Bending Genders: The Biology of Natural Sex Change in Fish

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Abstract

Sexual fate is no longer seen as an irreversible deterministic switch set during early embryonic development but as an ongoing battle for primacy between male and female developmental trajectories. That sexual fate is not final and must be actively maintained via continuous suppression of the opposing sexual network creates the potential for flexibility into adulthood. In many fishes, sexuality is not only extremely plastic, but sex change is a usual and adaptive part of the life cycle. Sequential hermaphrodites begin life as one sex, changing sometime later to the other, and include species capable of protandrous (male-to-female), protogynous (female-to-male), or serial (bidirectional) sex change. Natural sex change involves coordinated transformations across multiple biological systems, including behavioural, anatomical, neuroendocrine, and molecular axes. We here review the biological processes underlying this amazing transformation, focussing particularly on its molecular basis, which remains poorly understood, but where new genomic tech-

nologies are significantly advancing our understanding of how sex change is initiated and progressed at the molecular level. Knowledge of how a usually committed developmental process remains plastic in sequentially hermaphroditic fishes is relevant to understanding the evolution and functioning of sexual developmental systems in vertebrates generally, as well as pathologies of sexual development in humans.

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Natural Sex Change in Fish

The fundamental dichotomy that establishes an individual as either female or male in early embryonic development remains fixed throughout life in most organisms. However, in diverse plant and animal species, individuals begin life as one sex, changing sometime later to the other in a process called sequential hermaphroditism [Warner, 1975; Munday et al., 2006a; Vega-Frutis et al., 2014]. Teleost fishes are the only vertebrate lineage with sequentially hermaphroditic representatives. For such species, sex change is a usual event in the reproductive cycle and is typically cued by changes in social structure or attainment of a critical age or size [Warner, 1984; Godwin,

2009; Kobayashi et al., 2013]. Sequential hermaphroditism is documented in at least 27 families spread across 9 teleost orders and displays in 3 patterns [Avisé and Mank, 2009]: (1) protogynous (female-to-male), (2) protandrous (male-to-female), and (3) serial bidirectional sex change. In all cases, functional sex reversal entails radical restructuring of the gonad plus changes in morphology and behaviour [Warner, 1984; Nakamura et al., 2005; Godwin, 2009].

The biological processes and adaptive advantages of natural sex change have fascinated scientists for decades [Ghiselin, 1969; Robertson, 1972; Warner, 1975, 1984; Williams, 1975; Munday et al., 2006a]. The ecological and evolutionary contexts in which sequential hermaphroditism occurs in fishes are now well studied [reviewed by Nakamura et al., 2005; Avisé and Mank, 2009; Godwin, 2009; Lamm et al., 2015]. The behavioural, anatomical, and hormonal transformations that characterise protogynous, protandrous, and bidirectional sex change are described for several representative species [e.g., Nakamura et al., 1989; Warner and Swearer, 1991; Godwin and Thomas, 1993]. However, proximate causes of sex change remain largely speculative. How environmental or physiological cues are interpreted, processed, and transduced to initiate sex change remains unknown. How sex change is facilitated at the level of the genome, through transient and persistent changes in gene expression, is still largely unstudied. In particular, the trigger and ensuing molecular cascade that transforms one sex into another has not been described for any species.

Teleosts are incredibly diverse and plastic in the ways that sex is both determined and expressed. Sex may be determined genetically (e.g., XY, ZW, polygenic), environmentally (e.g., temperature, pH, population density), or through their interaction, and species may exhibit gonochorism (fixed separate sexes), sequential or simultaneous hermaphroditism, or even single sex populations [Devlin and Nagahama, 2002; Kobayashi et al., 2013]. All 3 expressions of sequential hermaphroditism occur sporadically across the teleost tree of life, implying multiple evolutionary origins and a high degree of evolutionary plasticity in reproductive mode among fishes. Therefore, fish are obvious models for studying the functioning and evolvability of sex determination and differentiation systems in vertebrates [Wilhelm and Koopman, 2006; Matson and Zarkower, 2012; Herpin et al., 2013; Herpin and Schartl, 2015]. Sex determination refers to the biological mechanisms that decide sexual fate, while sex differentiation describes the ensuing developmental processes that realise that fate. Knowledge of how

sex change occurs naturally in fish would greatly enhance our understanding of cellular differentiation, reprogramming, and developmental commitment [Orban et al., 2009; Koopman, 2008; Holmberg and Perlman, 2012], relevant to understanding both normal gonadal development in vertebrates and atypical sexual development in humans. Sequential hermaphroditism is also a dramatic example of developmental (phenotypic) plasticity in response to the environment [West-Eberhard, 2003; Aubin-Horth and Renn, 2009; Moczek, 2015]. Epigenetic regulation of gene expression provides a missing link between gene-by-environment interactions and plastic developmental responses [Jaenisch and Bird, 2003; Zhang and Ho, 2011] and is a nascent area of research with broad cross-disciplinary applicability [Feinberg, 2007].

Here, we review current knowledge of the biological and evolutionary processes underlying sex change in sequentially hermaphroditic fish. We highlight recent progress in our understanding of how sex change is controlled at the molecular level and discuss new opportunities afforded by emerging genomic technologies for elucidating the proximate triggers and ensuing molecular cascades that underlie natural sex change in fishes.

Why Change Sex (and When)?

Size Advantage and the Evolution of Sequential Hermaphroditism

The dominant theory explaining the adaptive significance of sequential hermaphroditism, and the timing and direction of sex change in teleost fishes, is the size advantage model (SAM) [Ghiselin, 1969; Warner, 1975; Munday et al., 2006a; Kazancioglu and Alonzo, 2010]. According to the SAM, sex change is adaptive when reproductive value is greater as one sex when small and another sex when older (and therefore larger), such that the timing of sex change should maximise expected lifetime reproductive success (i.e., combined male and female fitness) [Warner, 1988]. Whether the reproductive value of males or females increases more steeply with body size determines whether female-first or male-first hermaphroditism is adaptive, respectively, and is tightly associated with a species' mating system and social structure [Munday et al., 2006a]. Evolutionary transitions between reproductive modes, for example shifts from gonochorism to protogyny, are associated with changes in mating system dynamics [Erisman et al., 2013].

Fig. 1. Examples of ecological contexts for protogynous (A), protandrous (B), and serial bidirectional (C) sex change in social fishes. **A** The lek-like social structure of bluehead wrasse (*Thalassoma bifasciatum*) is typical of many protogynous hermaphrodites. A brightly-coloured terminal-phase male defends and courts a group of smaller females on a tropical reef in the Caribbean Sea. Loss of a terminal-phase male prompts one of the females to change sex and replace him. Image author: Kevin Bryant. **B** A colony of protandrous cinnamom clownfish (*Amphiprion melanopus*) inhabit-

ing a bubble anemone off the north coast of Timor-Leste in the Pacific Ocean. A monogamous breeding pair consisting of a dominant female and a smaller male share shelter space with several immature subordinates. Image author: Nick Hopgood. **C** A monogamous pair of Maori coral gobies (*Gobiodon histrio*), a species widespread on tropical reefs of the Indo-West Pacific. The sessile lifestyle of coral gobies living among spatially isolated coral habitats means that serial bidirectional sex change enables any 2 fish to form a heterosexual breeding pair. Image author: Richard Field.

Protogyny

A male size advantage on reproductive success should drive the evolution of protogynous sex change. Protogyny is the predominant expression of sequential hermaphroditism in fishes, occurring in 15 families, and is especially pervasive in social species with polygynous mating systems. In these species, large males use aggressive territorial defence to monopolise matings with females [Warner, 1984]. This leaves small males at a severe reproductive disadvantage, leading to strong selection for size-based protogyny [Warner and Swearer, 1991]. By reproducing initially as a female while small, an individual may significantly increase its lifetime reproductive success by changing sex to male and breeding with multiple females later in life after reaching a larger size.

Protogyny is ubiquitous in highly social wrasses (Labridae), of which the bluehead wrasse (*Thalassoma bifasciatum*) is an especially well-studied model. Bluehead wrasse are small, polygamous reef fish with a lek-like mating system, whereby dominant males defend spawning sites to which females have high fidelity (fig. 1A) [Warner and Schultz, 1992]. Loss of a dominant (terminal phase, TP) male stimulates sex change in (typically) the largest female of a social group and involves dramatic changes in behaviour, anatomy, and colouration [Warner and Swearer, 1991]. Although most juvenile bluehead wrasse develop as females, some develop directly as small, female-mimic (initial phase, IP) males, which employ a

‘sneaker’ mating tactic [Semsar and Godwin, 2004]. IP male development is also under social control: more IP males develop on high-density reefs where TP males can less-effectively monopolise mating opportunities [Munday et al., 2006b]. In these and other diandric fish, it remains unknown whether the same or different molecular pathways are involved in sex determination and differentiation of IP and TP males.

Proximate triggers of sex change are less obvious in group-spawning species that lack a well-defined social structure. Groupers (Epinephelidae) periodically form large breeding aggregations where typically one male and several females will break off to spawn. Protogynous sex change in such species may depend on attainment of a threshold age and/or size, as well as the population density and sex ratio at spawning [Shapiro et al., 1993; Bhandari et al., 2003].

Protandry

A strongly female-biased reproductive size advantage should favour the evolution of protandrous sex change. Protandry is less taxonomically widespread, occurring in 6 families, and is usually associated with monogamous or random mating systems without male territorial defence or intense sperm competition [Munday et al., 2006a]. In such cases, male-to-female sex change is adaptive because of the positive relationship between female fecundity and body size [Warner, 1975].

Protandrous sex change is socially controlled in monogamous anemonefish (*Amphiprion* and *Premnas* spp.) [Fricke and Fricke, 1977; Godwin, 1994] (fig. 1B). Anemonefish live in small social groups among venomous sea anemones. A single mating pair consists of a large dominant female and a small male plus smaller subordinate non-breeders. Loss of the dominant female prompts protandrous sex change in her partner and maturation of the most dominant immature fish as the new breeding male. In this limited shelter space scenario, restrictions on body size mean that a breeding pair will have greater reproductive value when the larger individual is female, and monogamous protandry is favored over polygamy-based protogyny [Hattori, 2012].

Many commercially valuable aquaculture species are protandrous, and there is considerable interest in controlling sex ratios by manipulating sex change in farmed fish [Budd et al., 2015]. Most research has focussed on black porgy (*Acanthopagrus schlegeli*, Sparidae), which reproduce as male for the first 2 years of life before roughly 50% of fish change sex to female [reviewed by Lee et al., 2001; Wu et al., 2010a; Wu and Chang, 2013]. Australian barramundi (*Lates calcarifer*, Latidae) and gilthead seabream (*Sparus aurata*, Sparidae) also sexually mature as male before becoming female at an older age and larger size [Guiguen et al., 1994; Liarte et al., 2007]. Whether a threshold age and/or size also triggers sex inversion in protandrous group-spawning species remains unclear [Guiguen et al., 1994].

Bidirectional Sex Change

Bidirectional hermaphrodites have the capacity for sex change in either direction, potentially repeatedly during their lifetime. Field evidence for bidirectional hermaphroditism is limited to 10 species in 5 families [Manabe et al., 2013; Kuwamura et al., 2015], and most reports are for species formerly thought to be protogynous. For example, in some socially polygamous and primarily protogynous species where social structure is highly unstable, sex-changed males may revert back to female should they find themselves competing with a larger male (e.g., Okinawa pygmy goby, *Trimma okinawae*, Manabe et al. [2007]; cleaner wrasse, *Labroides dimidiatus*, Kuwamura et al. [2011]). Natural bidirectional sex change has not been reported for any otherwise protandrous species.

True serial sex change is characteristic of monogamous coral-dwelling gobies (fig. 1C) (e.g., *Gobiodon* and *Paragobiodon*). Sex change in these fish is not well explained by the SAM and is thought to provide reproductive assurance in the face of niche specialisation and a

sessile lifestyle [Nakashima et al., 1995; Munday et al., 1998]. Coral gobies experience limited mating opportunities and a high risk of moving between spatially isolated coral colonies. The ability to change sex repeatedly in either direction allows any 2 fish to form a heterosexual breeding pair, thus reducing travelling distance and predation risk when finding a partner as well as time between breeding events [Munday, 2002; Munday et al., 2010].

Gonadal Sex Change

Natural sex change in teleost fishes involves radical restructuring of the gonad to transform a functional gonad of one sexual phenotype into that of the opposite sex [e.g., Bhandari et al., 2003]. Detailed histological descriptions of gonadal sex change have been made for numerous species representative of protandrous [Godwin, 1994; Guiguen et al., 1994; reviewed by Lee et al., 2001; Wu et al., 2010a], protogynous [Nakamura et al., 1989; Lo Nostro et al., 2003; Muncaster et al., 2013; reviewed by Liu et al., 2016], and bidirectional [Sunobe et al., 2005; reviewed by Cole, 2010, 2011; Kuwamura et al., 2015] hermaphrodites. There is considerable diversity in gonadal configuration and ontogeny among different sequentially hermaphroditic lineages, which closely follows phylogenetic lines and reflects the multiple independent evolutionary origins of hermaphroditism [Sadovy de Mitcheson and Liu, 2008; Cole, 2010]. In many sequential hermaphrodites, recognisable tissues of both sexes are present in the gonad prior to sex change, whereas in others, reproductive tissues are completely replaced by those of the secondary sex.

Gonadal restructuring is complete in protogynous wrasses, where no testicular tissues are detectable in the ovary prior to sex change, and secondary male testes have only a remnant ovarian lumen and lamellae structure as evidence of their former function [Warner and Robertson, 1978]. Protogyny has a common evolutionary origin in the wrasses, and key histological events during gonadal sex change are comparable across species [Nakamura et al., 1989]. Ovarian follicle atresia and oocyte degeneration heralds the onset of sex change, followed by proliferation of spermatogonia and Leydig cells in the peripheral ovarian lamellae, before commencement of spermatogenesis characterises the fully functional testis of a male fish capable of fertilising eggs (fig. 2A). This process is completed with impressive rapidity in the tropical bluehead wrasse, taking as little as 8 days [Warner and Swearer, 1991]. The process may take several weeks or months in temperate wrasses [Muncaster et al., 2013]. In other

protogynous species, including seabass and groupers, crypts of resting spermatogenic tissues are present within the ovarian germinal epithelia prior to sex change [Shapiro et al., 1993; Bhandari et al., 2003; Sadovy de Mitcheson and Liu, 2008]. In protandrous barramundi, reorganisation of gonadal morphology is similarly dramatic, and ovarian tissues develop inwards from the ventral periphery of the gonad to completely replace testicular tissues [Guiguen et al., 1994] (fig. 2B). Previtellogenic oocytes may also be present in testes of adult male barramundi prior to sex change [Guiguen et al., 1994].

In other sequential hermaphrodites, sex change proceeds from a bisexual gonad or ovotestis. Male and female regions are delimited by connective tissue in the ovotestis of porgies (Sparidae), which include protandrous and protogynous representatives [Sadovy de Mitcheson and Liu, 2008]. In protandrous black porgy, active testicular tissues dominate the ovotestis during the first and second spawning seasons when fish are functionally male [Wu et al., 2010a]. At this time, the small ovarian portion contains oogonia and a few primary oocytes but matures and expands during non-spawning (intersex) periods [Lee et al., 2008]. As the third breeding season approaches, testicular tissues redevelop in those fish that remain functional males but are completely replaced by ovarian tissue in fish that change sex to female [Lee et al., 2008]. Protandrous anemonefish also have a bisexual gonad when male, which is composed of active spermatogenic tissue and previtellogenic oocytes that are topographically distinct but not separated by boundary tissue [Shapiro, 1992; Godwin, 1994].

The ovotestis of bidirectional sex-changing gobies contains ovarian and testicular portions simultaneously (fig. 2C), with either portion being reproductively fully functional and so characterising the current sexual phenotype [Sunobe et al., 2005; Cole, 2010, 2011; Kuwamura et al., 2015]. Maintaining a bisexual gonad affords these species the flexibility to rapidly adjust their sexual phenotype (e.g., 7 days in *T. okinawae*, Sunobe et al. [2005]) and exploit any reproductive opportunity, consecutively if necessary. Sequential hermaphroditism has multiple evolutionary origins in gobiid fishes, and there is substantial taxon-specific variation in gonadal configuration. This varies from the undelimited ovotestis of *Eviota* gobies, where male and female cells are intermixed, to an increasingly complex and compartmentalised organ (as in *Lythrypnus*, *Trimma*, *Gobiodon* and *Paragobiodon* spp.) [for review, see Cole, 2010].

Even in many gonochoristic fishes, the juvenile gonad passes through a bisexual phase before developing as fully

Fig. 2. Histological details of the gonad of protogynous (A), protandrous (B), and serial bidirectional (C) sex-changing fish. **A** A transitional bluehead wrasse (*Thalassoma bifasciatum*) gonad in the later stages of protogynous sex change (stage 5 of Nakamura et al. [1989]), showing degenerating oocytes (DO) and crypts of proliferating spermatocytes (Sp). Scale bar = 20 µm. Image author: Hui Liu. **B** The ventral portion of a transitional barramundi (*Lates calcarifer*) gonad in the later stages of protandrous sex change, showing developing ovarian tissues including previtellogenic oocytes (PVO). Scale bar = 20 µm. Image author: Quyen Bahn. **C** The paired ovotestis of female-phase (i) and male-phase (ii) individuals of the bidirectional sex-changing goby, *Trimma kudo*. The female-phase ovotestis consists of an enlarged ovarian portion containing many vitellogenic (VO) and previtellogenic (PVO) oocytes, plus a regressed testicular portion. In male-phase individuals, testicular tissue in active spermatogenesis dominates the gonad, and a small ovarian portion contains mostly early-stage oocytes. The male-phase ovotestis also contains an accessory gonadal structure (AGS) not present in the female phase. Scale bar = 20 µm. Image authors: Tomoki Sunobe and Hisaya Manabe.

male or female (e.g., zebrafish, Orban et al. [2009]), while in others, all individuals initially develop an immature ovary [Devlin and Nagahama, 2002]. Sequential hermaphrodites may follow either of these early developmental patterns [Sadovy de Mitcheson and Liu, 2008]. That

germ cells of both sexes coexist at least transiently in the gonad of many gonochoristic fishes implies considerable evolutionary scope for plasticity in reproductive mode.

Cellular Origins during Complete Gonadal Restructuring

It is unclear what cell populations initiate differentiation of the gonad down the opposing sexual path in species where no germinal tissues of the secondary sex are discernible prior to sex change [Nakamura et al., 1989]. Tracing cellular origins and fates during gonadal sex change is made challenging by the difficulty of correctly identifying and staging germ cells in sexually plastic species [Lo Nostro et al., 2003]. In protogynous hermaphrodites, early male tissues are usually first observed at the periphery of the ovarian lamellae in the vicinity of the germinal epithelium [Lo Nostro et al., 2003]. However, it is difficult to establish whether proliferating spermatogonia arise from a dormant, but sexually differentiated, germ cell population (i.e., co-existence of oogonia and spermatogonia) or from sexually bipotent primordial germ cells that are undifferentiated or even reprogrammable [Lo Nostro et al., 2003; Liu et al., 2016].

Current evidence suggests that in protogynous species, testicular construction begins with bipotential germ (gonia) and somatic (epithelial) cells residing within the ovarian germinal epithelium [Lo Nostro et al., 2003]. At the initiation of sex change in freshwater swamp eel (*Synbranchus marmoratus*), gonial cells that formerly produced oocytes become spermatogonia, enter meiosis, and produce sperm [Lo Nostro et al., 2003]. Epithelial cells associated with these early spermatogonia become Sertoli cells, which previously differentiated as granulosa cells in the female ovary [Lo Nostro et al., 2003]. Granulosa cells have been shown to survive and even proliferate in the early transitioning gonad of three-spot wrasse (*Halichoeres trimaculatus*) as their associated oocytes undergo apoptosis and may contribute to testicular construction [Nozu et al., 2012]. Demonstrated bipotentiality of transplanted spermatogonia and oogonia in rainbow trout [Okutsu et al., 2006; Yoshizaki et al., 2010], a strictly gonochoristic species with an XY sex-determining system, indicates that gonial cells retain sexual plasticity in adult teleosts generally and are reprogrammable depending on the somatic microenvironment. Retained developmental bipotency of gonial and somatic cell populations into adulthood is not only significant in enabling sex change in the lifetime of individual sequential hermaphrodites but in the evolvability of sexual plasticity in teleosts generally.

Knowledge of how germinal and somatic cells interact in transitioning gonads to potentially influence sexual fate in a reciprocal manner will be key to understanding precisely how a gonad of one sexual phenotype can be re-engineered into that of the opposite sex. Developing molecular markers to accurately distinguish among cells at early developmental stages will be essential in achieving this goal.

Endocrine Regulation of Sex Change

Steroid Balance Controls Sexual Fate

The balance between gonadal oestrogen and androgen production directs sexual differentiation and gonadal development. In teleosts, 17 β -estradiol (E2) and 11-ketotestosterone (11-KT) are the principal oestrogen and androgen that promote ovarian or testicular function, respectively. The relationship between them is especially close, as production of either E2 or 11-KT depends on the bioconversion of testosterone (T) via the aromatase (*cyp19a1a*) or the 11 β -hydroxylase (*cyp11b*)/11 β -hydroxysteroid dehydrogenase (11 β -HSD, coded by *hsd11b2*) pathways, respectively [see Guiguen et al., 2010]. Therefore, relative expression of these opposing pathways in the gonad determines the sex steroid balance and ultimately controls gonadal fate.

In sex-changing fishes, dramatic shifts in plasma sex steroids accompany gonadal sex change (fig. 3), yet what tips the balance in favour of the opposing hormonal environment remains unclear. A precipitous drop in plasma E2 precedes ovarian degeneration and protogynous sex change and is followed by a gradual increase in 11-KT production at the onset of spermatogenesis [Nakamura et al., 1989; Bhandari et al., 2003, 2006; Ohta et al., 2008; Muncaster et al., 2013]. The opposite trend characterises protandrous sex change, whereby plasma E2 increases after 11-KT levels fall [Godwin and Thomas, 1993; Lee et al., 2001]. In bidirectional gobies, E2 follows this sexually dimorphic pattern, but 11-KT does not [Kroon et al., 2003, 2009; Lorenzi et al., 2012]. The role of 11-KT in gobies is unclear as there is no apparent association between concentrations of this steroid and gonadal development [e.g., Kroon et al., 2009]. Low 11-KT concentrations in gobies have been proposed to reflect the lack of secondary male characteristics in these species and to facilitate rapid switching between sexual phenotypes [Kroon et al., 2009; Godwin, 2010].

Exogenous manipulation of sex steroids causes masculinisation or feminisation in fish [e.g., Chang et al., 1995; reviewed by Devlin and Nagahama, 2002; Higa et al.,

Fig. 3. Shifts in steroid profiles and sex-specific gene expression accompany sex change in each direction. During protogynous sex change (**A**), steroid profiles shift from an oestrogenic to an androgenic environment (adapted from studies in saddleback wrasse [Nakamura et al., 1989] and blue-banded goby [Solomon-Lane et al. 2013]). **C** Following shutdown of the aromatase gene (*cyp19a1a*), female-specific gene expression is progressively down-

regulated before expression profiles become increasingly male-biased [based on Liu, 2016]. The opposite pattern is observed during protandrous sex change (**B**); there is transition from an androgenic to an oestrogenic environment (adapted from studies in anemone fish [Godwin and Thomas, 1993]), and from male-specific to female-specific gene expression (**D**) [based on Wu et al., 2013].

2003; Yeh et al., 2003; Budd et al., 2015]. Application of non-aromatisable androgens downregulates the aromatase pathway in female fish, leading to sex change [Govoroun et al., 2001; Bhandari et al., 2006; Li et al., 2006; Ohta et al., 2012]. Aromatase inhibitors (AI) disrupt ovarian E2 production in protogynous [Higa et al., 2003; Nozu et al., 2009], protandrous [Lee et al., 2001; Nakamura et al., 2015], and bidirectional hermaphrodites [Kroon et al., 2005]. Although AI treatment leads to complete sex change in protogynous species, rescue is possible through the co-administration of E2 [Higa et al., 2003]. That sex change is typically not sustained following withdrawal of hormonal treatments [e.g., Wu et al., 2015] indicates that, while sex steroids clearly regulate gonadal fate, a molecular switch is required to sustain the shift in hormone production and maintain sex change.

Neuroendocrine Control of Sex Change

The oestrogen-androgen balance is ultimately regulated through the hypothalamic-pituitary-gonadal (HPG) axis and its interaction with neighbouring axes (fig. 4). Gonadotropin releasing hormone (GnRH), released in pulses from the hypothalamus, stimulates the pituitary to produce and release the gonadotropins (GtHs) follicle stimulating hormone (FSH) and luteinising hormone (LH) into circulation. GtHs directly regulate gonadal steroidogenesis via receptor-mediated stimulation of ovarian follicle cells or somatic Leydig cells in the testis [reviewed by Devlin and Nagahama, 2002; Weltzien et al., 2004].

Manipulating GnRH or GtH signalling can induce partial or complete sex change in protogynous (e.g., honeycomb grouper, Kobayashi et al. [2010a]; rainbow wrasse, Reinboth and Brnslle-Sicard [1997]) and protan-

Fig. 4. Schematic illustration of neuroendocrine crosstalk between the HPG and HPI axes regulating steroidogenesis and behaviour in teleosts. Solid lines indicate interactions with support from fish models, and dashed lines indicate interactions with support from non-teleost systems. ACTH = Adrenocorticotrophic hormone; AVT = arginine vasotocin; CRH = corticotropin-releasing hor-

none; DA = dopamine; E2 = 17 β -estradiol; GnIH = gonadotropin-inhibitory hormone; GnRH = gonadotropin-releasing hormone; FSH = follicle-stimulating hormone; FSHR = FSH receptor; LH = luteinising hormone; LHR = LH receptor; MIH = maturation-inducing hormone; NE = norepinephrine; T = testosterone; 5-HT = serotonin; 11-KT = 11-ketotestosterone.

drous hermaphrodites [Lee et al., 2001]. Expression of GtH subunits and their receptors (LHR, FSHR) also fluctuates across sex change in protogynous [e.g., Kobayashi et al., 2010a], protandrous [e.g., An et al., 2009, 2010], and bidirectional [e.g., Kobayashi et al., 2009] species. However, the precise roles of GnRH and GtH signalling in controlling sex change is difficult to model as patterns are inconsistent even between closely related species, e.g., contradictory patterns of expression for GtH receptors are observed in protogynous grouper [Alam et al., 2010; Hu et al., 2011] and may reflect species-specific gonadotropin functioning in teleosts [Levavi-Sivan et al., 2010].

The perception and processing of external cues into the physiological responses that characterise sex change remain poorly understood but have been investigated

most intensively in socially protogynous wrasses [see recent review by Lamm et al., 2015; Liu et al., 2016]. Rapid neurochemical changes in the brain drive behavioural sex change and precede gonadal restructuring, which is coordinated via the HPG axis, by several days [Larson 2003a, b; Semsar and Godwin, 2003; reviewed by Godwin and Thompson, 2012; Lamm et al., 2015]. Crosstalk between these distinct, but likely overlapping, neuroendocrine pathways may mediate the effects of the social environment on gonadal state to coordinate sex change at the whole-body level.

Within minutes of TP male removal, dramatic neuroendocrine changes in the brain of large females elicit behavioural sex change [Godwin, 2010; Lamm et al., 2015]. These include fluctuations in several neurochemicals

known to modulate social rank and sexually dimorphic reproductive behaviours in fishes and other vertebrates [Godwin et al., 2000; Perrault et al., 2003; reviewed by Godwin and Thompson, 2012]. Norepinephrine (NE) and arginine vasotocin (AVT) appear to stimulate protogynous sex change, while dopamine and serotonin appear inhibitory [Kramer et al., 1993; Semsar et al., 2001; Larson et al., 2003a, b; Perreault et al., 2003; Semsar and Godwin, 2003, 2004]. In protogynous wrasses, AVT appears especially important in promoting male-typical behaviour such as courtship and aggression [see Godwin and Thompson, 2012; Semsar et al., 2001, 2004]. However, species-specific variations and methodological differences across studies highlight the need for further research that is more broadly representative of different sex change strategies.

There is growing interest in the role of kisspeptins and isotocin (IT) in early sex change. Kisspeptins regulate vertebrate reproduction by stimulating GnRH release [Elizur, 2009; Mechaly et al., 2013; Espigares et al., 2015] but may also mediate transitions in social status [Maruska and Fernald, 2011] via their receptors on AVT and IT neurons [Kanda et al., 2013]. IT, the teleost homologue of mammalian oxytocin, is also associated with social status and sex-specific reproductive behaviours [Goodson and Bass, 2000; Lema et al., 2015]. These are of interest, as rank may serve as a primer for sex change in socially polygynous breeders [Lamm et al., 2015; Liu et al., 2016]. In a recent whole-transcriptome analysis of bluehead wrasse forebrain, increased expression of the gene encoding IT was one of the few statistically significant changes detected across sex change [Liu, 2016]. However, the opposite trend was observed in bluebanded goby, where lower IT activity was recorded in the pre-optic area of males and late-stage sex-changers compared with females [Black et al., 2004]. To date, fluctuations in the expression of kisspeptin (*kiss2*) and its receptor (*kiss1r*) across sex change are reported only for orange-spotted grouper, *Epinephelus coioides* [Shi et al., 2010]. The social-rank hypothesis and a potential regulatory role for kisspeptin and IT in socially controlled sex change warrants further research. Neurochemical regulation of sex change in protandrous and bidirectional species, and species where sex change is not under social control, remains largely uninvestigated.

Sex Change and the Stress Response

The stress response, regulated through the hypothalamic-pituitary-interrenal (HPI) axis, modulates processes central to major life-history transitions, including changes in behaviour, metabolism, and growth [Wada,

2008; Solomon-Lane et al., 2013]. Through the actions of corticotropic releasing hormone (CRH) and glucocorticoid steroids (GCs), the HPI axis responds to environmental stressors, with potentially significant effects on gonadal fate (fig. 4). That these factors apparently also mediate agonistic behaviour and social status information implicates the HPI axis in linking social status to sex change in sequential hermaphrodites [Solomon-Lane et al., 2013].

Elevated temperatures and other environmental stressors cause gonadal masculinisation in various gonochoristic fishes via increased cortisol levels causing down-regulation of aromatase and activation of androgen pathways [Hattori et al., 2009; Hayashi et al., 2010; Yamaguchi et al., 2010; reviewed by Fernandino et al., 2013]. Cortisol is thought to stimulate 11 β -HSD expression, which catalyses the production of both 11-KT and cortisone, the deactivated metabolite of cortisol [Fernandino et al., 2012, 2013]. There is accumulating evidence that, by influencing steroidogenic gene expression and communicating environmental and social status information along the HPI axis, cortisol plays a role in regulating natural sex change in sequential hermaphrodites [Solomon-Lane et al., 2013]. A transient spike in serum cortisol has been recorded during protandrous sex change in cinnamon clownfish [Godwin and Thomas, 1993] and during protogynous sex change in bluebanded goby [Solomon-Lane et al., 2013], experiencing a 'permissive' social environment. Furthermore, long-term cortisol administration was recently shown to promote protogynous sex change in three-spot wrasse [Nozu and Nakamura, 2015]. Therefore, the cortisol pathway may interface between the HPI and HPG axes to promote sex change in sequential hermaphrodites.

Molecular Regulation of Sex Change: Switches, Triggers, and Antagonistic Networks

Sexual fate can no longer be considered an irreversible developmental commitment during early embryonic development but as a battle for primacy between male and female developmental trajectories (fig. 5). Master sex-determining genes are highly variable across vertebrates, yet a core set of downstream effectors act antagonistically in feminising (e.g., *cyp19a1a*, *foxl2*, *wnt4*) and masculinising (e.g., *dmrt1*, *amh*, *sox9*) networks to promote ovarian or testicular development, respectively [see Munger and Capel, 2012]. Therefore, sexual fate depends not only on activating one or other sex-specific network but on continued suppression of the opposing network to maintain

Fig. 5. Antagonistic sex-specific gene networks maintain sexual fate in fishes by promoting either an oestrogenic or androgenic environment. In females (left), *cyp19a1a* expression generates aromatase, which converts testosterone (T) to estradiol (E2) and maintains the autoregulatory feed-forward loop that sustains high oestrogen levels to support ovarian function [Guiguen et al., 2010]. Within this loop, the transcription factors Foxl2 and Sf1 interact to upregulate *cyp19a1a* expression [Wang et al., 2007], which is also controlled by gonadotropins like FSH through the synthesis of cAMP [Guiguen et al., 2010]. An oestrogenic environment reinforces female-specific gene expression while suppressing male-

promoting genes. In males (right), *cyp19a1a* expression and aromatase production is suppressed such that androgenesis prevails and supports testicular function and male-specific gene expression. Androgens have been reported to both inhibit and activate *amh* (+/-) expression [Pfennig et al., 2015]. Dmrt1 suppresses *cyp19a1a* promoter activity directly [Wang et al., 2010] as well as indirectly via its antagonistic relationship with Foxl2 (see text). Dax1 may also negatively regulate *cyp19a1a* expression through its suppression of *sf1* and *foxl2* expression [Wang et al., 2001; Nakamoto et al., 2007].

that fate throughout life [Herpin and Shartl, 2011a]. This makes sexual fate amenable to manipulation by factors that interrupt supremacy of the prevailing sexual network and allow a takeover by that of the opposite sex. These factors are yet to be determined but have presumably come under the control of environmental or physiological cues in sex-changing fish. The capacity to switch between opposing sexual developmental cascades in adulthood to produce the desired sex following the requisite stimulus has evolved repeatedly in teleosts. The molecular mechanism that triggers sex change remains unknown for any fish species.

In fishes, 2 widely conserved components of the molecular machinery essential for vertebrate sexual development are the genes *dmrt1* (*doublesex and mab-3 related transcription factor 1*) and *cyp19a1a/b* (coding for gonadal/brain aromatase that catalyses conversion of androgens to oestrogens). Each is indispensable within male- and female-promoting networks, respectively (fig. 5). In females, *cyp19a1a* expression maintains an auto-regulatory loop that sustains the high oestrogen environment necessary for ovarian function [Guiguen et al., 2010]. In males, Dmrt1 is a critical transcriptional regulator activating male-promoting genes (e.g., *sox9*, *sox8*) while sup-

pressing ovarian pathways (*foxl2* and *rspo1/wnt/β-catenin* signalling) [Herpin and Schartl 2011a, b]. Dmrt1 interacts antagonistically with the female-specific transcription factor Foxl2 to influence *cyp19a1a* expression and control oestrogen production and gonadal fate in teleosts [Kobayashi et al., 2013; Li et al., 2013]. Under- or over-expression of either *foxl2* or *dmrt1* induces sexual cell fate reprogramming and gonadal sex reversal in mice and fish [Uhlenhaut et al., 2009; Matson et al., 2011; Li et al., 2013; Lindeman et al., 2015]. In gonochoristic and sex-changing fishes, *cyp19a1a*, *foxl2*, and *dmrt1* expression is consistently sex-specific, depending on which gonadal phenotype is developing [Xia et al., 2007; Alam et al., 2008; Wu et al., 2008]. Expression of these genes also responds predictably to hormonal manipulations: *dmrt1* is upregulated and *cyp19a1a* and *foxl2* are downregulated in fish given androgens, aromatase blockers, or oestrogen antagonists, whereas oestrogen treatments have the opposite effect [reviewed in Guiguen et al., 2010; Herpin and Schartl, 2011b]. Therefore, a molecular and endocrine feedback loop becomes apparent through which *dmrt1* and *cyp19a1a* regulate the androgen/oestrogen balance to control sexual fate in fish (fig. 5). Evidence that *cyp19a1a*, *dmrt1*, and *amh* (anti-Müllerian hormone)

expression respond to external environmental fluctuations, most notably in fishes where rearing temperature influences sex ratios [Fernandino et al., 2008; Guiguen et al., 2010; Pfennig et al., 2015], raises the possibility that these and other key sex genes may be similarly sensitive to environmental cues that initiate sex change in sequential hermaphrodites.

In trying to understand the molecular control of sex change in sequential hermaphrodites, gene expression studies have focussed on these core genes and their potential proximate regulators. Most work has focussed on the protandrous black porgy [Wu and Chang, 2009; Wu et al., 2010a, b, 2012, 2015] and on protogynous wrasse and grouper [Li et al., 2006; Xia et al., 2007; Alam et al., 2008; Liu et al., 2009; Kobayashi et al., 2010b; Miyake et al., 2012; Horiguchi et al., 2013; Nozu et al., 2015]. Recently, and for the first time, whole-transcriptome expression analysis was used to comprehensively examine how expression landscapes alter across natural sex change in brain and gonad tissues of the protogynous bluehead wrasse [Liu, 2016]. Collectively, these studies have produced some surprising results. In the following sections we highlight several genes and potential regulatory factors that are emerging as important orchestrators, and potential initiators, of natural sex change in sequential hermaphrodites.

Given the now widely recognised antagonism among sex-specific gene networks in a range of systems, how a takeover by the secondary sex is orchestrated to enable sex change in fishes is of broad interest. Is the secondary network activated in order to override that of the primary sex, or must the primary network first be shut down by other factors, thus lifting suppression of the secondary sexual network? Whole-transcriptome expression profiling in bluehead wrasse reveals a clear trend whereby female-related gene expression gradually declines in the gonad (e.g., *dax1*, *figla*, *gdf9*, *hsd7b1*, *hsd11b3*, see key exceptions below) before expression profiles become increasingly masculinised (e.g., *dmrt1*, *gsdf*, *cyp11c1*, *sox9*) [Liu, 2016] (fig. 3A). The reverse pattern is evident from numerous candidate gene studies in protandrous black porgy: male-related gene expression (e.g., *dmrt1*, *amh*, *amhr2*) declines coincidentally with testis volume before expression profiles are increasingly feminised as the bisexual gonad becomes purely ovarian (e.g., *cyp19a1a/b*, *foxl2*, *wnt4*) [reviewed by Wu and Chang, 2013]. Broadly speaking, the greatest shift in sex-specific gene expression occurs during mid-to-late sex change (fig. 3). Therefore, it appears that shutdown of the prevailing sexual network is necessary to promote sex change, which progresses as

suppression of the opposing network is lifted. Genes whose expression changes prior to this shift are particularly interesting as candidate components of the switch that initiates sex change.

Aromatase – The Switch Initiating Protogynous Sex Change?

The gonadal aromatase gene, *cyp19a1a*, currently stands alone as being rapidly and completely shut down at early protogynous sex change. *Cyp19a1a* expression precipitously declines in bluehead wrasse from the first sign of ovarian atresia (histological stage 2) and prior to the collapse of the feminising expression landscape [Liu, 2016] (fig. 3A). *Wnt4a* and *sf1* expression drop off concurrently but are not halted completely. Arrested *cyp19a1a* expression during early female-to-male sex change is widely reported in single-gene studies of other protogynous species [e.g., Li et al., 2006; Zhang et al., 2008; Liu et al., 2009] and is consistent with arrested E2 production at this stage (fig. 3A). Therefore, if *cyp19a1a* downregulation is the switch that initiates gonadal sex change in protogynous fishes, upstream factors that negatively regulate its expression are potential components of the trigger mechanism.

The promoter regions of teleost *cyp19a1a/b* genes contain putative DNA-binding motifs for numerous transcription and endocrine factors with known roles in vertebrate sex differentiation, including Foxl2, steroidogenic factor 1 (Sf1), potential SRY-box (Sox9), Wilms tumor 1 protein (Wt1), GATA binding proteins, cAMP responsive elements (CRE), and response elements for glucocorticoids (GRE), oestrogens (ERE), progesterones (PRE), and androgens (ARE) [reviewed by Gardner et al., 2005; Guiguen et al., 2010]. Epigenetic modification of *cyp19a1a* promoter regions may be a means through which its responsiveness to these factors can be altered (see later section).

Evidence is accumulating for (and against) a role for several proximate regulators of *cyp19a1a* in the initiating stages of sex change. For example, several key sex pathway genes known to regulate *cyp19a1a* expression appear unimportant at early sex change, based on their expression patterns in several protogynous species. Recorded shifts in the expression of *dmrt1*, *dax1*, and *foxl2* occur downstream of *cyp19a1a* shutdown in bluehead wrasse [Liu, 2016]. Similarly in protogynous grouper [Bhandari et al., 2003; Alam et al., 2008] and three-spot wrasse [Nozu et al., 2015], changes in *dmrt1* and *foxl2* expression occur only after serum E2 levels have begun to fall. A spike in *foxl2* expression was even recorded at mid-sex change in

three-spot and bluehead wrasse [Kobayashi et al., 2010b; Liu, 2016]. Foxl2 expression may simply become decoupled from the oestrogen cycle once *cyp19a1a* is shut down by other factors.

One of the few studies examining gonadal gene expression across bidirectional sex change showed a sharp decline in *sf1* expression at early female-to-male sex change in the goby *T. okinawae* [Kobayashi et al., 2005]. Only ovarian *sf1* expression was examined, which also increased gradually during male-to-female sex change [Kobayashi et al., 2005]. In transitioning bluehead wrasse gonads, *sf1* expression also declined abruptly and concurrently with that of *cyp19a1a*, although it recovered thereafter [Liu, 2016]. The potential interaction of *Sf1* with the promoter region of *cyp19a1a* in the initiating stages of female-to-male sex change (and with *Dmrt1* in early protandrous sex change) warrants further investigation.

Dmrt1 and Protandrous Sex Change

Molecular control of protandrous sex change remains poorly understood, with research limited to single gene studies almost exclusively in black porgy. So far, it has been established that *dmrt1* expression decreases sharply at the onset of testicular degeneration in black porgy [Wu et al., 2012], as well as gilthead seabream [Liarte et al., 2007]. Shutdown of *dmrt1* may be important in initiating protandrous sex change, similar to *cyp19a1a* in protogynous species. Knocking down *dmrt1* expression in black porgy resulted in a loss of testicular germ cells and induced ovarian development in some experimental fish [Wu et al., 2012]. In the same study, testicular *dmrt1* expression was significantly lower in fish that several months later underwent sex change to female, relative to those that remained male. However, in an earlier study, reduced *amh* but not *dmrt1* expression was reportedly predictive of sex change [Wu et al., 2010b]. These data are intriguing but require further validation to clarify the prominence of *dmrt1* and *amh* downregulation at early protandrous sex change.

It remains unknown what factors interrupt *dmrt1* expression in early protandrous sex change. *Dmrt1* expression in the male testis is thought to be maintained by androgens and gonadotropin signalling (e.g., LH) via the HPG axis [Herpin and Scharf, 2011b; Wu et al., 2012; Wu and Chang, 2013]. More detailed expression studies in the brain and gonad during early sex change are required to identify potential upstream regulators of *dmrt1*. Functional studies are also necessary to address how negative regulation of *dmrt1* is achieved in early protandrous sex change.

Amh

Amh is a multifunctional growth factor and central player in vertebrate gonadal development in both sexes. The primary role of *Amh* is in early testicular differentiation, where it functions to inhibit germ cell proliferation and steroidogenesis to promote maleness [reviewed by Pfennig et al., 2015]. In protandrous black porgy, expression of *amh* in cells bordering ectopic oocytes has been suggested to suppress ovarian development in the ovotestes of male-phase fish [Wu et al., 2015].

Amh directly inhibits *cyp19a1a* expression in mammals, leading to an inverse relationship in the expression of these 2 genes [Pfennig et al., 2015]. In teleosts, an inverse pattern of *cyp19a1a* and *amh* expression is typical but not universal, as is inhibition of *amh* expression by oestrogens and FSH [see Pfennig et al., 2015]. In whole-transcriptome data from bluehead wrasse, *amh* and its receptor *amhr2* are the first male pathway genes upregulated in the gonad, and their initial increase occurs concurrently with the interruption of *cyp19a1a* expression [Liu, 2016]. Whether *Amh* contributes to the suppression of *cyp19a1a* at this time or is an early response to falling oestrogen levels and a proximate effector of male-specific pathways is unclear and requires finer-scale time series expression data. Early upregulation of *amh* was also observed in the transitioning gonad of ricefield eel [Hu et al., 2015]. However, the timing in relation to expression of other male pathway genes or *cyp19a1a* was not established.

While studies hint at the importance of *Amh* signalling in early protandrous and protogynous sex change, more experimental evidence is needed to elucidate its exact functions. In the gonochoristic tilapia, *amh* and *amhr2* expression in the brain and pituitary appears to modulate FSH and LH release, and occurs prior to *amh* upregulation in the gonad [Poonlaphdecha et al., 2011]. This may suggest a potential role for *Amh* in regulating brain-gonad communication via the HPG axis.

A New Role for Cortisol

Cortisol may be an important physiological mediator through which different environmental stressors or cues affect gonadal development and sexual fate (see above). Three, non-mutually exclusive, means are proposed through which cortisol may promote stress-induced gonadal masculinisation in gonochoristic systems [Fernandino et al., 2013; Pfennig et al., 2015]. Cortisol may act to (1) inhibit aromatase expression via preferential binding to glucocorticoid response elements in the *cyp19a1a* promoter, (2) upregulate *amh* to induce germ cell apoptosis and promote maleness, and (3) stimulate *hsd11b2*

(11 β -HSD) expression, which promotes 11-KT synthesis but also converts cortisol to inactive cortisone. In protogynous hermaphrodites, cortisol may act similarly to induce testicular developmental pathways. The early drop in *cyp19a1a* expression and concomitant activation of *amh* expression recorded in whole-transcriptome data from transitioning bluehead wrasse already supports this link [Liu, 2016]. Moreover, this data also showed subsequent upregulation of *hsd11b2* and downregulation of *hsd11b3* (genes whose products may be respectively responsible for metabolising cortisol to inactive cortisone and vice versa) at mid-sex change [Liu, 2016]. These results are consistent with evidence of a transient spike in serum cortisol levels during the first 3 days of female-to-male sex change in bluebanded goby [Solomon-Lane et al., 2013]. The significance of elevated cortisol in protandrous sex change, as observed in anemone fish [Godwin and Thomas, 1993], remains unclear. However, a dose-dependent effect on androgenesis has been observed [Vandenberg et al., 2012], and cortisol may enhance or suppress 11-KT production depending on species-specific response thresholds [Fernandino et al., 2013]. Functioning of cortisol as a proximate regulator of natural sex change deserves special research attention.

Epigenetic Mechanisms

Epigenetic regulation of gene expression is critical during development [Feng et al., 2010] and is a mechanism through which environmental cues can be translated into plastic phenotypic responses [Bosssdorf et al., 2008]. Epigenetic modifications to DNA (e.g., methylation of cytosine bases) and histones (e.g., acetylation) regulate gene expression by reversibly altering the availability of genes, or specific exons, to transcription and typically inhibit and promote transcription, respectively [West-Eberhard, 2003; Duncan et al., 2014].

There is now good evidence that epigenetic factors regulate sexual fate, including sex reversal, by adjusting the responsiveness of male- or female-promoting gene networks to activation. DNA methylation has been linked to environmentally sensitive sex reversal in several fishes (table 1). For example, methylation-induced downregulation of *cyp19a1a* expression was identified as mediating high-temperature masculinisation of genetically female European sea bass (*Dicentrarchus labrax*) [Navarro-Martin et al., 2011]. Whole-genome methylation data paired with whole-transcriptome expression analysis in half-smooth tongue sole (*Cynoglossus semilaevis*) – a species with ZW chromosomal sex determination and temperature-dependent sex reversal – revealed that sex pathway genes differ-

entially expressed between ovary and testis are also major targets of methylation [Shao et al., 2014]. Methylation signatures of males and sex-reversed ZW pseudomales were indistinguishable. Specifically, it was shown that following sex reversal, female-specific W chromosomal genes are suppressed in ZW pseudomales through methylation [Shao et al., 2014]. Therefore, environmentally-induced sex reversal likely involves sex-specific epigenetic reprogramming of the genome and may include pervasive DNA methylation to silence the opposing sexual pathway.

Epigenetic modifications altering the expression of key sex pathway genes is a plausible, and indeed likely, mechanism through which sex change can be both initiated and maintained in sequential hermaphrodites. However, to date this area has received little research attention. In whole-transcriptome data from bluehead wrasse, several genes encoding DNA methyltransferases (e.g., *dnmt3ab*, *dnmt4*) and histone acetyltransferases (e.g., *kat8*, *hat1*) or deacetylases (e.g., *hdac2*, *hdac10*) showed significant expression changes across gonadal sex change [Liu, 2016]. Specific functions for these genes during sex change cannot be inferred until more is known regarding epigenetic regulation of vertebrate sexual differentiation pathways.

At present, the only evidential link between epigenetic modification of sex pathway genes and natural sex change is described for protogynous ricefield eel. *Cyp19a1a* promoter regions were found to be hypermethylated and deacetylated in testis compared with ovary [Zhang et al., 2013]. DNA methylation was concentrated within putative binding sites for cAMP response elements and Sfl and was shown to block gonadotropin-induced cAMP activation of *cyp19a1a* expression in vitro. Importantly, *cyp19a1a* promoter methylation increased as *cyp19a1a* expression decreased during sex change to male in ricefield eel, and implantation with DNA methylation inhibitors (5-aza-2'-deoxycytidine) could prevent or reverse the process. Therefore, methylation of *cyp19a1a* is likely an essential component of the molecular sex change mechanism and necessary for maintaining a secondary male gonadal fate in protogynous hermaphrodites.

Towards a Mechanistic Model of Sex Change

A new model proposes how a normal female reproductive cycle may be interrupted to initiate sex change in socially protogynous fishes, featuring crosstalk between the HPG and HPI axes [Liu et al., 2016]. Upon loss of a dominant male, rapid neurochemical changes in the hypothalamus of large females promote behavioural sex change (see previous section). Specifically, elevated AVT and NE levels may perturb GnRH and LH dynamics via

Table 1. Evidence for epigenetic control of sex reversal in teleost fishes

Species and sexual system	Gene/s assayed	Epigenetic mechanism	Gonadal gene expression	Reference
European sea bass (<i>Dicentrarchus labrax</i>) XY GSD, high-temperature ♀-to-♂ sex reversal	<i>cyp19a1a</i>	DNA methylation	↓with sex reversal to ♂	Navarro-Martin et al. [2011]
Japanese flounder (<i>Paralichthys olivaceus</i>) XY GSD, high-temperature ♀-to-♂ sex reversal	<i>cyp19a1a</i> <i>dmrt1</i>	DNA methylation DNA methylation	↓testis vs. ovary ↓ovary vs. testis	Wen et al. [2014]
Half-smooth tongue sole (<i>Cynoglossus semilaevis</i>) ZW GSD, high-temperature ♀-to-♂ sex reversal	♀-pathway (e.g., <i>cyp19a1a</i> , <i>figla</i>) ♂-pathway (e.g., <i>gsdf</i> , <i>amh</i> , <i>dmrt1</i>)	DNA methylation DNA methylation	↓ZZ/ZW testes vs. ovary ↓ovary vs. ZZ/ZW testes	Shao et al. [2014]
Ricefield eel (<i>Monopterus albus</i>) protogynous hermaphrodite	<i>cyp19a1a</i> <i>cyp19a1a</i>	DNA methylation histone acetylation	↓testis vs. ovary ↓with sex change to ♂ ↑testis vs. ovary	Zhang et al. [2013]

GSD = Genotypic sex determination.

the HPG axis to induce follicle apoptosis in the ovary, while driving up serum cortisol via the HPI axis [Liu et al., 2016]. Elevated cortisol could block *cyp19a1a* transcription directly and via activation of specific transcription factors (e.g., *Amh*) to instigate the chain reaction of falling E2 levels that accelerate ovarian degeneration and interrupt female-specific gene expression. Thereafter, elevated cortisol levels may stimulate 11-KT production to activate male pathway genes and support testicular differentiation. Changes in the DNA methylation state of *cyp19a1a* and other key genes are likely also important. This is the first detailed mechanistic model linking neurochemical changes occurring in the brain associated with behavioural sex change, with changes in gene expression and endocrine production during gonadal sex change in sequential hermaphrodites.

Conclusions

A picture is beginning to emerge of how transformations across a variety of biological systems integrate to initiate and progress natural sex change. Sex change is clearly initiated in the brain, but where, by what, and how this is communicated to induce gonadal sex change remain areas of significant opacity. Re-direction of gonadal fate begins when expression of critical sex-maintenance genes (e.g., *cyp19a1a* in protogynous and *dmrt1* in pro-

tandrous species) is interrupted, causing a cascaded collapse of the prevailing expression landscape, endocrine environment, and gonadal anatomy. Once antagonistic suppression of the opposing sexual network is lifted, establishment of a new sex-specific expression and endocrine environment drives gonadal development towards the secondary sex. A multi-layered trigger mechanism is likely at the head of this cascade, ensuring that sex change proceeds only under specific circumstances. Recent research already implicates several factors that could act in consort to silence *cyp19a1a* expression in the initiating stages of protogynous sex change (e.g., cortisol, DNA methylation, *Amh*). However, current models are largely based on correlations in the timing of gene expression changes, and experimental validation is required to disentangle cause from effect before the functioning of these mechanisms can be fully appreciated. Also critical will be a deeper understanding of how complex neurological systems in the brain crosstalk with the stress (HPI) and reproductive (HPG) axes to translate external environmental signals into internal physiological responses.

Fishing for Answers

To date, our insights on sex change derive largely from investigations of a handful of protogynous systems and studies on the protandrous black porgy. Contrasting

across a broader spectrum of sex change strategies, using a diversity of study systems, will be critical in understanding how transition between sex-specific developmental networks is achieved during sex change, and whether common mechanisms trigger and regulate the process in both directions. Species capable of bidirectional sex change may be especially revealing regarding the flexibility of the molecular and epigenetic machinery at the heart of the sex change process.

Classically, the establishment of study systems, particularly those involving manipulative genetic approaches, is time consuming and costly. While such work remains challenging, the current revolution in genomic sequencing promises to make the establishment of model systems both easier and more cost-effective. RNA sequencing (RNA-seq) [Wang et al., 2009], especially, enables rapid discovery of the co-ordinated pattern of gene expression, including for uncharacterised genes and isoforms, across a consistent sample time series without the need for prior genetic resources. Whole-transcriptome profiling in bluehead wrasse already demonstrates the value of the RNA-seq approach for identifying candidate triggers of sex change and genes with novel and unexpected roles in vertebrate sexual development [Liu et al., 2015; Liu, 2016]. Single-cell RNA-seq technologies are especially exciting with regards to characterising genetic cascades involved in cellular reprogramming and cell fate determination. Coupling RNA-seq with genome-wide analysis of DNA methylation and histone modification will generate test-

able hypotheses in the continued search for components of the trigger mechanism used by sequential hermaphrodites to alter sexual fate in adulthood. However, manipulative studies will be required thereafter, and an integrative approach will be necessary to address how a common genetic toolkit can be flexibly adapted to achieve sexual plasticity, both within the lifetime of an individual fish and across the various teleost lineages that have independently evolved sequential hermaphroditism. Such in vitro manipulation has historically been confined to a handful of model systems, but it seems likely that new gene editing approaches [Cong et al., 2013] will soon enable us to undertake sophisticated experiments quickly and affordably to precisely document the pathway of events governing the transformation that is natural sex change.

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