation of *cyp19a1a* and *amh* DNA methylation of *dmrt1* and *gsdf*	↑ in ZW/ZZ testis versus ovary ↓ in ZW/ZZ testis versus ovary	19
Dicentrarchus labrax (European sea bass)	Gonochoristic—XY GSD subject to TIM	DNA methylation of *cyp19a1a*	↑ during ♀-to-♂ sex reversal	20
Kryptolebias marmoratus (mangrove killifish)	Simultaneous hermaphrodite	DNA methylation of *cyp19a1* (DQ339107.1) DNA methylation of *sox9a*	↓ in males and hermaphrodites incubated at ↑ T versus ↓ T ↑ in males versus hermaphrodites incubated at ↓ T	15
Lates calcarifer (barramundi)	Protandrous	DNA methylation of *cyp19a1a* and *amh* DNA methylation of *dmrt1* and *nr5a2* DNA methylation of *foxl2* and *sox8*	↑ in testis versus ovary ↓ in testis versus ovary ↓ in both males and females	16
Monopterus albus (ricefield eel)	Protogynous	DNA methylation of *cyp19a1a* Histone acetylation of *cyp19a1a*	↑ in testis and ovotestis versus ovary ↓ in testis versus ovary	17
Oreochromis niloticus (Nile tilapia)	Gonochoristic—XY GSD subject to TIM	DNA methylation of *cyp19a1a* DNA methylation of *fgf16*	↑ in testis versus ovary ↓ in testis versus ovary	23
Paralichthys olivaceus (olive flounder)	Gonochoristic—XY GSD subject to TIM	DNA methylation of *cyp19a1a* DNA methylation of *dmrt1*	↑ in testis versus ovary ↓ in testis versus ovary	21
Thalassoma bifasciatum (bluehead wrasse)	Protogynous	DNA methylation of *cyp19a1a* DNA methylation of *dmrt1*	↑ in testis versus ovary ↓ in testis versus ovary	18

Abbreviations: GSD, genetic sex determination; T, temperature; TIM, temperature-induced masculinization.

and stable modifications of DNA and its associated histone proteins appear to be major enactors of this process (11–13). Importantly, these stable modifications do not require any change in genetic sequence and as such are termed epigenetic. Epigenetic changes are linked to a wide range of phenotypic plasticity examples observed in many taxa (12); however, environmentally induced sex change in fish is among the most dramatic. To date, epigenetic regulation of sex change has been documented in both sequential hermaphrodites (14–18) and gonochoristic species undergoing environmental sex reversal (ESR) (**Table 1**) (19–23).

Here, we review the latest advances in our understanding of the genetic mechanisms governing sex change in fish and the potential role of epigenetics in the transduction of the environmental signals triggering this process. We also explore one of the field's greatest questions: How are fish so sexually plastic compared with other vertebrates?

Environmental sex reversal (ESR): a sexual pattern in which functional sex reversal occurs in otherwise GSD individuals as a result of specific external conditions (e.g., temperature)

The Knowns and Known Unknowns of Sex Change in Fish

Although the behavioral, gonadal, and morphological modifications involved in the process of sex change are now described for several species (3), the genetic cascade orchestrating this transformation needs deeper exploration. It has been hypothesized that the trigger of sex change at the neuroendocrine level is mediated by the cross talk between two physiological axes regulating reproduction and stress: the hypothalamic–pituitary–gonadal (HPG) and the hypothalamic–pituitary–interrenal (HPI) axes (3) (**Figure 2**). The HPG axis exerts control over reproduction and development in all vertebrates (24), and its interaction with the HPI or stress axis may be responsible for the transduction of environmental signals (e.g., social cues, changes in temperature) that can interrupt the reproductive cycle and initiate sex change (3, 10). In the case of socially protogynous fish, loss of the dominant male individual from a social group may increase arginine vasotocin (AVT) and norepinephrine (NE) levels in the hypothalamus of larger females, inducing

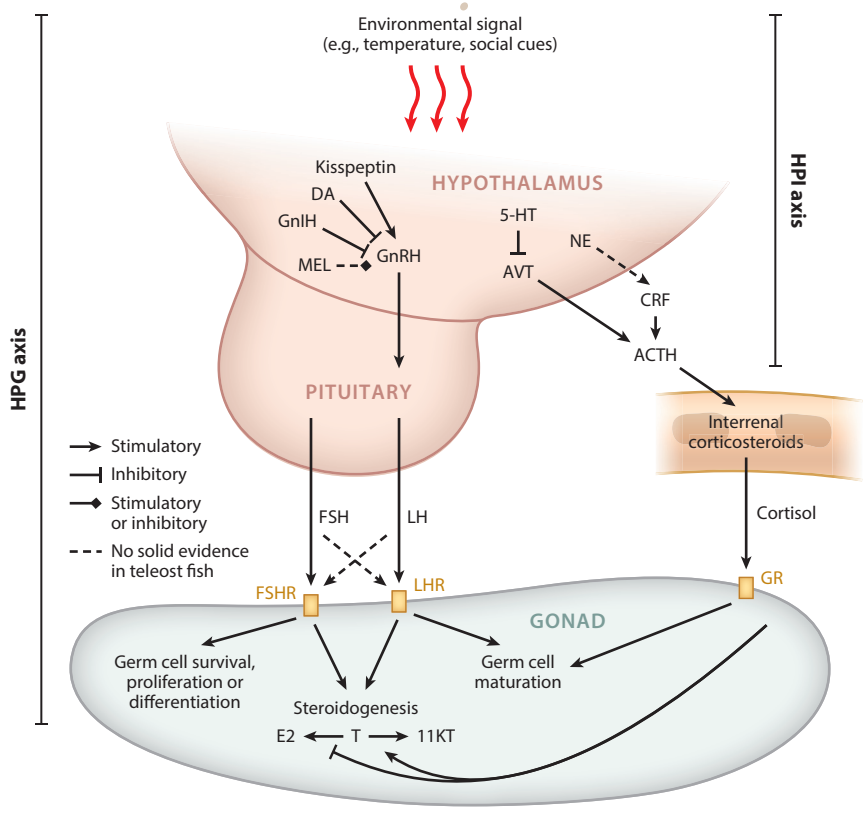


Figure 2

Cross talk between the neuroendocrine HPG and HPI axes controls sexual development and reproduction in fish. Solid lines indicate interactions with support from fish models; dashed lines indicate interactions with support from other systems that are yet to gain supporting evidence in fish. Abbreviations: 5-HT, serotonin; 11KT, 11-ketotestosterone; ACTH, corticotropin or adrenocorticotropic hormone; AVT, arginine vasotocin; CRF, corticotropin-releasing factor; DA, dopamine; E2, 17β-estradiol; FSH, follicle-stimulating hormone; FSHR, follicle-stimulating hormone receptor; GnIH, gonadotropin-inhibitory hormone; GnRH, gonadotropin-releasing hormone; GR, glucocorticoid receptor; HPG, hypothalamic–pituitary–gonadal; HPI, hypothalamic–pituitary–interrenal; LH, luteinizing hormone; LHR, luteinizing hormone receptor; MEL, melatonin; NE, norepinephrine; T, testosterone. Figure adapted with permission from References 3 and 8.

behavioral sex change (24). These rapid neurochemical changes, in turn, affect the liberation of gonadotropin-releasing hormones (GnRH) and luteinizing hormones (LH), promoting ovarian cell apoptosis and elevating cortisol levels (3, 10, 25). An increase in circulating cortisol, along with epigenetic factors, could inhibit the female pathway by suppressing 17β-estradiol (E2) production while boosting 11-ketotestosterone (11KT) secretion and switching on the male developmental pathway (3, 10, 25).

Even though numerous studies indicate sex change begins in the brain (26–28), recent transcriptomic studies seeking to characterize the effects of sex change on brain gene expression have thus far revealed limited variation between sexes compared with the gonads (29). Other fish, although not sex changers, also show restricted sex-biased brain gene expression (30, 31), suggesting that those differences in expression pattern that do arise among the sexes may be subtle and regionalized within the brain.

In the case of socially regulated sex change, behavioral changes precede alterations in gonadal morphology, and occur even in ovariectomized females (32), indicating that visual social cues set off neurochemical cascades that trigger behavioral changes important for establishing dominance and courtship behaviors in the secondary sex (33). A handful of genes encoding key neuronal signaling factors (e.g., *cyp19a1b*, *it*, *avt*, *kisspeptin*) have gained the interest of most brain-focused research on fish sequential hermaphroditism (3, 34). The role of brain aromatase (*cyp19a1b*) in fish sexual behavior is well established, and its expression is controlled by sex steroids (34), as well as components of the stress axis (35). Isotocin (*it*), the fish homolog of oxytocin in mammals, is known to mediate sociosexual interactions, but its role in sex change remains unclear (33). In the bluehead wrasse (*Thalassoma bifasciatum*), a diandric (i.e., two male phenotypes) protogynous hermaphrodite, isotocin was found to be overexpressed in socially dominant terminal-phase males (31), suggesting a role in mediating, and perhaps maintaining, the dominance behaviors specific to those males (29). Kisspeptin and its receptors (kiss2/kiss1r) also hold promise as regulators of sex change, as this neuropeptide is known to control reproductive function and puberty in mammals through its direct effects on GnRH neuron function (6). Nevertheless, to date, variation in the expression of this gene and its receptors during sex change has been reported only in the orange-spotted grouper (*Epinephelus coioides*) (36).

The brain is of course complicated, with regional specializations defined by a heterogeneous collection of specialized neurons and supporting cells. Thus, the limited resolution of prior work is likely a question of refinement; a fine-scale anatomically informed approach may be necessary to detect significant expression differences for neuropeptide genes in the brain (31) that earlier studies at a gross scale failed to identify. Such an approach, for example, coupling detailed immunohistochemical and single-cell sequencing methods, may allow us to identify region- and cell-specific differences and ultimately unravel the precise neural signaling pathways involved in perceiving social cues and triggering sex change in fish (31).

GENETIC ORCHESTRATION OF SEX CHANGE

Classical Sex Pathways

Sexual metamorphosis in fish is mediated by neuroendocrine and molecular pathways that exert control over the behavioral, physiological, and morphological changes underlying sequential hermaphroditism (3, 8, 24, 25, 33). Here, we focus on the latest developments in our understanding of the genetic regulatory systems driving this process.

It is now understood from mammalian systems that the female and male signaling networks act in competitive opposition (37). Thus, for sexual fate to be determined, not only does the appropriate sex-specific gene network need to be initiated, but the opposing sex-specific network must

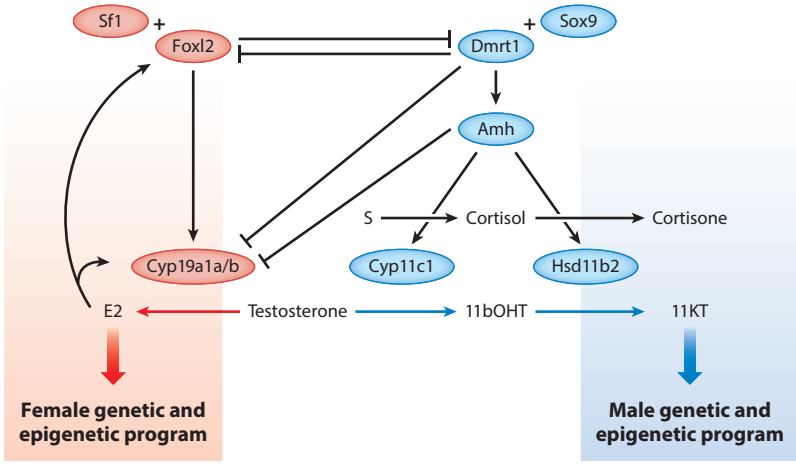


Figure 3

Model of antagonistic sex-specific gene networks controlling sexual fate in fish. Conserved downstream effectors promote the feminizing or masculinizing pathway, respectively, while actively inhibiting the opposing sexual network. Testosterone can be converted into either 17β-estradiol (E2, the most potent estrogen in fish) through gonadal aromatase (Cyp19a1a) or 11KT (the most powerful androgen in fish) by 11β-hydroxylase (Cyp11c1) and 11β-hydroxysteroid dehydrogenase type 2 (Hsd11b2) enzymes. Cyp11c1 and Hsd11b2 are critical not only for the synthesis of 11-oxygenated androgens but also for the metabolism of glucocorticoids (e.g., cortisol). Abbreviations: 11bOHT, 11β-hydroxytestosterone; 11KT, 11-ketotestosterone; Amh, anti-Müllerian hormone; Dmrt1, doublesex and mab-3 related transcription factor 1; Foxl2, forkhead transcriptional factor L2; S, 11-deoxycortisol; Sf1, steroidogenic factor 1; Sox9, SRY-related HMG box 9. Figure adapted with permission from Reference 8.

be actively suppressed (38). Although diverse master sex-determining genes have been identified in fish (39), the downstream effectors of sexual differentiation appear generally more conserved, acting within opposing feminizing and masculinizing gene pathways that promote either ovarian or testicular development, respectively (40). Several of the component genes have been investigated for their role in sex change in fish (e.g., *cyp19a1a/b*, *dmrt1*, *foxl2*, *amh*, *wnt4*, *sf1*, *sox9*) (41–48) (**Figure 3**).

Aromatase is responsible for the conversion of androgens (i.e., testosterone) to estrogens (i.e., E2) (encoded by *cyp19a1a* and *cyp19a1b*, in the gonad and brain, respectively) fundamental for the maintenance of ovarian function. Rapid downregulation of *cyp19a1a* expression at the initiation of female-to-male sex change has been recorded for several protogynous species (17, 49, 50), as well as for fish that undergo temperature-induced masculinization (TIM), a form of ESR (51–53). Aromatase downregulation is considered the potential trigger of female-to-male gonadal sex change, causing estrogen production to collapse and interrupting a positive feedback loop that maintains feminizing gene expression, thus lifting suppression of the masculinizing network (3, 8).

Temperature-induced masculinization (TIM): a form of ESR in which female-to-male sex-reversed individuals are obtained under artificial conditions induced by temperature changes

Evidence suggests that both epigenetic factors and cortisol, the hormone most directly linked to stress in fish, could be pivotal upstream mediators through which environmental stimuli can suppress the female sexual network through downregulation of aromatase and promote advancement of the male sex (3, 8, 33). An inverse relationship between *cyp19a1a* expression and DNA methylation has been reported in several protogynous (17, 18) and ESR (20, 21) species. Cortisol is now recognized as a likely key factor triggering gonadal sex change in protogynous fish (3). The inhibition of aromatase expression is considered one of the potential pathways by which cortisol could mediate sex change (10). In the olive flounder (*Paralichthys olivaceus*), treatment with E2

can suppress the masculinizing effects of high temperature or cortisol (53). Furthermore, in vitro studies in this species have shown that cortisol can bind glucocorticoid response elements in the *cyp19a1a* promoter, directly preventing transcription (54, 55). Moreover, there is evidence of cortisol inducing masculinization in TIM fish species by upregulating *amh* to promote maleness via germ cell apoptosis (54–56). The interaction between epigenetic factors and stress (measured in the form of cortisol) on key sex-pathway genes during sex change and, in particular, the potential cortisol-mediated methylation of *cyp19a1a* during TIM are areas worthy of more investigation (10, 57).

Transcription factors *foxl2* (*forkhead transcriptional factor L2*) and *sf1* (*steroidogenic factor 1*), which can act jointly to upregulate aromatase expression, have also generated interest as activators of the aromatase expression and feminizing pathways (58). The role of *foxl2* in the promotion of ovarian development and suppression of the male network is broadly established, and sex reversal following knockout of *foxl2* has been demonstrated in the Nile tilapia [*Oreochromis niloticus* (50)] and mice (59). However, the exact way in which *sf1* interacts with *cyp19a1a* during the initial stages of sex change is not fully understood (25). *Sf1* expression has been observed to plummet during early sex change in both the bidirectional orange-red pygmygoby (*Trimma okinawae*) and the bluehead wrasse, although in the latter its expression was rescued as sex change advanced (3, 60).

Interacting antagonistically with *foxl2a* to influence *cyp19a1a* expression, *dmrt1* (*doublesex and mab-3 related transcription factor 1*) is essential for the activation of the male-promoting gene network and the inhibition of those genes required for female development, such as genes within the ovary-specific Rspo1/Wnt/β-catenin signaling pathway (e.g., *wnt4a*, *ctnnb1*) (39, 61). However, changes in the expression of *foxl2a* and *dmrt1* during protogynous sex change occur in the later stages of transition (following a drop in E2 levels) (43), which suggests that these genes are more important at later stages of female-to-male sex change, rather than during its initiation (18). Dmrt1 may play a more prominent role in initiating male-to-female sex change in protandrous hermaphrodites (61). For example, a decrease in *dmrt1* expression coincides with the first signs of testicular tissue recession in the protandrous black porgy [*Acanthopagrus schlegelii* (62)], gilthead seabream [*Sparus aurata* (63)], and Red Sea clownfish [*Amphiprion bicinctus* (64)].

Interestingly, expression of another key male pathway gene, *amh* (encoding anti-Müllerian hormone), is more closely concordant with early sex change in both protogynous and protandrous systems. Expression of *amh* steadily increases at the first signs of ovarian atresia in the early stages of female-to-male sex change in the protogynous ricefield eel (*Monopterus albus*) and the bluehead wrasse, concurrent with the decrease in *cyp19a1a* expression (3, 65), whereas, in male-to-female sex change, *amh* expression decreases at the onset of sex change in the protandrous black porgy (47). More recently, data from a transcriptome study in protandrous clownfish, the first genome-wide study in a social sex-changing species, indicate that changes in *dmrt1* expression may occur prior to those in *amh* (64). Further studies of this nature, in both protogynous and protandrous species, may lead to increased refinement on the genes involved in sex change and the sequence and timing of their expression.

Sex-biased expression of the masculinizing gene *sox9* [*SRY* (*sex-determining region Y*)*-box 9*] was reported in transcriptomic data from bluehead wrasse (18, 29), which provides the most compelling data available today regarding the molecular drivers of protogynous sex change. In male fish, *sox9* transcription is activated by *dmrt1* (38, 61). *Cyp19a1a/b* gene promoter regions contain DNA-binding motifs that can be associated with *sox9* as well as several other transcription and endocrine factors (e.g., Foxl2, Sf1, glucocorticoid response elements) (8, 66). Although changes in the environment, such as variations in temperature or density, can affect the expression of *cyp19a1a*, *dmrt1*, or *amh* (25), the effect of these external factors on other sex-pathway genes should be investigated in greater depth.

Noncanonical Sex Pathways

In addition to genes known previously to be involved in sex determination and differentiation in other vertebrates, some that are less well known, or have not been as extensively examined, are emerging as potential key components of sex change in fish. Among these is *sox8*, which is vital for sex determination and testis differentiation in mice and known to regulate expression of *amh* (67). Recent evidence suggests Sox8 could also be an important driver of protandrous sex change in fish (64). Its expression, alongside that of *dmrt1* and *amh*, was observed to be greatly upregulated in male Red Sea clownfish compared with females, suggesting a role of this gene in testicular differentiation and spermatogenesis (64). Male-biased expression of *sox8* has also been reported in protandrous sharpsnout seabream (*Diplodus puntazzo*) (68), barramundi (*Lates calcarifer)* (69), and black porgy (45) and in the protogynous bluehead wrasse (29).

A member of the Fanconi anemia/BRCA DNA repair pathway, *fancl*, has also drawn interest as a candidate regulator for sex change. A mutation in this gene compromised the survival of developing oocytes in juvenile zebrafish (*Danio rerio*), a gonochoristic species, causing female-to-male sex reversal through Tp53-mediated germ cell apoptosis (70).

A recent transcriptomic analysis of the bluehead wrasse gonad provides evidence for a potential role for retinoic acid (RA) signaling in gonadal sex change (29). The RA pathway is necessary for ovarian development and regulates the sex-specific timing of meiosis initiation (71). Specifically, two RA pathway genes, *aldh1a2* (*retinaldehyde dehydrogenase type 2*) and *cyp26b1* (*cytochrome P450, family 26, subfamily b, polypeptide 1*), were found to be upregulated in male blueheads compared with females, whereas *cyp26a1* (*cytochrome P450, family 26, subfamily a, polypeptide 1*) was downregulated (29). Similar patterns are reported in Nile tilapia (72). Enzymes Aldh1a and Cyp26 play opposing roles in controlling RA levels and together determine the moment germ cells enter meiosis (72). In mice, there is evidence that *cyp26b1* is upregulated by Sf1 and Sox9 to maintain the male pathway, whereas Foxl2 inhibits its expression in the ovaries (73). In addition, a study on flounder TIM found that *cyp26b1* was upregulated by cortisol, which hindered germ cell meiosis to promote maleness (55). Further research across a diversity of fish is needed to shed light on the role of RA signaling in sexual plasticity and its association with the stress axis.

Gonadal soma-derived factor (*gsdf*), belonging to the Tgf-β ligand superfamily, has emerged as a potential key player in the battle between the male and female trajectories to define sexual fate, after being identified as the male sex-determining gene in the Luzon rice fish (*Oryzias luzonensis*) (74). Gsdf has also been associated with sex determination in sablefish (*Anoplopoma fimbria*) (75). However, loss of its function was found to have no effect on sex ratios in zebrafish, a species in which Gsdf seems to play a role similar to that of Amh (76). A deeper study of Gsdf function in diverse fish will shed light on its importance during fish sex change.

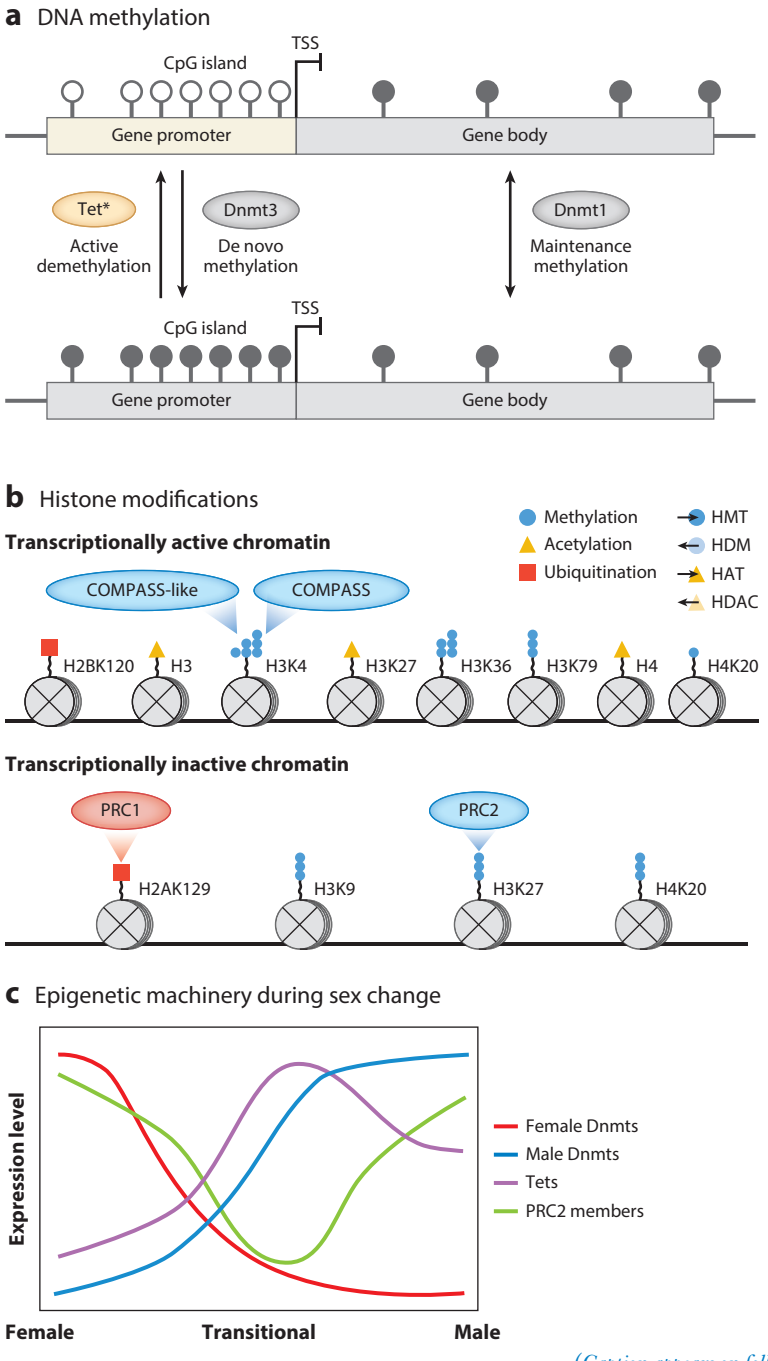
Work in other vertebrate systems continues to reveal genes with unexpected roles in sexual fate, and whose homologs in fish warrant investigation. Novel work on mice has also revealed a defining role for E3 ubiquitin ligase *znrf3* in the determination of sexual fate. Mutant male mice lacking *znrf3* underwent partial or complete gonadal sex reversal, potentially through a key role for this gene in the inhibition of pro-ovarian RSPO1/WNT-β-catenin/FOXL2 pathways that are essential for female development in both mammals and fish.

EPIGENETIC MEMORY AND SEXUAL COMMITMENT

Epigenetic Regulation of Sex Change

When Conrad H. Waddington coined the term epigenetics, he used it to describe the process of how genotypes give rise to phenotypes during development (77). The scope of epigenetics has narrowed and adapted since then, and a modern definition states, "An epigenetic trait is a stably

heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (78). Modern epigenetic studies focus on heritable modifications of DNA, histones, and chromatin structure (**Figure 4**) (79). Those modifications can regulate gene expression through preventing or favoring the binding and access of transcription factors (80, 81) or regulating



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Epigenetic mechanisms involved in sex change. (*a*) In vertebrates, most CG sites are methylated (*gray filled lollipops*), except for short CG-rich regions, termed CpG islands, where CG are commonly unmethylated (*white lollipops*). Near the transcription start site (TSS), methylation of CpG islands is associated with transcriptional silencing. DNA methylation patterns are maintained and remodeled by a dedicated group of enzymes. The maintenance DNA methyltransferase Dnmt1 copies the methylation patterns to newly synthesized DNA strands. Remodeling may occur through de novo DNA methyltransferase Dnmt3, passive demethylation produced by lack of DNA methylation maintenance during cell division, or active demethylation mediated by Tet proteins and further steps (Tet*). (*b*) Posttranslational histone modifications include a large number of histone changes, such as methylation, acetylation, and ubiquitination. Specific changes in residues are associated with active and inactive chromatin states. A large set of enzymes, including histone methyl-transferases (HMT), histone demethylases (HDM), histone acetyl-transferases (HAT), histone deacetylases (HDAC), polycomb repressive complexes (PRC1/2), and complex proteins associated with Set1 (COMPASS and COMPASS-like), have been linked to such changes. (*c*) Sex change in bluehead wrasse shows a marked shift in epigenetic machinery. This includes sex-biased expression of Dnmt paralogs, overexpression of Tet proteins during intermediate stages, and changes in the dynamics of polycomb repressor group 2 (PRC2) protein members, altogether suggesting a genome-wide epigenetic reprogramming event (18).

chromatin remodeling proteins (82), ultimately modulating the availability of genes to transcription machinery. Even though somatic cells in an organism possess almost entirely the same genomic content, they have different identities, which are defined by patterns of gene expression. Because epigenetic modifications are mitotically stable, they represent an essential memory module allowing faithful maintenance of unique cell identities (12, 83).

Sexual phenotype is the result of a coordinated interplay of genetic pathways, environmental influences, and epigenetic regulations (39) and results in a largely binary fate—individuals are either female or male. In species with a genetic sex determination (GSD) system, genetic differences between the sexes occupy a primary role in determining sexual fate. In species with environmental sex determination (ESD), this decision is more plastic, and therefore, epigenetic modifications are expected to play a strong role in initiating and maintaining sexual identity. For sex-changing fish, in which a profound phenotypic change occurs without any obvious modification in the DNA sequence or content, this plasticity is even more remarkable. Currently, GSD and ESD are not considered mutually exclusive, but rather they are two ends of a continuum (13, 39). Species with GSD, for example, can experience sex change under extreme environmental conditions (19, 84). Conversely, in species with the ability to sex change, or in those with ESD, plasticity in response to environmental stimulus is underpinned by genetic factors. Epigenetic modifications provide not just a mechanism to preserve sexual identity but an interface to integrate environmental signals during sex determination and sex change. Exposure to environmental stimuli such as temperature (19, 20, 22), chemical compounds (17, 85), and social cues (18) is shown to result in epigenetic modifications of key genes in sex determination (discussed in detail below). Assuming genetic content is stable in sex-changing fish, the switching of sex pathways is possible only in light of a high degree of epigenetic plasticity.

Genetic sex determination (GSD): a mechanism by which sexual fate is determined at fertilization by inherited genetic elements, differs between males and females (e.g., XX/XY)

Environmental sex determination (ESD): a mechanism where sexual fate is determined during early development by external experimental conditions

Epigenetic Landscapes and Canalization

Waddington's landscape remains a powerful metaphor in developmental biology, and despite its clear limitations (e.g., static nature and dimensional limitations), analogies can be established with sex determination and sex change (**Figure 5**). The sensitivity or robustness of a phenotype to perturbations can be represented as the slope in the landscape or the degree of "canalization" (86). In gonochoristic species, the robustness of the sex program to environmental perturbations

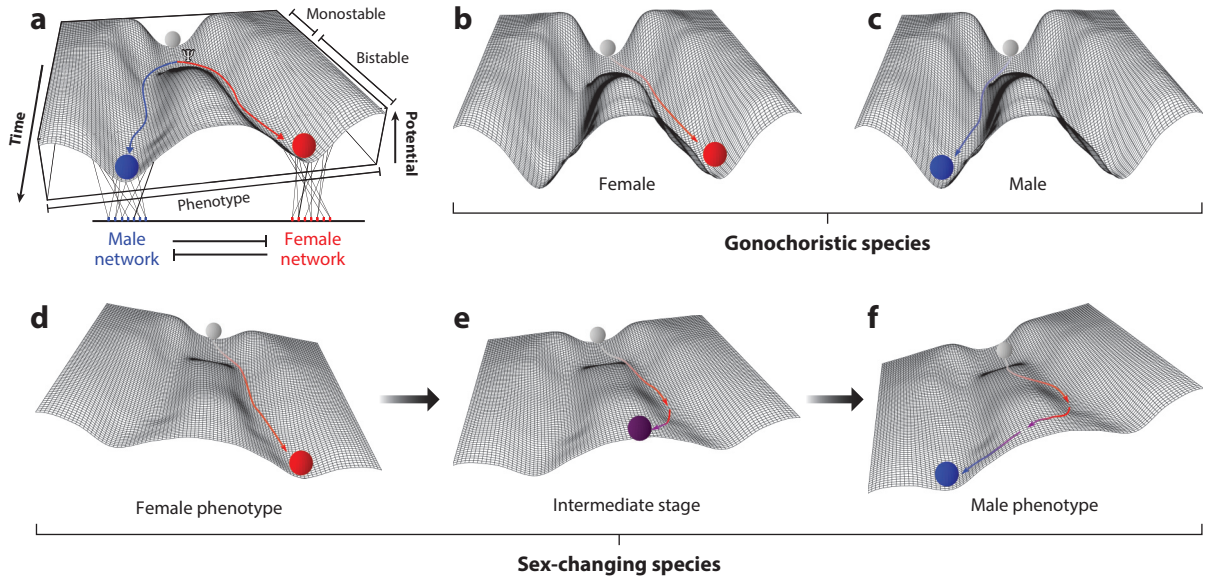


Figure 5

Waddington's epigenetic landscape during sex determination and sex change. (*a*) Starting from an undifferentiated state in the bipotential gonad, cells roll down through a bifurcating valley (Ψ) influenced by external stimuli, such as temperature or chemical compounds, or internal influences, such as master sex-determining genes. As the marble progresses, the cellular potential decreases, and the cell is committed to a particular sexual fate. The landscape is shaped by interactions of several interconnected ropes (gene regulatory networks–environment) attached to dowels fixed in the ground (genes). (*b*,*c*) In gonochoristic species, the landscape remains static in time, and strong genetic and epigenetic barriers separate (*b*) female and (*c*) male phenotypes. (*d*–*f*) In sex-changing fish, phenotypic plasticity during adulthood allows the individual to surpass such barriers and change sexual phenotype.

is high, suggesting a high degree of canalization and strong epigenetic barriers. In contrast, in sex-changing fish, environmental perturbations can overcome those barriers, enabling sexual fate reprogramming. Whether such epigenetic plasticity is the result of rapid genomic evolution, gene duplications, or selective pressure from changing environmental conditions remains unanswered (5). In the same way, the nature of the reprogramming process remains unclear. New cell types may emerge from transdifferentiation, where differentiated cells switch directly into another differentiated lineage, or it may involve dedifferentiation, in which a differentiated cell temporarily reverts to a less-differentiated stage before recommitting to an alternative fate. It is also possible that a population of germline cells remains plastic (undifferentiated) in adult gonads and proliferates to produce the gonadal tissues, structures, and gametes of the opposite sex.

DNA Methylation and Sex Determination

Cytosine methylation was the first epigenetic modification of DNA described and is currently the most studied and best understood (87). The biochemical stability and heritability of cytosine methylation, coupled with its reversibility and flexibility, provide an additional module of information critical during cell lineage commitment and organ development (83). In eukaryotes, DNA methylation occurs exclusively at the C5 position of cytosine (5-mC), mostly in the context of palindromic CpG dinucleotides (CG) (88). The enzymes catalyzing DNA methylation are referred to as DNA methyltransferases (89). Whereas the maintenance methyltransferase Dnmt1

recognizes hemi-methylated DNA and adds a methyl group to the newly synthetized strand, Dnmt3 is implicated in de novo establishment of methylation marks (**Figure 4*a***).

One of the most astonishing examples of epigenetic plasticity is environmentally sensitive sexual development in the flatfish half-smooth tongue sole (*Cynoglossus semilaevis*) (19). In this species, ESR coexists with a relatively young ZW sex-determination system (~30 million years old) (90). Under normal temperature conditions (22°C), ~14% of ZW genetic females are reversed to phenotypic males (pseudomales) (90). Exposure to higher temperatures (28°C) during a sensitive developmental period increases the sex-reversal rate to ~73%. Sex-reversed pseudomales are fertile, and their ZW-F1 offspring exhibit an extremely high reversal rate (~94%) at normal temperatures (22°C). Interestingly, methylation patterns in pseudomales (ZWm) resemble those in true males (ZZ) and are accurately transmitted to the offspring. Detailed analysis of testis methylomes identified differentially methylated regions (DMRs) in less than 0.01% of the genome for either ZWm and ZZ or ZWm and ZW-F1. In contrast, DMRs between testis and ovary represent approximately 4% of the genome and are enriched in sex-determining pathways (e.g., *dmrt1*, *gsdf*, *amh*, *amhr2*, *wt1a*, and *wt1b*). These results indicate that environmental sex reversal can override sexual fate determined by genetic factors through epigenetic regulation.

For other gonochoristic species, promoter regions of genes critical during sex determination have methylation levels inversely correlated with expression. In the European sea bass (*Dicentrarchus labrax*), another GSD species with temperature-sensitive sex reversal, gonadal methylation levels within the *cyp19a1a* promoter of juvenile males are significantly higher than for juvenile females (20). Interestingly, high temperatures during the critical sex-determination window increased *cyp19a1a* promoter methylation in females. In vitro studies showed that methylation of the *cyp19a1a* promoter blocks the ability of Sf1 and Foxl2 to induce transcription. In olive flounder and Nile tilapia, which use a XX/XY determination system, promoter regions of *cyp19a1* are also differentially methylated and correlated with gene expression (21–23). Methylation in promoters of other candidate genes, such as *dmrt1* and *foxl2*, shows a similar pattern (21).

Modifications in DNA methylation patterns have been identified in several sequentially hermaphroditic fish. In the protogynous ricefield eel, the *cyp19a1a* promoter is hypermethylated in testis compared with ovary, blocking its activation by gonadotropins through a cAMP-dependent pathway (17). During female-to-male sex change, methylation of response elements and Sf1 binding elements progressively increases and is inversely correlated with *cyp19a1a* expression. Interestingly, treatment with 5-aza-2′-deoxycytidine, a global DNA methylation inhibitor, reversed natural sex change in this species (17). In the protogynous bluehead wrasse, sex change involves dynamic shifts in expression of genes encoding the DNA methylation machinery (18). Tet proteins, enzymes responsible for removal of DNA methylation, show a peak in expression at intermediate stages of sex change. Moreover, female-specific Dnmt proteins are replaced with male-specific Dnmts, thus suggesting a genome-wide event reprogramming DNA methylation patterns (**Figure 4*c***). In line with these observations, DNA methylation progressively increases as ovaries become testes. Notably, DNA methylation was coupled to gene expression at all stages, and methylation in promoter regions of key sex-defining genes, such as *cyp19a1* and *dmrt1*, was inversely related to gene expression (18).

Similar observations have been reported for protandrous and synchronous hermaphrodites. In the protandrous black porgy, demethylation of the *cyp19a1a* promoter in the transitionary gonad was identified as an early sign of individuals transitioning to female during natural sex change (14). Likewise, induction of femaleness through testis excision produced *cyp19a1a* promoter demethylation. Importantly, treatment with E2 was able to induce transient ovarian development (during which the ovary reverts to testis after steroid withdrawal) and did not induce demethylation. In protandrous barramundi, Domingos et al. (16) found *cyp19a1a* and *amh* promoters are

hypomethylated in ovaries compared with testes, whereas *dmrt1* and *nr5a2* (alt. *sf1*) are hypermethylated. Interestingly, the authors found promoter DNA methylation was inversely related to gene expression only for *dmrt1* and *nr5a2*, and alternative splicing resulted in nonfunctional sex-specific isoforms for *dmrt1* in females and *cyp19a1a* in males, suggesting these alternative forms of epigenetic control and posttranscriptional modifications can also regulate sexual fate. In the mangrove killifish (*Kryptolebias marmoratus*), a partially simultaneous hermaphrodite that is capable of self-fertilization, temperature affected sex ratios (male/hermaphrodite) and methylation patterns of genes associated with sex differentiation, including *cyp19a1* (DQ339107.1), *sox9a*, and *dmrt1* (15). Taken together, these observations reveal that DNA methylation dynamics are critical for both maintaining sexual identity and reprogramming sexual fate in sex-changing fish.

Histone Modifications and Other Epigenetic Mechanisms

The nucleosome is the functional unit of chromatin and is composed of a histone octamer (two of each of four core histones: H2A, H2B, H3, and H4) around which DNA is wrapped. The nucleosome is a dynamic structure and can undergo extensive changes in conformation and composition that determine DNA accessibility and control gene expression (91). Posttranslational modifications (PTMs) of histones may exert those effects through two mechanisms: first, by influencing directly the structure of chromatin architecture in short or long distances, and second, by regulating the recruitment of specific effector molecules, such as chromatin remodeling factors and transcriptional regulators. A large number of enzymes that direct histone PTMs, including histone acetyl-transferases (HATs), histone deacetylases (HDACs), histone methyl-transferases (HMTs), and kinases, have been described and linked to active chromatin remodeling (**Figure 4*b***). Both the histone PTMs and the enzymes that direct those modifications have been hypothesized as finely tuned sensors for environmental and metabolic cues that influence gene expression (92).

Although many aspects of histone modifications and nucleosome architecture remain unclear, they are increasingly recognized as critical factors of ESD in reptiles (57, 93), sex reversal in mammals (94, 95), and sex determination and sex change in fish (13). In European sea bass, for example, exposure of larvae to high temperatures increased the expression of genes encoding histone-modifying enzymes (*ehmt2* and *hdac11*) and polycomb group (PcG) proteins (*pcgf2*, *jarid2a*, and *suz12*) in early-differentiating female gonads (96). In ricefield eel, DNA methylation in the promoter of *cyp19a1a* is associated with histone 3 (Lys9) deacetylation and trimethylation in testis, suggesting that epigenetic control of key sex genes plays a critical role in initiating and maintaining the sex-specific expression programs in sex-changing fish (17). In agreement with those observations, transcriptomic analysis in Sparidae hermaphrodites showed sex-biased gonadal expression for histone PTM enzymes, such as HATs (*ep300a*, *hat2b*) and HDACs (*hdac2*, *hdac8*, *hdac10*, and *hdac11*) (97). Similarly, in bluehead wrasse, HATs (e.g., *ep300a/b*, *hat1*, *kat8*) and HDACs (e.g., *hdac2*, *hdac7*, *hdca10*) were found to be dynamically expressed across gonadal sex change (18). This study also found that *ezh2*, *suz12*, *eed*, and their cofactor *jarid2*, components of the polycomb repressor group 2, were dynamically regulated during female-to-male sex change (18). These data support a central role for histone modifications and chromatin remodeling in shaping gonadal phenotype (**Figure 4*c***).

GENE/GENOME DUPLICATION AND SEXUAL PLASTICITY

Why fish exhibit such diverse and plastic sexual developmental patterns remains unresolved but may be linked to their accelerated rate of genomic evolution and gene duplication relative to other

vertebrates (5). Gene duplication provides raw material for evolutionary innovation through mutational processes that partition ancestral gene functions between copies (subfunctionalization) or confer novel function to either copy (neo-functionalization) (98, 99). A whole-genome duplication event at the base of the teleost tree (teleost whole-genome duplication, or TWGD), plus frequent tandem and regional duplications in different lineages, has expanded the developmental genetic tool kit of fish (100, 101) and promoted flexibility within sexually dimorphic gene pathways (102).

Many sex-pathway genes are duplicated in teleosts and show evidence of functional divergence between copies. Most obviously, neo-functionalization has repeatedly elevated duplicated sex-pathway genes into master sex-determining roles, contributing to the diversity and rapid turnover of teleost sex-determining systems (5, 103). Examples include *dmy*/*dmrt1y* from *dmrt1* in medaka (*Oryzias latipes*) (104, 105) and *amhy* from *amh* in Patagonian pejerrey (*Odontesthes hatcheri*) and Nile tilapia (106, 107). Perhaps more surprising is the secondment of a gene originally unrelated to sex into the sex-determining role in salmonids, where the sdY gene derives from the immune-related gene *interferon regulatory factor 9* (108).

Functional diversification of gene paralogs is also evident in downstream sexual networks. For example, spatial subfunctionalization of duplicate aromatase genes, arising from the TWGD, has likely partitioned estrogen biosynthesis function between the gonad (*cyp19a1a*) and brain (*cyp19a1b*) (109, 110). Strikingly, *cyp19a1* paralogs look to have assumed different evolutionary trajectories in two derived cichlid lineages, where they seemingly have separately acquired novel functions in the testis (111). Other paralogous sex-pathway genes also show evidence of functional shifts in different cichlid lineages based on unexpected tissue-specific expression patterns (e.g., *sox9a*/*sox9b*, *wnt4a*/*wnt4b*) (111). Neo-functionalization through rapid sequence divergence of a duplicated androgen receptor gene (AR-B) has produced a hyperactive subtype in the Acanthomorpha (112, 113), which encompasses most modern teleosts, including the vast majority of hermaphroditic species (4).

To what extent sub- and neo-functionalization have facilitated the repeated evolution of sex change in fish remains unclear. However, recent transcriptomic studies in sequential hermaphrodites reveal surprising, divergent sex-specific expression patterns for several paralogous sex-pathway genes. In the protogynous bluehead wrasse, several critical female-pathway genes, notably *foxl2a*/*foxl2b* (alt. *foxl3*) and those in the Rspo1/Wnt/β-catenin signaling pathway (*wnt4a*/*wnt4b*, *fstl4*), are duplicated, with one copy exhibiting the expected ovary-specific expression pattern that declines across sex change and the other becoming upregulated as testicular structures appear, suggesting such duplicates have acquired new roles associated with male development (18). In contrast, paralogous male-pathway genes (e.g., *sox9a*/*sox9b*) appear to have retained male-specific expression patterns (18). Moreover, in contrast to mammals, in which only three DNA methyltransferase genes (*Dnmt1*, *Dnmt3a*, and *Dnmt3b*) have been identified, genome duplications have affected *dnmt* evolutionary history in fish (114). In zebrafish, for example, one maintenance *dnmt1* and six de novo methyltransferases (*dnmt3–8*) exist (115). Despite the overlapping functions of Dnmt proteins, differences in the expression of Dnmt3 paralogs suggest gene subfunctionalization (115). As mentioned, *dnmt* genes show sex-specific expression patterns in blueheads that become inverted as sex change progresses (**Figure 4*c***) and a female gonadal methylation pattern is replaced with a male pattern (18, 29).

Comparative transcriptomic analyses across five sparid fish (Spariformes) with diverse reproductive modes (rudimentary hermaphroditism, protogyny, protandry, or gonochorism) similarly revealed striking species differences in sex-biased gonadal gene expression for many paralogous and single-copy sex-related genes, including follistatin-like genes, steroid receptors, and epigenetic regulators (97). Divergent sex-specific expression of paralogous sex-pathway genes, but also epigenetic regulators, may facilitate sexual plasticity in sequential hermaphrodites.

Overall, these data suggest that even key sexual developmental genes, notably those central to the sex steroid and Wnt/β-catenin pathways, are duplicated and have undergone functional diversification in teleost fish, supporting the link between sexual and genomic plasticity in fish (5, 102). Notably, examples of originally masculinizing genes gaining feminizing functions appear to be rarer. Functional and comparative genomic studies required to test these ideas further are becoming increasingly feasible with new technologies (e.g., clustered regularly interspaced short palindromic repeats, or CRISPR) and the growing availability of high-quality genome assemblies for diverse fish.

APPLICATIONS

Aquaculture

Several commercially valuable aquaculture species (e.g., grouper, barramundi, sea bass) naturally change sex or undergo temperature-sensitive ESR. Thus, uncontrolled sex change in fish farms can have a direct impact on the economic potential of these ventures. In certain cases, developing monosex populations is desirable for boosting growth rates or securing availability of broodstock of a particular sex (116). That is why the technology necessary to accomplish control over sex ratios of commercially valuable species for aquaculture has become critical to successfully obtaining profitable stocks (116, 117). Sex control in fish can also be useful for conservation, as a tool to induce reproduction in endangered populations, or to prevent pest species from propagating (118, 119). Genetic or epigenetic tools for controlling sex ratios would offer an efficient, low-cost alternative to the current widespread use of hormonal treatments to produce monosex stocks that also risk steroid contamination of the environment (10).

Possible Effects of Climate Change on Sex-Changing Species

For species in which temperature exerts an effect on sexual fate, rising global temperatures are expected to impact sex ratios, raising concerns regarding the adaptation and survival of such species under future global warming. For example, rapid changes in ocean warming and acidification could cause nonadaptive, highly skewed sex ratios in some fish (19). This would primarily affect populations with temperature-dependent sex determination (TSD), or those species liable to ESR. In reptiles, for example in the case of the Australian central bearded dragon (*Pogona vitticeps*), high temperatures can promote epigenetic modifications that alter the function of the *Jumonji* family genes *jarid2* and *jmjd3*, overriding chromosomal sex-determining cues and inducing sex reversal in this species (57). In fact, several TSD fish and reptile species have been used as a proxy for the measurement of the biological impact of temperature fluctuations (120, 121). Effects of climate change have already been investigated in some TSD sea turtle species, with modeled scenarios predicting highly skewed sex ratios in some turtle populations, as well as an increase in mortality rates (122). The effects of habitat and temperature on sex ratios of wild populations of juvenile southern flounder (*Paralichthys lethostigma*) have also been described (121). These studies lead us to infer that similar repercussions could threaten other fish species (123). Among these, potential effects in addition to those on sex ratios may include shifts in distribution, modifications in developmental timing and larval dispersal, and physiological and behavioral alterations (123).

FUTURE PERSPECTIVES

Single-Cell Sequencing

The gonad is a unique and complex organ, whose high cellular heterogeneity makes it difficult to fully understand the molecular and cellular basis underlying sex determination and sex change

in fish (124). The arrival of single-cell technologies has opened a new and exciting front in this area. Although single-cell transcriptomics has illuminated the process of cell-fate commitment in mammalian sex determination, to date, there are no single-cell studies during sex change in fish or any other vertebrate (125). Technical issues, such as sample collection, cell preparation, and lack of genomic resources for nonmodel species, have made its adoption challenging. We expect the growing ease of access to single-cell platforms and the rapid development of sequencing technologies will stimulate research in this area. Key questions remain to be addressed with single-cell technologies: What are the developmental origin and trajectory of the germline and soma populations in the transitioning gonad? How is the sex-specific network rewired at a single-cell level? Is there evidence of rare and unknown cell populations at differentiated or intermediate stages? As single-cell technologies mature and new methods to coexamine epigenetic, transcriptional, and proteomic information from individual cells, in a spatially explicit manner, become more widely used, they promise to revolutionize our knowledge of gonadal development and sexual plasticity in fish, and vertebrates more generally.

Tissue Culture

The ex vivo culture of living cells, tissues, or organoids for the study of sex change is a largely unexplored but promising area of research. Tissue culture is an important tool that offers a wide range of advantages, such as the ability to carefully control experimental conditions in a well-defined environment that is easily manipulated. Moreover, tissue culture allows for a greater number of experimental replicates while minimizing animal usage, in comparison to in vivo studies. Successful manipulations of Japanese eel (*Anguilla japonica*) (126) testes explants and three-spot wrasse (*Halichoeres trimaculatus*) (127) ovaries have been demonstrated, which suggests a potential application of this technique in the study of cell dedifferentiation and lineage reprogramming in sex-changing fish.

Genetic and Epigenetic Editing

Technological advances in genome engineering, including precise gene editing, hold promise for dissecting the molecular mechanisms behind complex developmental processes like sex determination and development (128). Those tools, including ZFNs (zinc finger nucleases), TALEN (transcription activator-like effector nuclease), and CRISPR/Cas9, allow a reverse genetic approach by analyzing the effect of engineered DNA modifications on phenotype. Numerous genes involved in sex determination and reproduction have already been targeted in model fish species, such as zebrafish and medaka. Such experiments have uncovered unexpected divergent roles of known sex genes in mammals and new mechanisms driving sexual fate (128, 129). More recently, targeted epigenetic editing by programmable DNA binding domains fused to an enzymatic or scaffolding effector domain (e.g., DNMT3 and TET1 catalytic domains, VP64, KRAB) have been implemented successfully in cellular and animal models, leading to new insights into the function of epigenetic modifications in gene expression (130). Limitations in genomic research and experimental manipulation of sex-changing fish species have thus far prevented application of these techniques in such species. Nevertheless, we expect that such limitations will soon be overcome, and that analyzing the role of specific genes or sequences using genetic and epigenetic editing tools will lead to important insights into sexual plasticity and sex change in fish.

CONCLUSIONS

Recent years have seen new and surprising insights into the fundamental molecular processes that underlie sexual plasticity. Across several systems, we now appreciate that redirection of gonadal

fate begins when the expression of key sex-maintenance genes is interrupted (e.g., *cyp19a1a* in protogynous, and *drmt1* in protandrous, species), prompting collapse of the prevailing sex-specific expression network, endocrine environment, and gonadal anatomy, and enabling establishment of an opposing expression landscape that promotes gonadal development of the secondary sex (8, 18). The upstream molecular triggers of this cascade remain unclear. However, recent studies implicating the stress axis and epigenetic modifications in linking environmental cues with sexual fate decisions, in fish and reptiles, have been major breakthroughs. What specific roles different epigenetic changes play in initiating, regulating, and maintaining sex change, and the degree to which these are conserved across species, warrants particular research attention. How environmental cues for sex change are perceived and integrated in the brain to initiate gonadal metamorphosis also remains a central question, and one that is poorly understood in phenotypic plasticity research and developmental biology generally.

Going forward, to fully appreciate how transformations across multiple biological axes coordinate to initiate and progress sexual metamorphosis, and whether common mechanisms regulate this transformation in both directions, will require integrative approaches. In the post-omics era, abundant high-throughput technologies now permit the study of biological molecules and their interactions at exceptional scale and resolution, and applying multi-omics approaches on the same samples enables us to measure molecular changes across multiple systems simultaneously. For example, combining mRNA expression data [e.g., RNA-seq, CAGE-seq (cap analysis gene expression sequencing)] with data on DNA methylation (e.g., RRBS, or reduced representation bisulfite sequencing), active chromatin regions (ATAC-seq), and histone modifications and transcription factor binding (e.g., ChIP-seq) can link candidate epigenetic regulators with expression changes. Technological advances enabling application of multi-omics technologies to single cells (131), and which provide spatially resolved information (132), are now also a reality and hold particular promise for understanding how sexual metamorphosis is cued, triggered, progressed, and maintained at a molecular and cellular level. Successful integration of multi-omics technologies will depend on overcoming challenges regarding the statistically powered design, statistically rigorous analysis, and biologically appropriate interpretation of such experiments (133) but can be expected to drastically advance our understanding of sexual plasticity.

DISCLOSURE STATEMENT

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