DNA methylation memory: Understanding epigenetic reprogramming in vertebrates

by

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Declaration

Except where specific reference is made to other sources, the work presented in this thesis is the work of the author. It has not been submitted, in whole or in part for any other degree.

Oscar Javier Ortega Recalde

Abstract

DNA methylation is an epigenetic mark critical for vertebrate development and is associated with numerous cellular and organismal processes including X-chromosome inactivation, genomic imprinting, and regulation of gene expression. Importantly, DNA methylation patterns are faithfully inherited during cell division, providing an information memory module additional to the DNA code. This mark, along with other epigenetic modifications, plays an essential role in establishing and maintaining cell identity. DNA methylation dynamics has been studied in detail in eutherian mammals, where two major waves of demethylation, the first in the early embryo and the second during germline development, remove most marks. Erasure of epigenetic memory is associated with cell reprogramming, and in mammals, is inextricably linked to increased developmental potency. For divergent vertebrate models, this dynamic is largely untested and indirect evidence suggests epigenetic memory may be retained in the germline. Furthermore, the role of epigenetic memory and reprogramming in major cell fate transitions, such as sex determination and sex change, is underexplored despite being apparently driven by epigenetic mechanisms in at least some species.

In order to understand how epigenetic memory is maintained, erased, and reprogrammed in divergent vertebrates, I have focused on two fish species. I have analysed the epigenome of the germline during gonad development in zebrafish (*Danio rerio*), and the transcriptome and methylome of bluehead wrasse (*Thalassoma bifasciatum*) during female-to-male sex change. Using a combination of techniques, including isolation of germline cells, whole genome bisulfite sequencing, and comparative epigenomics and transcriptomics, I explored

epigenetic memory and reprogramming in these species. This thesis is presented as a collection of research and review papers, as well as a discussion synthesising my results.

In the first paper, I assessed the DNA methylation dynamics during zebrafish germline development (Ortega-Recalde, Day, Gemmell, & Hore, 2019). Using a low-coverage whole genome bisulfite sequencing technique, I found that germline DNA methylation levels remain similar to somatic cells throughout development. Additionally, I discovered a remarkable amplification of ribosomal DNA during the critical period of gonad transformation, principally in females, associated with demethylation. Thus, in stark contrast to mammals, the zebrafish germline preserves global levels of DNA methylation and undergoes an enigmatic sex-specific amplification which may be related to sex determination in this species.

The broader significance of my findings regarding DNA methylation memory in the zebrafish germline are examined in the second manuscript (Ortega-Recalde & Hore, 2019). In this review, I presented contrasting evidence concerning epigenetic memory and amnesia in vertebrates. In addition to my work, I reviewed new results supporting that non-mammalian species do not undergo DNA methylation erasure, and that epigenetic memory may be an underappreciated reservoir of information between generations. I discuss several hypotheses to explain such divergence and the consequences of retention of germline DNA methylation for transgenerational epigenetic inheritance.

The third paper reports a study of socially controlled sex-change in bluehead wrasse (Todd et al., 2019). By using transcriptomic and methylome approaches,

this study revealed the molecular events through which a mature ovary is reprogrammed to a functional testis. In addition to identifying the primary triggers and subsequent molecular cascade of this transformation, this study showed that sex change involves a genome-scale epigenetic reprogramming event, including a marked shift in epigenetic molecular machinery, reminiscent of mammalian naïve pluripotent stem cells and primordial germ cells, and extensive changes in DNA methylation patterns. Thus, epigenetic reprogramming plays a central role orchestrating gonad transformation in this species despite not undergoing global DNA methylation erasure.

The significance of the third paper is discussed alongside other contemporary work in the fourth manuscript (Ortega-Recalde, Goikoetxea, Hore, Todd, & Gemmell, 2019). This exhaustive review presents current evidence regarding the genetic and epigenetic basis of natural sex change in fish. The extraordinary sexual plasticity behind such phenomena shows that sexual fate is a reversible process, initiated by male and female signalling networks, with sexual identity likely preserved long-term by epigenetic modifications in the absence of any underlying genetic changes.

Finally, in the last chapter I discuss the broader implications of my results, consider the limitations of my research, and integrate my findings into a comprehensive body of work. Together, my research shows that epigenetic memory dynamics, in particular DNA methylation, is heterogeneous in the germline of vertebrates, but likely plays a central role in defining sexual fate for at least some fish species.

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Luis Humberto, and my brothers, **Betho** and **Diego**, for their support and encouragement.

This thesis is dedicated to my parents and brothers

We shall not cease from exploration,

and the end of all our exploring

will be to arrive where we started

and know the place for the first time

T.S Eliot (1942)

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11KT	11-ketotestosterone
5-aza	5-aza-2′-deoxycytidine
5caC	5-carboxycytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ATAC-seq	Assay for Transposase-Accessible Chromatin using sequencing
AVT	vasotocin
CGI	CpG island
ChIP-seq	Chromatin Immunoprecipitation Sequencing
CpG	CG dinucleotide
CRISPR	clustered regularly interspaced short palindromic repeats
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DMR	differentially methylated region
dpf	days post-fertilization
E	embryonic day
E2	17β-estradiol
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ERV	endogenous retrovirus
ESD	environmental sex determination
ESR	environmental sex reversal

FACS	fluorescence activated cell sorting
GnRH	gonadotropin-releasing hormones
GSD	genetic sex determination
H2A	histone 2A
H2A.Z (FV)	histone 2A variant H2A.Z (FV)
H3K27me1	trimethylation of histone H3 at lysine 27
H3K4me1	monomethylation of histone H3 at lysine 4
H3K4me3	trimethylation of histone H3 at lysine 4
HAT	histone acetyl-transferases
HDAC	histone deacetylases
HDM	histone demethylases
HMT	histone methyl-transferases
hpf	hours post-fertilization
HPG	hypothalamic–pituitary–gonadal
HPI	hypothalamic–pituitary–interrenal
HPLC-UV	high performance liquid chromatography-ultraviolet
IAP	intracisternal A particle
ICM	inner cell mass
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
LH	luteinizing hormones
LUMO	luminometric methylation assay
MBD	methyl-CpG binding domain
MeDIP	methylated DNA immunoprecipitation
NE	norepinephrine
PBAT	post-bisulfite adaptor tagging
PCA	principal component analysis

PcG	polycomb group
PCR	polymerase chain reaction
PGC	primordial germ cell
PRC	polycomb repressive complex
PRDM	PRDI-BF1 and RIZ homology domain containing
PTM	post-translational modifications
RAD-seq	restriction site associated DNA sequencing
RRBS	reduced representation bisulfite sequencing
RA	retinoic acid
rDNA	ribosomal DNA
RNA	ribonucleic acid
UHRF1	ubiquitin-like containing PD and RING finger domains 1
TALEN	transcription activator-like effector nuclease
TET	ten-eleven translocation
TIM	temperature-induced masculinization
TSD	temperature-dependent sex determination
TWGD	teleost whole-genome duplication
UAS	upstream activator sequence
WGBS	whole-genome bisulfite sequencing
ZFN	zinc finger nucleases

Chapter 1

Introduction

1.1 DNA methylation

Cytosine methylation has been described as an archetypal form of epigenetic modification (Goldberg, Allis, & Bernstein, 2007). 5-methylcytosine (5mC) was first described as part of nucleic acids by Johnson and Coghill in 1925, however, it was not until 1975 that two groups independently suggested that this nucleotide could regulate stable changes in gene expression and cell fate (Holliday & Pugh, 1975; T. B. Johnson & Coghill, 1925; Riggs, 1975). Although it is not the only epigenetic mark known, it has been studied the longest and is likely the best understood (Stricker, Köferle, & Beck, 2017). The biochemical stability and heritability coupled with reversibility and flexibility of this "5th base" provide an additional layer of information to the DNA code, a memory module critical to understand how genetic information is regulated and

interpreted to generate an individual (Heather J. Lee, Hore, & Reik, 2014; Lister & Ecker, 2009).

In eukaryotes, DNA methylation occurs almost exclusively at the C5 position of cytosine and, although it is not obligatory for all species, it is essential for many others (Colot & Rossignol, 1999; Feng, Jacobsen, & Reik, 2010; Raiber, Hardisty, van Delft, & Balasubramanian, 2017). The prevalence and distribution of DNA methylation differ widely among organisms, however, epigenome architecture seems to share an ancient logic (Feng, Cokus, et al., 2010; Long et al., 2013; Zemach, McDaniel, Silva, & Zilberman, 2010). Three different sequence contexts have been described: palindromic CpG (CG), CHG, and CHH (where H = A, C or G). In mammals, 5mC is almost exclusively restricted to the symmetric CG context, and it has been estimated that it occurs at ~ 70% - 85% of CG dinucleotides (Ehrlich et al., 1982; Hon et al., 2013). Methylation is heterogeneous among the genome, being principally located in transposons, gene bodies and intergenic regions, whereas high CpG density regions (CpG islands, CGI) are generally demethylated (P. A. Jones, 2012). Importantly, however, when methylation is found at CGIs which overlap gene promoters, the affected gene is generally transcriptionally silenced (P. A. Jones, 2012).

The molecular machinery required to write, maintain and erase this epigenetic mark is highly conserved in vertebrates (Feng, Jacobsen, et al., 2010; H. Wu & Zhang, 2014) (**Figure 3.1**). *De novo* methylation in mammals is established by DNA methyltransferases 3A and 3B (DNMT3A and DNMT3B) and it is maintained during mitosis by DNA methyltransferase 1 (DNMT1) and UHRF1 (ubiquitin-like containing PD and RING finger domains 1) (H. Wu & Zhang, 2014). UHRF1 is a DNMT1-cofactor that recognizes hemi-methylated DNA

strands (Bostick et al., 2007). DNA demethylation can occur passively, by replication-dependent loss of 5mC in the absence of methylation maintenance (passive dilution); or actively, by enzymes capable of removing and modifying the cytosine methyl groups (H. Wu & Zhang, 2014). Enzymes involved in active DNA demethylation include DNA repair enzymes, cytosine deaminases and DNA glycosylases, however, the initiators of methylation erasure are thought to be the TET (ten-eleven translocation) enzymes. TET enzymes iteratively oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) (Rasmussen & Helin, 2016). These oxidized cytosines can be actively removed from DNA by the base-excision repair pathway without the need for DNA replication (active modification – active removal). Alternatively, because none of the oxidized cytosine variants (i.e. 5hmC, 5fC, 5caC) is recognized by DNMT1, DNA demethylation can occur by dilution following replication (active modification – passive removal) (H. Wu & Zhang, 2014). Together, recent studies of these enzymes have driven significant progress towards understanding the mechanistic process controlling the dynamic regulation of DNA methylation.

Functions of 5mC are critical in many biological processes, including regulation of gene expression, genome stability, genomic imprinting, X-chromosome inactivation, chromatin architecture organization and cell fate decisions (Jaenisch & Bird, 2003; P. A. Jones, 2012; Heather J. Lee et al., 2014). From a developmental point of view, a fertilized egg with a unique genome, generates approximately 200 different types of cells, each one of these with specific functions, location in time and space, and surrounded by a unique environment (Ficz, 2015). This process is achieved through differentiation of totipotent cells in the early embryo, and loss of stem cell potential as they commit to specific cell lineages (Jaenisch & Young, 2008). Two biological systems have shown to be critical in this process.

On one hand, cell-specific transcription factors drive the differentiation process, guiding cell fate decisions and repressing alternative differentiation paths (Graf & Enver, 2009; K. Takahashi & Yamanaka, 2006). On the other hand, DNA methylation and other epigenetic marks maintain cell identity in the long term, constituting a cell fate memory module and a barrier between different cell states (Bird, 2002; Heather J. Lee et al., 2014; Watanabe, Yamada, & Yamanaka, 2013). This epigenetic landscape was envisioned by Waddington in 1975 who proposed a series of ridges and valleys in which the cell should cross to differentiate (Conrad Hal Waddington, 1957). The cell starts at the top of a slope in an undifferentiated state and descends through the valleys until it reaches a differentiated state at the bottom (**Figure 5.5**). The ridges canalize cells, preventing a cell-fate switch. Modern experimentations in cell reprogramming have supported this analogy, and highlight the central role of epigenetic modifications in developmental biology (Kim et al., 2010; X. S. Liu et al., 2016; Simonsson & Gurdon, 2004).

1.1.1 Methods to assess DNA methylation

Methods quantifying DNA methylation can be classified according to their resolution level. Techniques such as high performance liquid chromatography-ultraviolet (HPLC/UV), liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), enzyme-linked immunosorbent assay (ELISA) and luminometric methylation assay (LUMO), are highly reproducible and are used to provide a single value for 5mC within an entire genome (Kurdyukov & Bullock, 2016). Despite being much less common in the genome, 5hmC, 5fC and 5caC can also be detected (Y. Tang, Zheng, Qi, Feng, & Yuan, 2015). While these techniques are informative, they do not resolve the context in which cytosine modification is occurring, nor their relationship to genes and other genomic sequences. Given that DNA methylation is a key regulator of transcription on a

gene-by-gene basis, understanding where DNA methylation occurs within specific genomic loci is often considered important.

On an intermediate level, DNA methylation can be identified by hybridization with microarrays which can detect a fraction of CpGs (Schumacher et al., 2006). Alternatively, pull-down of methylated DNA by antibodies against 5mC binding proteins (MBD) or 5mC (MeDIP) can be used to enrich CpG rich sequences and used for microarray hybridization or high-throughput sequencing (Mohn, Weber, Schübeler, & Roloff, 2009; Nair et al., 2011). In contrast, reduced representation bisulfite sequencing (RRBS) uses a combination of restriction enzymes and bisulfite treatment to enrich sequences with high CpG content (Meissner et al., 2005). RRBS displays considerable sensitivity and enables interrogation of the genomic regions most likely to hold differentially methylated regions in a cost-effective manner.

Finally, whole-genome sequencing techniques can detect changes in DNA methylation at a single-base resolution level (Laird, 2010; Raiber et al., 2017). Among these, whole-genome bisulfite sequencing (WGBS) is the most comprehensive because it allows quantification of cytosine modification with single base-pair resolution throughout the genome (Lister et al., 2008, 2009). For this reason, it is now commonly used and also seen as the gold-standard for methylation analysis (N. Li et al., 2010).

WGBS is primarily based on the fact that cytosine modifications respond differently to the bisulfite chemical (Frommer et al., 1992; Huang et al., 2010). Unmodified cytosine undergoes rapid deamination to uracil in response to bisulfite treatment. 5fC and 5caC, which are unstable and extremely rare within

the genome, are also rapidly deaminated to uracil following bisulfite treatment (Frommer et al., 1992). In contrast, the most commonly modified cytosines, 5mC and 5hmC, are largely unaffected by bisulfite over short incubation periods. During hybridization and sequencing, uracil behaves as thymine, leading to a recognizable difference between the major modified bases (5mC and 5hmC) and unmodified cytosine. One limitation of conventional bisulfite sequencing is that 5mC and 5hmC cannot be distinguished, however, further bisulfite-based sequencing techniques such as oxidative bisulfite sequencing (Booth et al., 2012) and TET-assisted bisulfite sequencing (Yu et al., 2012) have been developed to overcome this obstacle. Nevertheless, given that these methods are technically challenging, they require more than double the amount of sequencing and 5hmC is at least an order of magnitude less common in the genome than 5mC, they are often not undertaken.

1.1.2 The challenge of low-cell number analysis

All methods of quantifying DNA methylation are difficult in a low-cell number context. Nevertheless, some significant progress has been made developing WGBS protocols that can work on material as limited as a single cell (Heather J Lee & Smallwood, 2018; S. a Smallwood et al., 2014). The technological advancements allowing this quantification exploits the ability of bisulfite to fragment DNA prior to high-throughput library construction (for conventional WGBS the library is created first, and then bisulfite treated) (**Figure 1.1**). The net result is the creation of bisulfite treated sequencing libraries from subnanogram quantities of DNA in a technique called post-bisulfite adaptor tagging (PBAT) (Miura, Enomoto, Dairiki, & Ito, 2012; Miura & Ito, 2015).

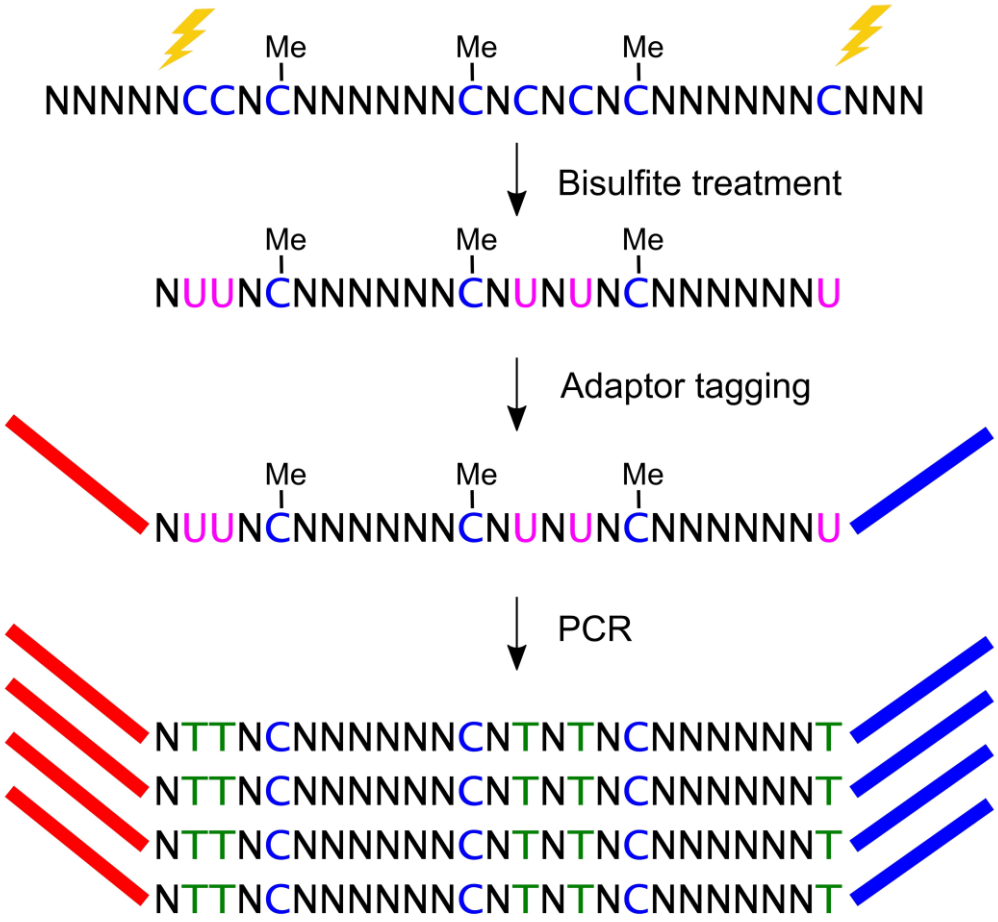

Figure 1.1 Post-bisulfite adaptor tagging. Bisulfite treatment is used to deaminate unmethylated cytosines to uracils and fragment DNA. This step is followed by adaptor tagging and PCR, where uracil is replaced by thymine. Library sequencing allows identification of modified and unmodified cytosines (Miura et al., 2012).

Given that PBAT is so successful in making libraries with scarce input material, perhaps the biggest limitations of the WGBS techniques are the costs related to sequencing and the difficulties in the analysis of high-throughput sequencing data. One way to reduce costs is to undertake low-coverage bisulfite sequencing (Feng, Cokus, et al., 2010; Popp et al., 2010), however the sequencing depth required to accurately measure genomic elements is largely unexplored.

1.2 DNA methylation dynamics during major developmental transitions

The human genome contains 20,000 protein-coding genes, however, in most tissues only 11,000-13,500 of these are active at any one time (Ramsköld, Wang, Burge, & Sandberg, 2009) and the exact pattern of gene expression is unique to a given tissue. At least part of the mechanism used to define these unique expression patterns, and therefore cellular identity, is specific DNA methylation profiles (Heather J. Lee et al., 2014). It, therefore, follows that if a cell or cell lineage changes its identity during development, then changes in DNA methylation patterns will also occur.

Reprogramming of DNA methylation during periods of dramatic changes in cellular identity is a major theme of this thesis. During development of the mammalian germline, when gametic nuclei are reorganized post-fertilization, and at the time of reanimation of the germline from somatic progenitors, major reprogramming of DNA methylation occurs (reviewed, Iurlaro, von Meyenn, & Reik, 2017; Heather J. Lee et al., 2014; Reik & Surani, 2015). In both cases, reprogramming involves global loss of DNA methylation and subsequently, acquisition of new cell-specific DNA methylation patterns. These developmental transitions will now be described in detail, and specifically what is known about DNA methylation reprogramming during this period for mammals and other vertebrates. First, however, some description of what the germline is and how it arises in different vertebrate species is required.

1.2.1 Germline specification

The germline is comprised of the cells giving rise to the next generation, and its development culminates in the production of gametes, the only cells capable of acquiring totipotency at fertilisation (Magnúsdóttir & Surani, 2014). During early development in almost all metazoans the germline is set aside (or "specified"), differentiating into highly specialized cells in charge of transmitting genetic (and epigenetic) information from one generation to the next, ensuring the survival of the species. The first stage of germline development is specification of the primordial germ cells (PGCs). PGC specification has been particularly well described in mammalian organisms such as mice and humans, where a set of unique epigenetic changes have profound consequences in cell reprogramming (Saitou & Yamaji, 2016; W. W. C. Tang, Kobayashi, Irie, Dietmann, & Surani, 2016).

The mechanism of germ cell specification is not universal across all animal species (**Figure 3.3**). One mechanism is through the inheritance of cytoplasmic determinants present in the egg (preformed germ plasm), the other is through signal induction by nearby cells during gastrulation (epigenesis) (Extavour & Akam, 2003). The specification through germ plasm was first proposed by the German biologist August Weismann and it has been observed in several model organisms such as *Caenorhabditis elegans* (*C. elegans*), *Drosophila melanogaster* (*D. melanogaster*), zebrafish (*Danio rerio*) and *Xenopus laevis* (*X. laevis*) (Extavour & Akam, 2003; Weismann, 1893). Germ plasm is maternally inherited and asymmetrically segregated during early stages of development. At the ultrastructural level, it is characterized by electron-dense structures named polar granules which contain RNA and protein molecules, among these, vasa, tudor, piwi and nanos (Juliano, Swartz, & Wessel, 2010). These components repress somatic differentiation programs activated in adjacent cells, mediating early

germ line commitment (Seydoux & Braun, 2006). In contrast, in epigenesis, cytoplasmic determinants have not been identified and cell-cell interactions are critical to inducing cells to become PGCs. This specification mechanism has been observed in urodele amphibians and a large number of mammals, including mice and humans (W. W. C. Tang et al., 2016). From an evolutionary point of view, epigenesis seems to be more ancestral in metazoans, while germ plasm appears to have evolved by convergence multiple times. Different hypotheses have been proposed to explain this convergent evolution (Evans, Wade, Chapman, Johnson, & Loose, 2014; Extavour & Akam, 2003; Whittle & Extavour, 2016, 2017).

In most species (including zebrafish, *X. laevis*, and mammals) PGC commitment occurs far from the somatic cells of the developing gonad and they have to migrate to reach their functional location (Raz, 2003; Richardson & Lehmann, 2010). For zebrafish specifically, Weidinger *et al.* (1999) described the migration of these cells using *vasa* as a specific molecular marker (Weidinger, Wolke, Köprunner, Klinger, & Raz, 1999). Soon after PGCs specification (between 500 - 1k cells stage, 2.75 – 3 hours post-fertilization hpf), cells become motile and use a chemotactic guidance system to begin their relocation (Dosch, 2015; Richardson & Lehmann, 2010). The full process begins at dome stage at 4.3 hpf with four clusters, each one composed of approximately four cells, and finishes at 24 hpf with two discrete clusters situated at the anterior end of the yolk extension, containing approximately 24-50 PGCs (Raz, 2003; Weidinger et al., 1999). Finally, after successive mitosis in the gonads, germline cells are able to start meiosis and gamete differentiation to produce sperm or eggs and generate a new organism upon fertilization.

1.2.2 Epigenetic reprogramming in the germline

The early developmental decision to separate PGCs from somatic cells is accompanied by a series of far-reaching changes in cell morphology, physiology and biochemistry (Magnúsdóttir & Surani, 2014). One of the major changes described in mammalian PGCs is genome-wide demethylation (Messerschmidt, Knowles, & Solter, 2014). This epigenetic "resetting" seems to be intrinsically associated with pluripotency acquisition and, following that, lineage commitment (Surani, Hayashi, & Hajkova, 2007). In mice, for example, after PGCs specification during embryonic days 6.5 – 7.5 (E6.5-7.5) in the epiblast, they lose approximately 90% of all epigenetic marks by E13.5 (Hajkova et al., 2002; Seisenberger et al., 2012).

Mouse PGCs seem to employ a combination of active and passive mechanisms to undergo genome-wide demethylation (Hackett et al., 2013). Soon after PGCs specification, Prdm1 and Prdm14 repress the expression of *de novo* DNA methyltransferases (Dnmt3a and Dnmt3b) and Uhrf1, critical for the maintenance of DNA methylation (Kurimoto, Yamaji, Seki, & Saitou, 2008; Magnúsdóttir et al., 2013). Repression of *de novo* and maintenance DNA methylation machinery leads to passive dilution of 5mC (Kagiwada, Kurimoto, Hirota, Yamaji, & Saitou, 2013). In addition, active demethylation principally induced by Tet enzymes has been observed in PGCs (Hackett et al., 2013; Yamaguchi et al., 2013). Upregulation of Tet1 and Tet2 during E9.5-11.5 has been associated with genome-wide reduction of 5mC and increase in 5hmC in PGCs during E9.5-E11.5 (Hackett et al., 2013). Interestingly, although Tet1 and Tet2 knockout models are viable, they show increased neonatal lethality and fertility reduction associated with defects in genomic imprinting and PGC development (Dawlaty et al., 2013; Yamaguchi et al., 2013).

The other known period of genome-wide DNA methylation erasure occurs in the mammalian zygote (F. Guo et al., 2014; Messerschmidt et al., 2014). Immediately after fertilization, the paternal genomes are separated in form of pronuclei (Wenqiang Liu et al., 2014). Like other vertebrates, the paternal pronucleus is hypermethylated in relation to its maternal counterpart. The paternal genome is demethylated before the onset of DNA replication by an active modification - passive removal process (Abdalla, Yoshizawa, & Hochi, 2009; Inoue & Zhang, 2011). A maternal factor, Stella, protects the maternal pronucleus from being demethylated during the same period in mice (Han, Ren, Zhang, Shu, & Wang, 2019; Nakamura et al., 2007). Additional to paternal active demethylation, both genomes are demethylated by passive mechanisms, and at blastocyst stage in mice (E3.5) DNA methylation levels in the inner cell mass (ICM) reach a minimum of ~25% (H. Kobayashi et al., 2012; S. A. Smallwood et al., 2011). DNA methylation levels follow similar dynamics post-fertilization in humans, however, the major wave of demethylation is completed by the 2-cell stage, with maximum demethylation occurring at ICM/blastocyst stage (~29%) (H. Guo et al., 2014). Importantly, some resistant regions, such as retrotransposons and imprinted loci, escape this wave of demethylation. STELLA and other maternal factors including ZFP57 and KAP1, are responsible for the protection of imprinted loci (X. Li et al., 2008; Nakamura et al., 2007, 2012; Quenneville et al., 2011). Finally, methylation levels increase progressively after this point and reach basal levels at epiblast stage (Hon et al., 2013; Smith et al., 2012).

One of the few non-mammalian models where DNA methylation reprogramming has been studied is zebrafish. For this species, global hypermethylation of sperm relative to the oocyte has been reported (Fang, Corrales, Thornton, Scheffler, & Willett, 2013; Mhanni & McGowan, 2004). Immediately after fertilization DNA is moderately demethylated and, soon after,

methylation *de novo* starts at the 32-cell stage (1¾ hpf) (Jiang et al., 2013). Intriguingly, just the maternal epigenome seems to be reprogrammed, being the embryo methylome virtually identical to the sperm methylome by mid-blastula stage (~ 4 hpf) (Jiang et al., 2013; Potok, Nix, Parnell, & Cairns, 2013). Recently, it has been reported that "placeholder" nucleosomes, containing histone H2A variant H2A.Z (FV) and H3K4me1 occupy hypomethylated regions in both sperm and cleavage embryos and, upon zygote genome activation, become either active or silent (Murphy, Wu, James, Wike, & Cairns, 2018). Zebrafish possess the basic molecular machinery responsible for regulation of DNA methylation including Dnmt and Tet enzymes, however, there is no expression of Tet and 5hmC quantities are very low in these early stages (Jiang et al., 2013; Potok et al., 2013). Conversely, active DNA demethylation at enhancers induced by Tet has been found during the phylotypic period (24 hpf), a critical stage for body planning and organ development (Bogdanović et al., 2016; Hyung Joo Lee et al., 2015). Specific PGC erasure has not been tested systematically in zebrafish and, given the specification method used for this species, it is feasible the germline does not undergo epigenetic erasure (**Figure 1.2**).

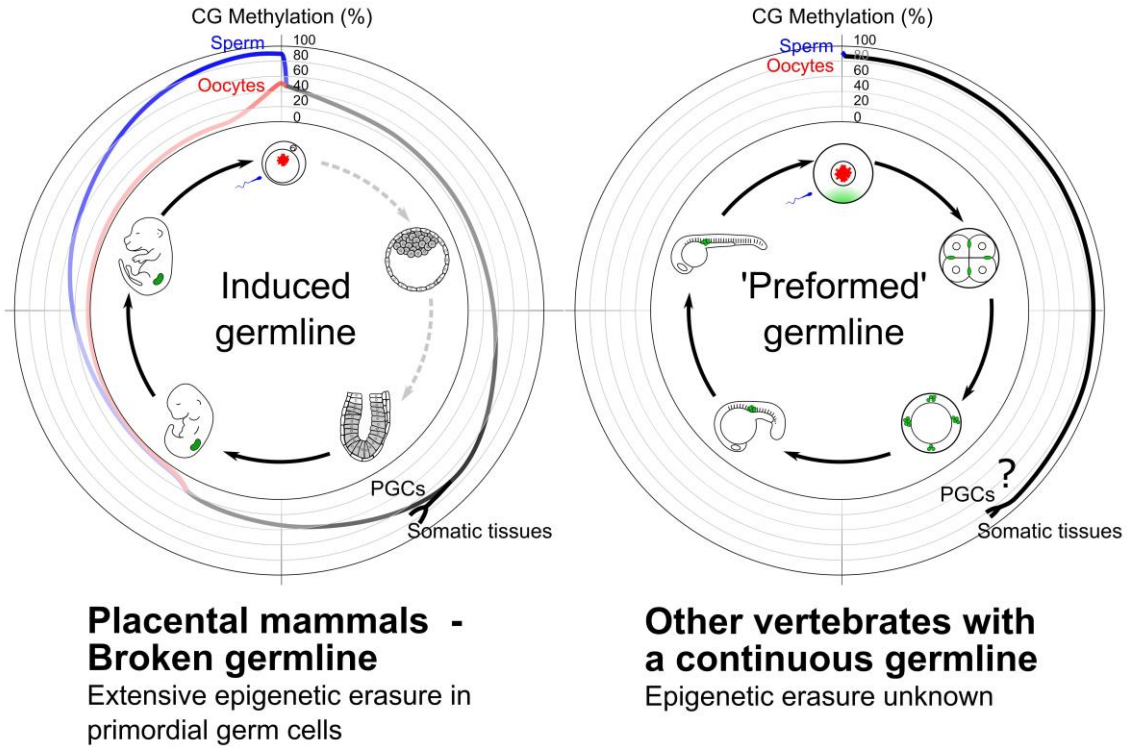

Figure 1.2 Germline development and epigenetic dynamics in mice and zebrafish. The mouse germline is discontinuous (broken arrows): PGCs are reprogrammed from the epiblast following inductive signalling from extraembryonic cells. In addition to extensive demethylation soon after fertilization, methylation marks are erased in PGCs after specification. In contrast, the zebrafish germline is continuous (solid arrows): PGCs inherit cytoplasmic determinants called germ plasm during early embryogenesis (labelled green). Although the methylome inheritance pattern in the embryo post-fertilization is known, the existence of genome-wide DNA methylation erasure in the germline has not been tested.

1.3 Epigenetic memory and transgenerational epigenetic inheritance

The unidirectional relationship between genotype and phenotype lies at the heart of the modern synthesis theory. In clear distinction to Jean-Baptiste Lamarck,

who proposed inheritance of acquired characteristics, August Weismann strongly rejected this hypothesis, postulating that hereditary information cannot pass from the soma to the germline and on to the next generation (Weismann barrier) (Sabour & Schöler, 2012; Weismann, 1893). Early experiments in genetics, developmental biology, and the later discovery of Mendelian inheritance have largely consolidated Weismann's point of view (Mayr, 1972).

Epigenetic information has added another layer of complexity to classic Darwinian evolution theory (Y. Wang, Liu, & Sun, 2017). As mentioned before, epigenetic marks are robustly erased in mammalian PGCs preventing at the same time acquired epimutations to be inherited by offspring. However, there is growing evidence that environmental factors can produce epigenetic changes in the germinal line, which, in turn, can be inherited by the next generations (Anway, D; Cupp, Uzumcu, Skinner, & K, 2005; Kelly, 2014; Radford et al., 2014; Skvortsova, Iovino, & Bogdanović, 2018). This process is known as transgenerational epigenetic inheritance and it has been proposed as a form of "soft inheritance", a dynamic inheritance in response to fluctuating conditions (Dickins & Rahman, 2012; Richards, 2006).

It has been suggested that epigenetic inheritance enables environmentally induced phenotypes to be transmitted between generations (Pál & Miklós, 1999). Conrad Waddington, even without knowing the biochemical nature of the gene or epigenetic mechanisms, proposed that under the influence of natural selection, development tends to become canalized against environmental perturbations, an adaptive character "so far canalized that it appeared even when the conditions returned to the previous norm", a process he referred to as "genetic assimilation" (C. Waddington, 1953; C. H. Waddington, 1959). Although further work showed

such phenotypic plasticity has a strong genetic basis, the exact mechanisms underlying genetic assimilation are unclear (Rutherford & Lindquist, 1998). Furthermore, the importance of transmission of epigenetic memory in the germline and the exact mechanisms of genetic assimilation continue to be intensely debated (Bonasio et al., 2015; Crispo, 2007; Pigliucci, Murren, & Schlichting, 2006; Skvortsova et al., 2018).

Despite the intrigue and speculation, very few examples of genuine transgenerational epigenetic inheritance have been described in mammals (Heard & Martienssen, 2014; Kazachenka et al., 2018; Miska & Ferguson-Smith, 2016). Among the few cases functionally validated are the Agouti viable yellow (*Avy*) and Axin fused (*AxinFu*) phenotypes, both related to endogenous retroviruses of the intracisternal A particle (IAP) class (Dickies, 1962; Michaud et al., 1994; Rakyan et al., 2003; Vasicek et al., 1997). The *avy* metastable epiallele is produced by the insertion of a murine IAP retrotransposon upstream of the transcription start site of the *Agouti* gene, leading to ectopic production of Agouti protein and yellow fur, obesity and tumorigenesis (Dolinoy, Weinhouse, Jones, Rozek, & Jirtle, 2010). Methylation levels of the retrotransposon correlate inversely with ectopic Agouti expression. Hypomethylation is associated with ectopic Agouti expression (yellow coat), whereas hypermethylation is associated with normal Agouti expression (pseudoagouti). The diverse phenotypes can be found in isogenic lines and are inherited transgenerationally after maternal transmission (Morgan, Sutherland, Martin, & Whitelaw, 1999). The *AxinFu* metastable epiallele resulted from the insertion of an IAP sequence in intron 6 of the *axin* gene (Rakyan et al., 2003). The insertion leads to a truncated version of Axin, a cytoplasmic protein a that regulates embryonic axis formation and is related to a kinky tail phenotype. Similar to the Agouti phenotype, the Axin fused phenotype is correlated with methylation levels in the IAP retrotransposon and

can be inherited transgenerationally after paternal and maternal transmission (Rakyan et al., 2003). Despite the importance of metastable epialleles as potential reservoirs of transgenerational epigenetic information in mammals a recent study showed those cases seem to be the exception rather than the rule (Kazachenka et al., 2018). Other studies in mammals suggest environmental factors such as in utero undernourishment, smoking during pregnancy, and cold stress in sperm can lead to intergenerational inheritance (Joubert et al., 2014; Radford et al., 2014; W. Sun et al., 2018). Again, whereas interesting those examples suggest limited potential for epigenetic inheritance between generations in mammal species.

The proposed reasons why DNA methylation is erased in mammals include: reacquisition of pluripotency, reestablishment of genomic imprinting, and erasure of epimutations (Heather J. Lee et al., 2014; Seisenberger et al., 2013). Under any circumstance, global DNA epigenetic erasure can be considered a molecular manifestation of the Weissman barrier, and strongly precludes transmission of epigenetic marks by the offspring (Miska & Ferguson-Smith, 2016).

While mammals appear to have a limited capacity for transgenerational epigenetic inheritance, an interesting observation in fish adds further fuel to an already fiery discourse regarding the generality of this phenomenon. The half-smooth tongue sole (*Cynoglossus semilaevis*) is a marine fish that has both genetic (ZW) and environmental sex determination systems. Pseudo-male individuals (ZWm) can be induced by high temperatures during the juvenile phase. Pseudo-male methylomes resemble true males (ZZ) (S. Chen et al., 2014; Shao et al., 2014). Strikingly, pseudo-male methylation modifications are inherited by the

offspring, giving rise to pseudo-males, despite a lack of a new temperature stimulus. Therefore, at least in this case, an environmental stressor has a major impact in the epigenome, and this trait is inherited by the offspring.

1.4 Sex Determination and Sex Change in Fish: an unexplored epigenetic paradigm

In most vertebrates, sexual phenotype is defined early during development in a process called sex determination (Capel, 2017). During this period genetic or environmental factors determine the sexual fate of the individual, either female or male (Capel, 2017). This period is followed by the subsequent differentiation of the bipotential gonad into an ovary or a testis. This trait is dichotomous and remains static in most organisms, nevertheless, a reduced number of species have the ability to modify their sexual phenotype in post-embryonic stages (Devlin & Nagahama, 2002; Schärer, 2017). Examples of this are the development of a transitory "juvenile ovary" in male zebrafish and female-to-male sex change in adult bluehead wrasse (*Thalassoma bifasciatum*) (H. Takahashi, 1977; R R Warner & Swearer, 1991). Both cases are major developmental transitions during the natural lifecycle of those organisms and show evidence of remarkable phenotypic plasticity. In mammals, the closest cases of reacquisition of cell plasticity are the early zygote post-fertilization and the germline during early differentiation (Heather J. Lee et al., 2014). Importantly, as mentioned before, cell reprogramming in mammals involves extensive erasure of epigenetic memory (Hajkova et al., 2002; F. Santos, Hendrich, Reik, & Dean, 2002). Upon embarking on this research project, it was unknown if the same dynamics is followed by other non-mammalian vertebrates during cell reprogramming events. In this section, key concepts about gonad and sex development for the two species used to examine this question will be introduced.

1.4.1 Sex determination and differentiation in zebrafish

After PGCs reach the gonadal primordium, around 24 hpf, they remain quiescent until 7 days post-fertilization (dpf) (Leerberg, Sano, & Draper, 2017; Tzung et al., 2015). At this stage, expression of genes related to sex differentiation or determination is not detected and the gonad is bipotential/undifferentiated (Leerberg et al., 2017). This stage finishes around 8 – 10 dpf, when germline cells begin to proliferate and diverse genes related to sex differentiation are expressed (e.g. *amh, cyp19a1a*, *soxb9*) (Rodríguez-Marí et al., 2005). Importantly, all zebrafish develop a "juvenile ovary" characterized by early meiotic oocytes since 13 – 14 dpf (H. Takahashi, 1977; Tzung et al., 2015; Uchida, Yamashita, Kitano, & Iguchi, 2002). This early cell commitment suggests germline cells follow an initial female pathway. The "juvenile ovary" stage finishes around 20 – 25 dpf, when zebrafish undergo gonad transformation (Orban, Sreenivasan, & Olsson, 2009). During this transitional period oocytes in males undergo apoptosis, whereas oocytes in females continue maturating (Orban et al., 2009; D. Sun et al., 2013; Uchida et al., 2002). At ~30 dpf a great reduction in the number of oocytes can be observed in presumptive males, and around 55 dpf the ovary-to-testis transformation period is completed (D. Sun et al., 2013; Uchida et al., 2002). Notably, the timing and extension of these stages show inter-individual differences (X. G. Wang, Bartfai, Sleptsova-Freidrich, & Orban, 2007). Despite the importance of zebrafish as a model organism and numerous studies focused on the sex differentiation process, the primary sex determination mechanism in zebrafish remains elusive.

Sex-determination systems are heterogeneous among vertebrates, ranging from chromosomal (e.g. most mammals and birds), to environmental (e.g., temperature, pH, population density), and including the intervening spectrum (e.g. reptiles and fishes) (Capel, 2017). Zebrafish is a gonochoristic species, which means individuals are either females or males and this trait is static during their

lifetime (Devlin & Nagahama, 2002). Several studies have shown domesticated strains of zebrafish lack sex chromosomes and suggested a polygenic sex-determination system (Amores & Postlethwait, 1998; Liew et al., 2012; Liew & Orbán, 2014). The important role of genetic factors in this process can be demonstrated in highly skewed and reproducible sex ratios in a family-specific manner (Liew et al., 2012; Ribas, Liew, et al., 2017). In addition, several environmental factors, such as temperature, rearing density, and chemical compounds, affect sex ratios (Ribas, Liew, et al., 2017; Ribas, Valdivieso, Díaz, Piferrer, & Noelia, 2017; D. Santos, Luzio, & Coimbra, 2017). Thus, sex determination in domesticated zebrafish strains is regulated by genetic and environmental factors.

Contrasting results regarding a polygenic sex determination system have been found in non-domesticated and recently domesticated strains (wildtype) (Anderson et al., 2012; R. Sharma, Sharma, Tripathi, & Sharma, 1998; Wilson et al., 2014). Early evidence by cytogenetic analysis suggested wildtype zebrafish possess a chromosomal sex determination system, compatible with heterogametic chromosomes (ZZ/ZW) (R. Sharma et al., 1998). Additionally, studies involving sex reversed populations and genome-wide linkage analysis on strains with a wildtype background pointed towards a female determinant factor and a major sex-linked locus (Anderson et al., 2012; Tong, Hsu, & Chung, 2010). A detailed examination by restriction site-associated DNA sequencing (RAD-seq), found that this major determinant can be traced to the end of the long arm of chromosome 4 in natural strains (Cooch Behar, EkkWill, Nadia and Wild India Kolkata) (Wilson et al., 2014). Importantly, the major locus was required, but not sufficient to develop a female phenotype, which suggests either a female promoting factor is located in chromosome W (dominant-female-allele hypothesis) or a dose-dependent male promoting factor is located in

chromosome Z (two-dose-male – one-dose-female hypothesis) (Wilson et al., 2014). While the exact molecular nature of the sex determinant remains unknown, those results showed commonly used strains (AB and Tübingen) lost the natural sex determinant locus during domestication.

Zebrafish germline has emerged as a critical regulator of sex fate in zebrafish. It has been suggested that a bimodal proliferation of PGCs starts around 8 dpf, and afterwards, this dimorphic trait is strongly correlated with zebrafish sexual fate (Wei Liu et al., 2015; Tzung et al., 2015). An increased number of PGCs is correlated with feminisation, whereas complete ablation of PGCs invariably leads to a male phenotype (Slanchev, Stebler, de la Cueva-Méndez, & Raz, 2005; Tzung et al., 2015). Gene expression studies have shown germline deficient lines exhibit reduced expression of pro-female genes (i.e. *cyp19a1a* and *foxL2*), whereas pro-male genes (i.e. *sox9a* and *amh*) remain expressed by somatic tissues (Siegfried & Nüsslein-Volhard, 2008). Consistent with these findings, numerous mutants of genes important for PGC and oocyte development (e.g. *fancl*, *nanos3*, *vasa, ziwi/piwil1*) have shown failure to follow a female differentiation pathway (Draper, McCallum, & Moens, 2007; Hartung, Forbes, & Marlow, 2014; Houwing et al., 2007). The mechanism by which oocytes and PGCs promote feminisation remains unclear.

Nevertheless, perinucleolar oocytes seem to be critical to the process of feminisation, and ultimately sex determination, in domesticated zebrafish strains (Rodríguez-Marí & Postlethwait, 2011; Uchida et al., 2002). These oocytes are characterized by a large nucleus with numerous marginal micro-nucleoli (Maack & Segner, 2003). The nucleolus is a nuclear compartment formed around the ribosomal DNA (rDNA) repeats and is responsible for the ribosome biogenesis

(Lindström et al., 2018). Perinucleolar oocytes appear in both sexes at the beginning of the gonad transformation stage (21-23 dpf), however, the ratio of those in total germ cells is significantly different between presumptive males and females (Uchida et al., 2002). One of the most insightful results to demonstrate the importance of perinucleolar oocytes comes from the *fancl* mutant (Rodríguez-Marí et al., 2010). Mutants for *fancl* have a normal number of germline cells during early stages of development, including "juvenile ovary" stage. However, during gonad transformation, apoptosis in perinucleolar oocytes is significantly increased and gonads in all mutants develop as a testis. Remarkably, *tp53* mutations rescue the phenotype suggesting oocyte apoptosis leads to masculinization of the gonad (Rodríguez-Marí et al., 2010). Therefore, perinucleolar oocyte survival is considered critical to support ovary differentiation (Rodríguez-Marí et al., 2010; Rodríguez-Marí & Postlethwait, 2011; Tzung et al., 2015) (**Figure 1.3**).

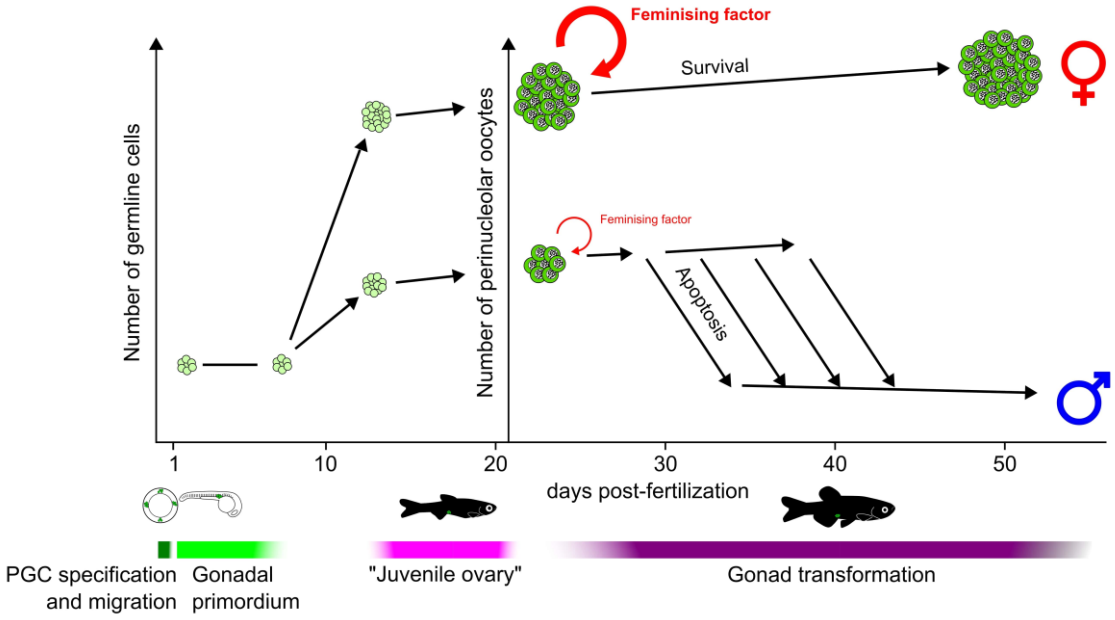

Figure 1.3 Zebrafish germline development and sex determination. PGCs become differentiated around 2.75 – 3 hpf. After arriving at the gonad, around 24 hpf, they remain quiescent until ~7 dpf. This period is followed by a bimodal

proliferation of germline cells and development of a "juvenile ovary" stage around 13 – 14 dpf. The "juvenile ovary" stage is characterized by early meiotic oocytes and finishes around 20 – 25 dpf, when zebrafish undergo gonad transformation. In individuals with a high number of perinucleolar oocytes those cells continue through meiosis, whereas in individuals with low numbers, oocytes suffer apoptosis and are replaced by testicular tissue. The ovary-to-testis transformation period in males is completed around 55 dpf (Orban et al., 2009; Tzung et al., 2015).

1.4.2 Sex change in fish

Sexual phenotype is a static and dichotomous trait in most vertebrates; nevertheless, a number of species have the capacity to express both female and male phenotypes during adulthood (hermaphroditism) (Todd, Liu, Muncaster, & Gemmell, 2016). Hermaphroditism is widely distributed in teleost fishes, where 27 families across 9 orders can switch their sexual phenotype as a natural process during their life cycle (de Mitcheson & Liu, 2008). The most common form of hermaphroditism is sequential, defined as functional sex change in sexually differentiated individuals (Todd et al., 2016). Sequential hermaphroditism occurs in three forms: female-to-male (protogyny), male-to-female (protandry), and bidirectional (Avise & Mank, 2009). Given the energetic cost of undergoing gonad reorganization, sex change must provide some adaptive advantage to those species undertaking it. Several theories, including size-advantage, have proposed that hermaphroditism evolved to maximize the reproductive success of a species (Ghiselin, 1969; Munday, Buston, & Warner, 2006).

Sex change is a remarkable example of phenotypic plasticity in vertebrates (H. Liu et al., 2017). Extensive changes in reproductive behaviour, external morphology, and most importantly, gonad structure and function, should take place during a short time frame to modify the sexual phenotype of an individual (Godwin, 2009; Lamm, Liu, Gemmell, & Godwin, 2015; H. Liu et al., 2017). Sex change occurs as a response to environmental cues, such as perturbations in social structure, or reaching a critical size or age. (Godwin, 2009; Y. Kobayashi, Nagahama, & Nakamura, 2013; Robert R. Warner, 1984). The molecular mechanisms controlling how those signals trigger a change in sexual phenotype are not completely understood, and stress has been proposed as a key catalyst (Goikoetxea, Todd, & Gemmell, 2017). On a structural and molecular level, sex change involves a profound modification in the expression of female and male expression programs, associated with changes in cell identity and developmental commitment (Todd et al., 2016).

The coral reef fish, bluehead wrasse, presents one of the most striking and well-studied examples of sex change (R R Warner & Swearer, 1991). These fish live in groups, composed of a large dominant terminal-phase male (blue-headed), numerous females (yellow), and initial phase males (**Figure 1.4**) (Godwin, 2009). Most bluehead wrasses begin their reproductive cycle as females; however, in absence of a socially dominant male the largest female or initial phase male, which mimics a female phenotype, takes the role of dominant male (R R Warner & Swearer, 1991). Changes in behaviour and pigmentation can be detected in a matter of days, whereas progressive ovary atresia in females is followed by complete replacement by testicular tissue in 8 – 10 days (R R Warner & Swearer, 1991). Absence of male sexual tissue in ovaries suggests an event of cellular reprogramming, although this hypothesis has not been tested. Additionally, whereas large-scale transcriptomic changes are identified in differentiated

stages, the gene expression and its regulation by DNA methylation have not been assessed during such transition (H. Liu et al., 2015).

Figure 1.4 Sexual dimorphism in bluehead wrasse. A dominant male (blue colouration) defends a spawning territory and a group of females (yellow colouration). Loss of the dominant male triggers a major reprogramming event leading to female-to-male sex change. Courtesy of Kevin Bryan.

1.5 Thesis aims

The overarching aim of this thesis was to advance the understanding of DNA methylation dynamics during major cellular transitions in divergent vertebrate species. To do this, I focussed upon three major questions:

- How can I assess accurately and inexpensively global DNA methylation levels?
- Does the zebrafish germline undergo extensive DNA demethylation like its mammalian counterpart?
- What is the DNA methylation dynamics during gonad transformation/sex change in fish? To answer this question, I used two model systems:
    - Gonad transformation in zebrafish, whereby the bipotent "juvenile ovary" undergoes differentiation into either the testes or continues development into the mature ovary.
    - Sex change in bluehead wrasse, where adult female fish undergo dramatic reprogramming of the ovary to testis.

I, therefore, divided my project into three specific objectives and presented my results in two research chapters published in peer-reviewed journals and two review chapters, currently under revision.

Objective 1: Computational modelling and simulation of low-coverage genome-wide bisulfite sequencing

Low-coverage whole-genome bisulfite sequencing (WGBS) has previously been used to characterize DNA methylation changes at low cost (Feng, Cokus, et al., 2010; Popp et al., 2010). Nevertheless, the amount of sequencing required to

accurately predict DNA methylation levels genome-wide has not been properly defined. Using large WGBS datasets from a range of species, I simulated the effect of different sample sizes to estimate genome-wide methylation. My findings support the presence of a sampling threshold of around 10,000 methylation calls (Margin of error ±2.5%). In addition to being critical for my project (See **Chapter 2** Margin of error estimation for low-coverage WGBS), these results have been integral to other studies conducted in the Hore laboratory (See **Appendix 1** Bootstrap sampling to determine margin of error in low-coverage WGBS) (Ortega-Recalde, Day, et al., 2019; Peat, Ortega-Recalde, Kardailsky, & Hore, 2017).

Objective 2: DNA methylation dynamics in the zebrafish germline

In order to test the hypothesis that species with a preformed germline do not undergo extensive epigenetic reprogramming, I employed a transgenic zebrafish line, where a fusion between the *vasa* promoter and the enhanced green fluorescent protein (EGFP) has been created (vasa:EGFP), and used to specifically mark germline cells (Krøvel & Olsen, 2002). A key objective during my project was to accurately and efficiently isolate PGCs from zebrafish embryos and young fish. For this purpose, I used fluorescence-activated cell sorting (FACS) in developmental stages between 24 hpf to 70 dpf. Using low-coverage PBAT sequencing on PGCs I analysed the genome-wide methylation dynamics during early and late development in zebrafish. My main findings enabled me to comprehensively asses global changes in zebrafish germline methylation patterns and were published in a peer-reviewed journal presented in **Chapter 2**, (Ortega-Recalde, Day, et al., 2019). The preservation of global DNA methylation in PGCs highlights the importance of germline memory as a mechanism of transgenerational inheritance in non-mammalian organisms, a concept explored in **Chapter 3** (Ortega-Recalde & Hore, 2019).

Objective 3: Epigenetic memory and epigenetic reprogramming during sex determination and sex change in fish

Sex change in fish represents a remarkable example of sexual and phenotypic plasticity. Some of this plasticity is evident early in development, such as in species like zebrafish, where all young fish develop a "juvenile ovary" and during gonad transformation the gonad takes either a female or male sexual fate. Even more extreme plasticity can be seen in adult sex change, as occurs in bluehead wrasse. To examine the dynamic of epigenetic memory and reprogramming during these transitions, first, low-coverage WGBS was performed in the zebrafish germline during gonad transformation (**Chapter 2**) (Ortega-Recalde, Day, et al., 2019).

To further this work in a different model system, I, along with other collaborators, used a combination of transcriptomic and methylome approaches to study sex change in bluehead wrasse (**Chapter 4**) (Todd et al., 2019). Integrative data analysis showed that environmental stimulus produced an early stress response associated with repression of the aromatase gene and subsequent collapse of the feminising expression program. Importantly, epigenetic reprogramming of the gonad, evidenced by extensive changes in the methylome and expression of epigenetic molecular machinery reminiscent of early development cells of mammals, showed epigenetic memory plays an essential role in maintaining and regulating the sexual fate of vertebrates. The main results of this project were published in a peer-reviewed journal presented in **Chapter 4** (Todd et al., 2019). The importance of epigenetic memory and epigenetic modifications in sex change are reviewed in **Chapter 5** (Ortega-Recalde, Goikoetxea, et al., 2019) and **Appendix 2** (Gemmell, Todd, Goikoetxea, Ortega-Recalde, & Hore, 2019).

Chapter 2

"Zebrafish preserve global germline DNA methylation while sex-linked rDNA is amplified and demethylated during feminisation"

Contribution

This chapter consists of a full accepted research manuscript published in *Nature Communications* (2019).

Zebrafish preserve global germline DNA methylation while sex-linked rDNA is amplified and demethylated during feminisation. **Oscar Ortega-Recalde**[1], Robert C. Day[2], Neil J. Gemmell[1] and Timothy A. Hore[1]. *Nature communications*. Vol. 10, Article number: 3053. 16 July 2019, doi: 10.1038/s41467-019-10894-7

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I was the only experimental author of this paper and, as such, I standardized the empirical bootstrapping method to quantify global DNA methylation, developed protocols to sort zebrafish germline cells, prepared low-coverage WGBS libraries from ultra-low concentrations of DNA (<100 cells), performed sequencing, analysed the data, and prepared the manuscript and figures. This work was supervised by Dr Timothy A. Hore, who also conceived and funded the study and contributed to experimental design and management. Professor Gemmell provided the transgenic zebrafish expressing vasa::EGFP and comments on experimental design, while Dr Robert C. Day helped with training for the sequencing machine.

My results show that, in stark contrast to mammals, zebrafish germline preserves DNA methylation during all the stages evaluated. Additionally, I found a remarkable amplification of oocyte-specific ribosomal DNA (rDNA) during the gonad transformation period. The absence of DNA methylation erasure in the germline is an important finding in stem cell biology and it is a potential mechanistic explanation for transgenerational epigenetic inheritance. The amplification of rDNA is a provocative observation which may be linked to the enigmatic sex determination mechanism in zebrafish. While this publication was in preparation, another manuscript, assessing DNA methylation during the early stages of germline development, independently provided support for the absence of demethylation in the zebrafish germline (Skvortsova et al., 2019).

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Zebrafish preserve global germline DNA methylation while sex-linked rDNA is amplified and demethylated during feminisation

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The germline is the only cellular lineage capable of transferring genetic information from one generation to the next. Intergenerational transmission of epigenetic memory through the germline, in the form of DNA methylation, has been proposed; however, in mammals this is largely prevented by extensive epigenetic erasure during germline definition. Here we report that, unlike mammals, the continuously-defined 'preformed' germline of zebrafish does not undergo genome-wide erasure of DNA methylation during development. Our analysis also uncovers oocyte-specific germline amplification and demethylation of an 11.5-kb repeat region encoding 45S ribosomal RNA (fem-rDNA). The peak of fem-rDNA amplification coincides with the initial expansion of stage IB oocytes, the poly-nucleolar cell type responsible for zebrafish feminisation. Given that fem-rDNA overlaps with the only zebrafish locus identified thus far as sex-linked, we hypothesise fem-rDNA expansion could be intrinsic to sex determination in this species.

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The germline is established during early development of almost all metazoans with the founding of primordial germ cells (PGCs)[1]. Despite the importance of these in the animal life cycle, the mechanism of PGC specification is not universal for all species. In mammals and urodele amphibians, signal induction from extraembryonic tissue reprograms epiblast cells to PGCs; a process known as epigenesis or induction. In contrast, most other vertebrates including reptiles, fish, birds, and anuran amphibians have an 'immortal' or 'preformed' germline whereby PGCs are specified by cytoplasmic determinants called germplasm. Germplasm is mitotically-inherited from the egg and is asymmetrically segregated during early stages of development[2]. Germplasm components repress somatic differentiation programmes activated in adjacent cells, mediating early germline commitment and helping preserve their developmental potency[3].

The process of PGC specification has been particularly well described in mammals. One of the most astonishing features of PGC specification in mammals is simultaneous genome-wide erasure of DNA methylation marks. In mice, global CG methylation decreases from 71% at PGC specification around day 6.5 (E6.5) to approximately 14% and 7% in male and female at E13.5 PGCs, respectively[4]. DNA demethylation in human PGCs shows a similar dynamic—PGC specification occurs around E12–E16 and DNA methylation drops to approximately 4.5% by week 7[5]. Global methylation reprogramming in marsupial mammals apparently occurs between 10 and 200 days post-partum, when PGCs are well-established in the gonad[6].

Global DNA demethylation in the mammalian germline occurs in sexually undifferentiated PGCs, and is essential for safeguarding against precocious germline differentiation[7,8]. A consequence of extensive erasure in the germline of mammals is that acquired DNA methylation is very rarely inherited[9,10]. Indeed, retention of epigenetic memory in the mammalian genome appears to be largely restricted to imprinted genes and methylated repeats[4]. Of the latter, many appear to be from the intra-cisternal A-type particle class, including those implicated in the transmission of the Agouti viable yellow mouse phenotype[11].

Currently, it is not known if germline erasure of DNA methylation is universal amongst vertebrates, or if it is restricted to species with an induced germline. Indirect evidence suggests that in at least some fish species, epigenetic marks are not erased and can be inherited from one generation to the next. For example, stable silencing of a methylated enhanced green fluorescent protein (EGFP) transgene (*GAL4-VP16,UAS:EGFP*), is heritable in zebrafish and correlates with DNA methylation levels[12]. In the half-tongue sole (*Cynoglossus semilaevis*), genetically female fish with ZW sex chromosomes can switch to a phenotypically 'pseudo-male' state (ZWm) by exposure to high temperatures during the juvenile phase. Strikingly, pseudo-males possess high levels of genomic methylation which are inherited to offspring, giving rise to further pseudo-males even without temperature stimulus[13]. These results suggest epigenetic erasure between generations is not prevalent in fish, yet, experiments have not been conducted to test this hypothesis. DNA methylation was not found to undergo extensive erasure in whole zebrafish embryos immediately after fertilisation[14–16], however, these experiments did not involve germline isolation and only sampled the first few days of development.

In addition to being the conduit for inheritance between generations, the germline has been identified as a driver of sex determination in several fish species, including zebrafish[17]. Specifically, zebrafish develop a 'juvenile ovary' around 11–21 days post-fertilisation (dpf)[18–20]. In fish with reduced numbers of germline cells, oocytes undergo apoptosis and male differentiation occurs. In contrast, greater germline cell numbers promote continued female development. Although environmental triggers, rearing density and small molecules targeting epigenetic modification can influence this process, the primary driver of differential germline proliferation in females and males remains elusive[21–24].

Here, we use a low-cell number bisulfite sequencing pipeline to assess the DNA methylation dynamics in the zebrafish germline. In contrast to mammals, we do not observe genome-wide methylation erasure at any germline stage from 24 h post fertilisation (hpf) until sexual maturity. In addition, we find amplification and demethylation of an 11.5-kb region located in the major sex-linked locus. This region encodes for a type of female-specific ribosomal RNA expressed in oocytes (fem-rDNA) and may play a role in oocyte survival and proliferation. These results provide evidence that the preformed zebrafish germline does not erase epigenetic memory in the form of DNA methylation, and suggests fem-rDNA amplification is implicated in sex determination.

Results

Isolation of zebrafish germline cells and low-coverage WGBS. To obtain germline cells from zebrafish we used the transgenic line *Tg(vasa:EGFP)*[25]. The reporter gene for this line contains the promoter region of *vasa*, an RNA binding protein component of the germplasm and well-described germline marker[26]. As such, vasa:EGFP protein is expressed in oocytes and segregated with PGCs during embryogenesis. At 24 hpf, when PGC migration is finished, we found a compact cluster of cells between the yolk ball and yolk extension in the gonadal region (Fig. 1a–d). Given there are few germline cells per individual fish at this developmental stage, ten fish were pooled for each replicate, dissociated with trypsin and prepared for cell sorting. The EGFP +ve cells were isolated with fluorescence-activated cell sorting (FACS) and accounted for approximately 0.01% or less of all cells analysed (Fig. 1e). The gating strategy is exemplified in Supplementary Fig. 1. This percentage is similar to values previously reported for teleost species (0.02–0.04%)[27]. To determine the purity of the population isolated, sorted cells were visualised under an inverted fluorescent microscope. The proportion of EGFP +ve cells ranged from 93.8 to 100% and resembled PGCs in terms of size and shape (Supplementary Fig. 2).

In order to maximise the number of samples tested, we used a low coverage whole genome bisulfite sequencing (WGBS) pipeline to uncover genome-wide methylation levels[28,29]. To be sure we sampled sufficiently, the number of CG calls required to accurately predict global methylation was undertaken using empirical bootstrap sampling[30] of previously published datasets[15]. As expected, we found that increasing the number of CG calls reduces the margin of error for global methylation (Supplementary Fig. 3A). However, beyond a certain threshold, we found increasing the number of CG calls had a minimal effect reducing the margin of error. An asymptotic model described by the equations $y = 1.207/\sqrt{x}$ and $y = 2.109/\sqrt{x}$, for sperm and muscle respectively, was used to fit a curve to the data. At our minimum sequencing depth of 10,000 CG calls, bootstrap sampling predicts a margin of error (99% confidence interval) of approximately ±1.2–2.1 methylation percentage points (Supplementary Fig. 3B).

Zebrafish germline preserves global DNA methylation. In mice, epigenetic reprogramming of PGCs occurs in two sequential steps, the first during PGC expansion and migration to hindgut endoderm, the second upon entry of PGCs into the gonads[4]. In marsupials, epigenetic reprogramming occurs postnatally when PGCs have finished migration to the gonad[6]. To capture the full spectrum of these reprogramming windows we measured

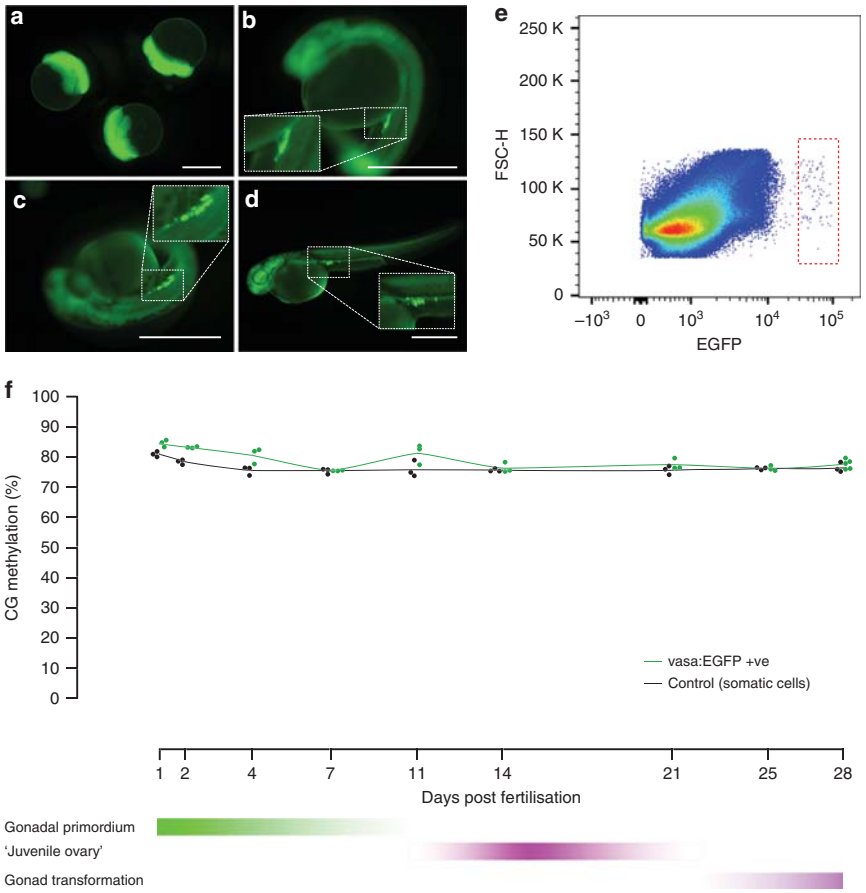

Fig. 1 Isolation and quantitation of DNA methylation in the zebrafish germline. **a**–**d** Fluorescence microscopy of *tg(vasa:EGFP)* zebrafish embryos and larvae. 1.5 h post-fertilisation (hpf) (**a**), 24 hpf (**b**), 48 hpf (**c**–**d**). 1.8× view of EGFP +ve labelled cells is shown inset (dashed lines). Scale bars are 500 µm. Forward scatter height (FSC-H) **e** Flow cytometry plot of 10 zebrafish larvae at 48 hpf. The red dashed square indicates the EGFP +ve population gated for isolation. Blue dots indicate discrete data points (i.e., cellular events), whereas green, yellow and red colouring indicate increasing data density. **f** Percentage of methylation in CG context from 1 to 28 days post-fertilisation (dpf) in both vasa:EGFP +ve germline cells (green line) and control cells (black line). For each sample type and timepoint, $n = 3$ independent biological replicates were used, except for 28 dpf vasa:EGFP +ve, which has $n = 5$ independent biological replicates

methylation from 24 hpf until gonadal transformation, 25–28 dpf. At 24 hpf, EGFP +ve cells were slightly more methylated than control cells, however, both showed high levels of CG methylation (84.06% and 81.41%, respectively). Thus, in stark contrast to mice which experience a massive loss in CG methylation, zebrafish PGCs have preserved global CG methylation upon arrival in the gonad.

Next, we measured methylation levels through gonadal primordium (2–11 dpf), 'juvenile ovary' (11–21 dpf) and early gonad transformation (25–28 dpf) stages. Genome-wide erasure of DNA methylation was not present at any of the time points assessed (Fig. 1f). Average levels of 5-methylcytosine (5-mC) in the CG context were 78.42% and 76.08%, respectively, for EGFP +ve and control cells. Detailed sequencing results are provided in Supplementary Data 1.

DNA methylation levels during gonad transformation. Mature germ cells in zebrafish possess sex-specific methylation programmes. In sperm, nearly 95% of CG dinucleotides are methylated, while oocytes are 75% methylated[14,15]. To explore the onset of this differentiation, we analysed germline methylation from gonad transformation until early gametogenesis. From 25 to 55 dpf, zebrafish gonads undergo an ovary-to-testis transformation in males or further ovarian maturation in females[19,31]. The vasa protein is expressed in male and female germline stem cells[32], yet the intensity of vasa:EGFP expression is correlated with the number of oocytes and can be used to distinguish presumptive females from males[33]. Accordingly, prior to sexual differentiation at 21 dpf, embryos retained a 'juvenile ovary' with low levels of fluorescence detected. At later stages, presumptive male gonads retained low fluorescence whereas female gonads displayed intense fluorescence (Fig. 2a–f).

We isolated germline cells from individual females and males at four time points during gonad transformation (35, 40, 45, 50 dpf). Despite the high expression of vasa:EGFP in mature oocytes, cell filtering prior to FACS restricted cell size to 40 µm. Thus, we were able to collect germinal stem cells (GSCs), oogonia, and stage IA and early IB oocytes[34]. For males, vasa:EGFP expression decreases as germ cells progress through gametogenesis[33], meaning just GSCs and cells during early male gametogenesis were assessed. The male and female germline cells we tested were

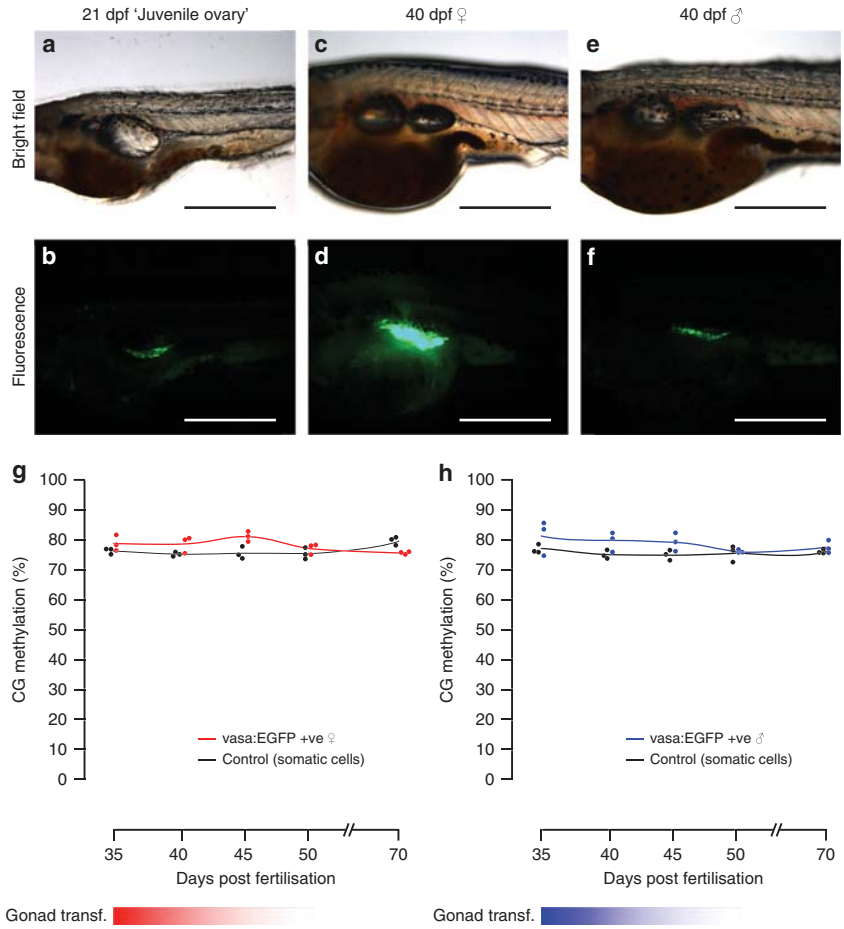

Fig. 2 Fluorescence microscopy of germline cells and their methylation during gonad transformation. **a**–**f** Phenotypic sex in zebrafish can be identified using vasa:EGFP expression: during the 'juvenile ovary' stage, expression of EGFP is low but consistent between individuals (**a**, **b**). Later, expression of EGFP vastly increases in presumptive females (**c**, **d**) relative to presumptive males (**e**, **f**). This enables sex phenotyping in early stages of sexual differentiation. Scale bars are 500 µm. **g**, **h** Methylation in the vasa:EGFP +ve germline cells of female (**g**) and male (**h**) fish from the gonad transformation stage until sexual maturity (35–70 dpf). Non-germline control cells were also tested (black lines and dots). For each sample type and timepoint, $n = 3$ independent biological replicates were used

not globally differentially methylated in any of the stages evaluated. The average levels of methylation in CG context for EGFP +ve cells were 78.9% and 79.06%, respectively, for presumptive females and males. Furthermore, global differences in methylation were not found in sexually mature individuals (70 dpf) with average 5-mC levels of 75.64% for females and 77.51% for males. This suggests that hypermethylation of the male germline relative to the female germline, occurs during the final stages of spermatogenesis.

Our low-coverage analysis cannot quantify methylation at single-copy loci, yet, we were able to analyse some genomic subsets. On average 54.31% ($n = 116$, ±SD 1.01) of CG calls mapped to repeated regions (very similar to the overall repeat level of 52%, from Howe et al.[35]). Not surprisingly, we found repetitive regions had greater methylation (mean 86.94%, ±SD 1.88) for all the samples compared to non-repetitive sequences (mean 67.64%, ±SD 4.29) ($p < 0.01$, Wilcoxon signed-rank test). Importantly, there was no hypomethylation of germline samples in either repetitive or non-repetitive subsets relative to non-germline controls (Supplementary Data 1).

Amplification and demethylation of oocyte-specific rDNA. Our initial analysis of germline methylation was performed using non-overlapping sliding windows (Figs. 1, 2), however, when all mapped reads were analysed irrespective of their location, methylation levels were greatly reduced for 9 EGFP +ve samples at 28–50 dpf (Supplementary Data 1). One explanation for this was that a lowly methylated region (or regions) was over-represented in our dataset for either technical or biological reasons. When we measured the occurrence of mapped CG calls within sliding 1 Mb windows throughout the genome, we found that the tip of chromosome 4 (Chr4:77,000,001–78,000,000; GRCz11) possessed a surprisingly high density of CG calls (Fig. 3a). Closer inspection revealed that the over-represented reads mapped to both strands of a 17.3-Kb region (chr4: chr4:77,549,891–77,567,278) containing an 11.5-Kb repeat unit encoding 45S ribosomal DNA (chr4:77,555,720–77,567,278) (Fig. 3b). It has been recently reported that at least 2 clusters of rDNA exist within the zebrafish genome[36]. One of these clusters contains the canonical rDNA expressed in all somatic cells. The other cluster is a maternal-specific rDNA type, which we term

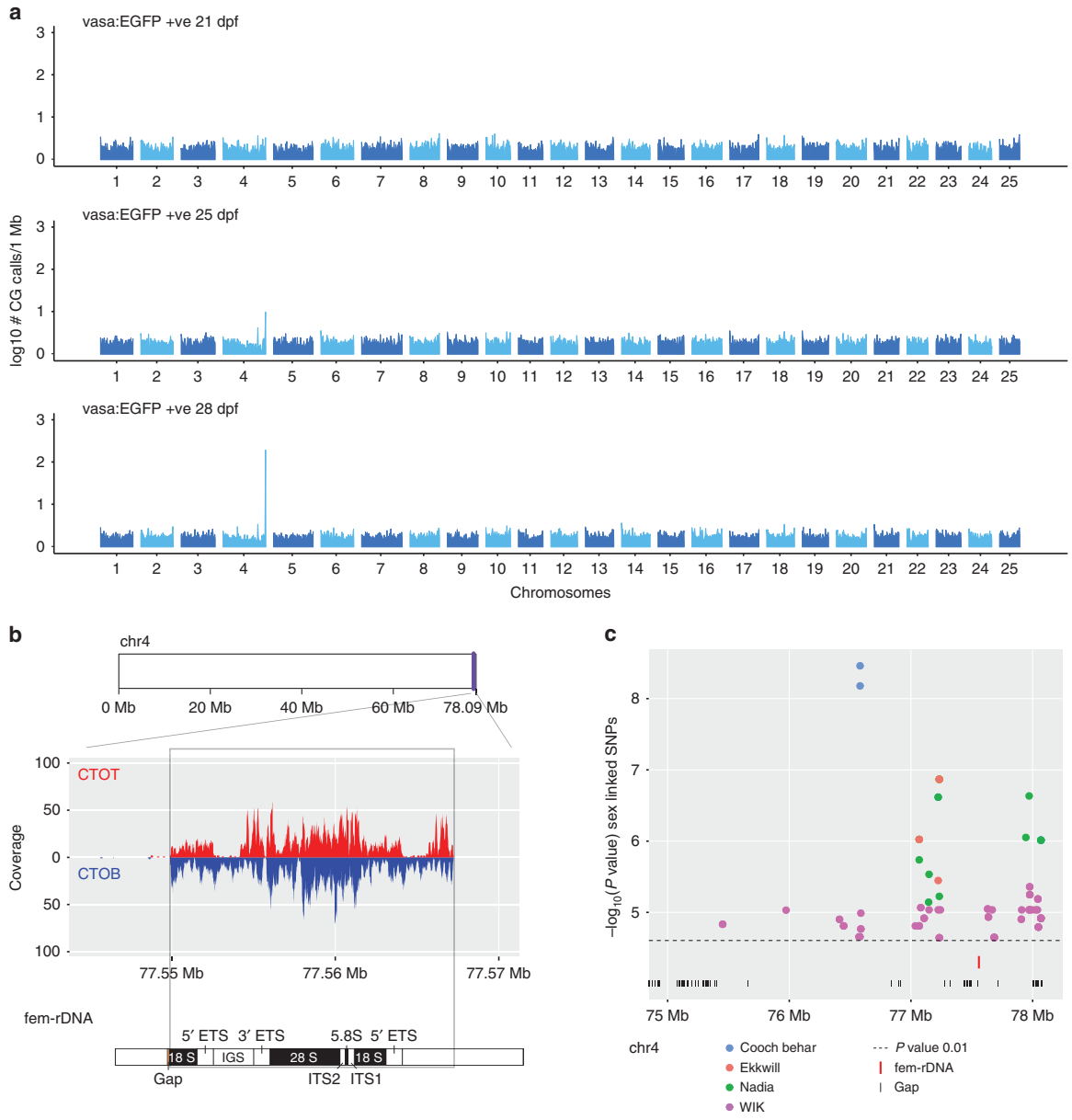

Fig. 3 Amplification of oocyte-specific fem-rDNA in a previously identified sex-linked region. **a** Number of CG calls mapping to windows of 1 Mb in the whole zebrafish genome. A peak is observed at the right tip of chromosome 4 following 25-dpf and 28-dpf. **b** Reads map to both the complementary original top (CTOT, red) complementary original bottom (CTOB, blue) strand of the 45S fem-rDNA unit on chromosome 4. Components of the rDNA repeat are indicated (External transcribed spacer (ETS), Internal transcribed spacer (ITS), Intergenic spacer (IGS)). **c** The amplified region is located within the most significant sex-linked SNPs from non-domesticated zebrafish strains, Cooch Behar, Ekkwill, Nadia and WIK

fem-rDNA, which overlaps with our overrepresented reads on chromosome 4. The genome reference has assembled only one unit of likely several fem-rDNA copies, separated by intergenic spacers[37].

The tip of chromosome 4 in zebrafish is notable for its close linkage to a previously identified locus associated with sex in natural strains[38]. Analysis of SNPs with the strongest statistical support for sex-linkage in two natural laboratory strains (WIK and EKW) and two recently-sourced wild isolates (Nadia and Cooch Behar) revealed that amplified 45S fem-rDNA is located within the major sex-determining region in chromosome 4 (Supplementary Data 2). The high and variable number of rDNA copies and its location in a poorly-assembled section of the genome makes it difficult to establish the true length of the fem-rDNA repeat. Nevertheless, sex-linked SNPs located at both ends of this gap suggest the complete fem-rDNA cluster is embedded within the sex-determining region (Fig. 3c).

Using our low-coverage BS-seq data, we measured fem-rDNA amplification and methylation levels in both germline and control samples from females, males and sexually indeterminate fish. In the non-germline control samples, we found that fem-rDNA reads, on average, comprised 0.032% of total reads sequenced

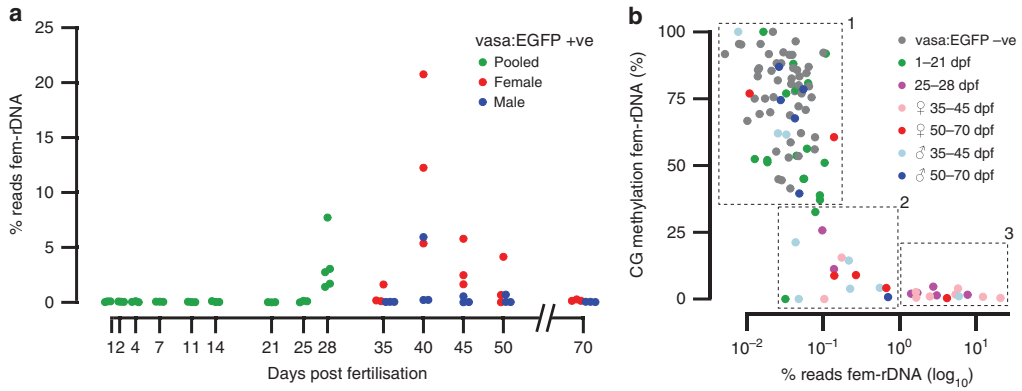

Fig. 4 Amplification and methylation of oocyte-specific fem-rDNA during gonad transformation. **a** Percentage of reads mapping to fem-rDNA in the germline prior to sexual differentiation (green), and in presumptive males (blue) and females (red) during gonad transformation and after sexual maturation. **b** Relationship between the amplification and methylation of fem-rDNA for vasa:EGFP −ve control samples (grey dots), and vasa:EGFP +ve germline cells from sexually undifferentiated fish at 1–21 (green dots) and 25–28 dpf (magenta dots); presumptive female fish at 35–45 (pink) and 50–70 (red) dpf; presumptive male fish at 35–45 (light blue) and 50–70 (blue) dpf. Samples have been divided into 3 clusters based on rDNA level and methylation; (1) 'background' non-amplified and methylated (2) moderately amplified and lowly methylated, (3) highly amplified and unmethylated. These consist of $n = 75$, $n = 15$, and $n = 14$ samples, respectively

(Supplementary Data 3). These levels were similar to the proportion of fem-rDNA reads in adult muscle tissue from other datasets (0.049%, Supplementary Data 4)[15]. As such, we considered this range to represent non-amplified, background levels of fem-rDNA. Fem-rDNA levels were also at the background level for 41 out of 55 germline samples but there was clear enrichment for fem-rDNA in 8 females and one presumptive male (>1% reads mapping to fem-rDNA), peaking at 40 dpf (Fig. 4a). For one female, 20.75% of reads mapped to fem-rDNA, representing amplification of at least 170-fold compared to non-germline controls. To validate this finding, we performed quantitative PCR using somatic-rDNA as a similar multi-copy genomic control and independently grown fish. We found fem-rDNA was amplified relative to non-germline cells in 9 out of 12 female germline samples (>5-fold) at 35 and 40 dpf (range 5.89–93.54-fold amplification relative to background), but found no amplification in a further three males (Supplementary Fig. 4).

When we analysed fem-rDNA methylation, we found three clear groups. All non-germline controls and many male and female germline samples were highly methylated (mean 73.2%, ±SD 17.29%), and did not show fem-rDNA amplification (mean 0.04%, ±SD 0.02%, see group 1, Fig. 4b). In contrast, those with strong amplification of fem-rDNA (i.e., >1% of total reads) were fully demethylated (mean 1.72%, ±SD 1.29%), and except for one individual, were either phenotypically female or late in the sexually indifferent phase (see group 3, Fig. 4b). An intermediate group of germline-only samples showed modest amplification of fem-rDNA (0.032–0.703% reads mapping to fem-rDNA, mean 0.23%, ±SD 0.22%) and were lowly methylated (mean 10.08%, ±SD 10.1%). Together this shows that fem-rDNA amplification and demethylation is highly correlated with feminisation of the zebrafish gonad.

Discussion

DNA methylation represents a stable yet flexible gene expression control system that is critical for formation of cell identity during development[39,40]. In mammals, global erasure of DNA methylation is closely related to the acquisition of developmental potency in the early embryo and during re-animation of the bipotential germline during PGC definition (reviewed in ref. [41]). In species with a preformed germline, where PGC specification relies on heritable maternal factors and not dedifferentiation of somatic cells, existence of epigenetic erasure and reprogramming is unknown. In this study, we employed low coverage WGBS-seq to evaluate DNA methylation dynamics in the zebrafish germline throughout development. In stark contrast to mammals, we find DNA methylation is not erased at any stage of germline development ranging from 24 hpf until sexual maturity. While we could not test germline cells for demethylation less than 24 hpf (oocyte-derived vasa:EGFP is found in somatic tissues at this time), Skovortsova et al.[42], in this issue, isolated germline cells from multiple time-points less than 36 hpf using an alternative transgenic line, and also found no global reprogramming. Indeed, compared to somatic cells, deep-sequencing of PGCs by Skovortsova et al[42]. revealed very few unique germline methylation patterns, despite markedly divergent transcription.

In mammals, epigenetic memory in the form of CG methylation is carefully maintained at 70–85% in adult somatic tissues, with significant demethylation only occurring in pathological situations[43,44]. Global DNA demethylation in the early embryo is tightly linked to acquisition of naïve pluripotency[45–47], with a second and more dramatic erasure event occurring in PGCs, where reprogramming helps activate the germline programme[8,48]. In contrast to mammals, species with a preformed germline such as *Xenopus* and zebrafish do not require de novo formation of PGCs at each generation and instead use inherited cytoplasmic determinants to continuously define germline cells[49]. In line with the lack of cellular reprogramming required, our study shows that global DNA methylation erasure is not a feature of germline specification in zebrafish (Fig. 5a). Given the vast majority of vertebrate species similarly define PGCs using this mechanism[49], it seems likely that bulk intergenerational preservation of DNA methylation exists in other non-mammalian vertebrates, but this remains to be tested. The absence of DNA methylation erasure at early zebrafish embryo stages[14–16] and in the germline (this study and Skovortsova et al.[42]) provides a mechanistic explanation through which DNA methylation at transgenes can be stably inherited between generations[12]. While transgenerational epigenetic inheritance appears to be a rare (but potentially important) mode of mammalian inheritance[9,10], our data suggest adaptive epigenetic changes in response to

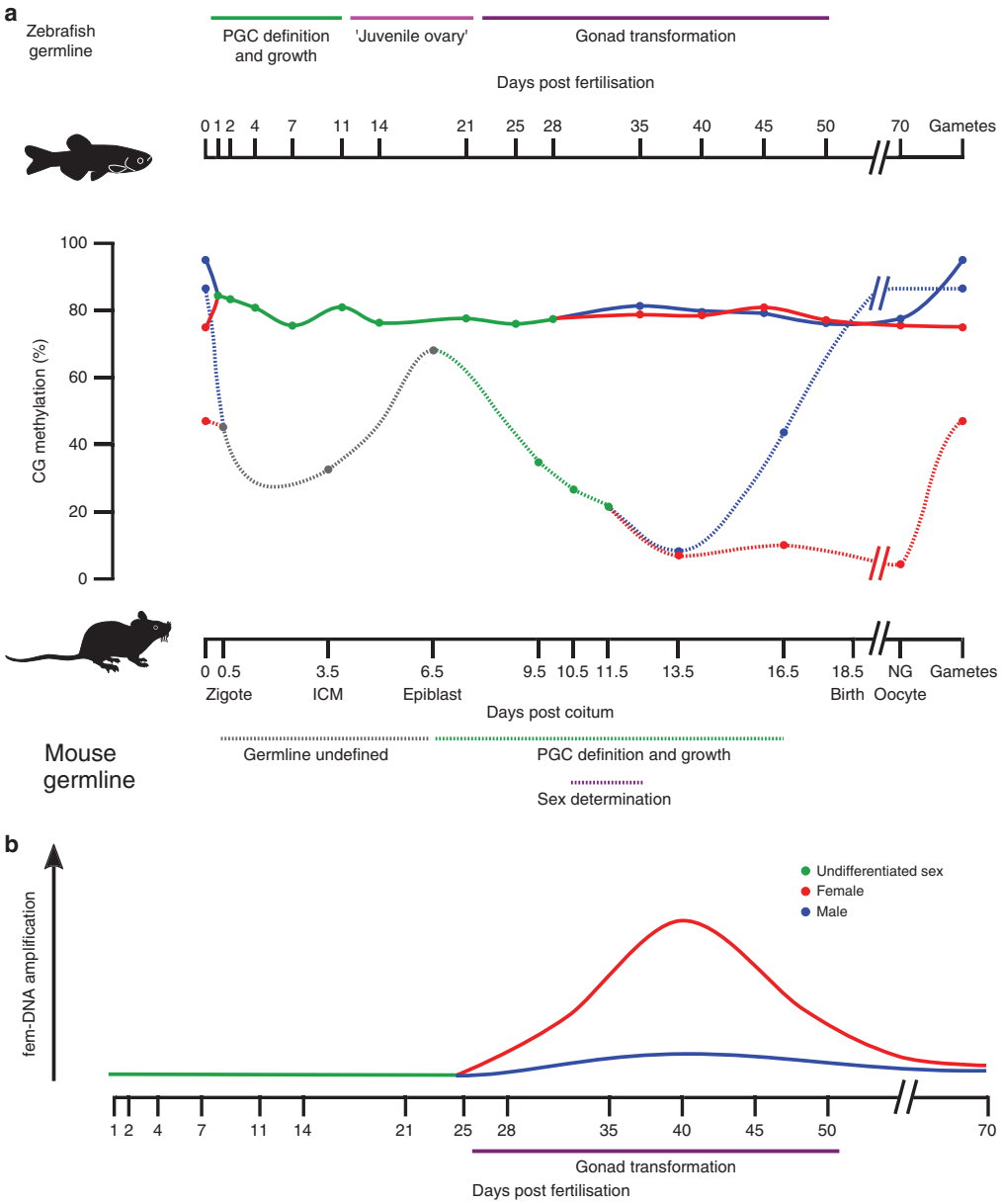

Fig. 5 Global DNA methylation and fem-rDNA dynamics in the zebrafish germline. **a** CG methylation in the mouse and zebrafish germline. In stark contrast to mice, the zebrafish germline does not undergo extensive erasure of DNA methylation and germline DNA from females (red), males (blue) and fish of undifferentiated sex (green) are similar. Note, data for mouse were taken from the meta-analysis provided by Lee, Hore and Reik[41]. **b** Striking amplification of fem-rDNA occurs in germline cells during the critical period of gonad transformation in females. Beginning around 28 dpf perinucleolar oocytes amplify in at least 2 orders of magnitude oocyte-specific 45S rDNA. ICM, inner cell mass; non-growing (NG) oocyte

environmental cues may be comparatively more significant in non-mammalian vertebrates.

Sexually dimorphic DNA methylation emerges early during gonadal development in mammals. For mice, global methylation levels in PGCs at day 13.5 post-coitum, soon after the beginning of sex differentiation, drop to 7% in females and 14% in males[4]. The oocyte epigenome remains significantly hypomethylated in relation to sperm in mice being 40.0% and 89.4%, respectively[50]. In zebrafish global levels of methylation in sperm are 95% whereas mature oocytes are 20% lower[14,15]. These gamete-specific methylation patterns appear to be generated relatively late in development compared to mice (Fig. 5a)—we find consistent methylation levels in both the male and female zebrafish germline from the gonad transformation period until the point of sexual maturity for cells in the early stages of gametogenesis. Isolation of male germline cells from late gametogenesis, using either single cell analysis or a marker more versatile than vasa:EGFP[51], will refine our understanding of when gross sexually-dimorphic methylation patterns become apparent in zebrafish. In addition, full-coverage sequencing during the gonad transformation period

may uncover sex-specific methylation at single-copy loci, however, the fact that global methylation remains similar between sexes supports previous claims that germline stem cells remain plastic in adults and can rapidly switch to producing the alternative gamete[52].

Extrachromosomal amplification of rDNA by rolling circle intermediates has been described in several animal species including *Xenopus* and zebrafish[36,53–55], and is thought to primarily help support the unique metabolic demands of the oocyte. Our study used detailed quantitative sequencing techniques in the germline to uncover >170-fold amplification of maternal-specific 45S rDNA (called here 'fem-rDNA'). We found fem-rDNA first appears in the germline at 28 dpf (Figs. 4a, 5b). At this stage, perinucleolar oocytes become particularly abundant in presumptive females[20,31] and are characterised by the proliferation of nucleoli, a structural manifestation of ribosome biogenesis[19,56]. Perinucleolar oocytes are critical for sex determination in zebrafish; as the central signalling cell responsible for feminisation of the gonad, decreased numbers of perinucleolar oocytes lead to a male gonadal fate[20,22,57]. By definition, an inability to amplify fem-rDNA would block formation of perinucleolar oocytes, and presumably therefore, suppress female differentiation. Interestingly, we found one presumptive male showed fem-rDNA amplification. The ovary-to-testis transition in male zebrafish involves degradation of perinucleolar oocytes[33], a process potentially still ongoing in this individual.

In addition to creating the defining characteristic of perinucleolar oocytes, the genomic location of fem-rDNA suggests it may be implicated in sex determination. A 1.5-Mb region overlapping fem-rDNA is strongly linked to sex phenotype in non-domesticated zebrafish strains[38], and as yet, no candidate sex-determining genes have been identified within it. Sex-linked machinery or regulatory elements that control fem-rDNA transcription or amplification, or even differential fem-rDNA repeat number, could contribute to sex determination in wild strains of zebrafish.

In domesticated zebrafish strains, where no regions of the genome appear to be sex-specific, non-genetic factors may contribute to rDNA amplification or transcription. Accessibility and transcription of ribosomal genes is strongly associated with epigenetic regulation. For example, loss of methylation in the spacer region of rDNA is inextricably linked with transcriptional activation in *Xenopus*[58], and methylating a single CG within the mouse rDNA promoter represses transcription in vitro[59]. It is tempting to speculate that epigenetic modification of fem-rDNA may help explain skewed or non-mendelian sex ratios in domesticated zebrafish strains. While this possibility remains to be tested, it is intriguing that the demethylating agent 5-azacytidine induces feminisation of zebrafish, as would be predicted if epigenetic modification of fem-rDNA played a central role in sex determation[24].

In conclusion, our work demonstrates (i) the absence of global DNA methylation erasure in the zebrafish germline and (ii) extensive amplification and demethylation of the oocyte-specific fem-rDNA cluster during gonad transformation. By showing that epigenetic memory in the form of DNA methylation is not erased in the germline from 24 hpf until sexual maturity, we provide a mechanistic explanation for transgenerational epigenetic inheritance in species with a preformed germline and suggests DNA methylation therefore may have an underappreciated role in heredity and evolution. In addition, the amplification and demethylation of fem-rDNA in peri-nucleolar oocytes, the key cell type signalling feminisation of the zebrafish gonad, suggests fem-rDNA has a critical function in sex determination for this species.

Methods

Zebrafish husbandry and collection. Use of zebrafish in this study was approved by University of Otago Animal Ethics Committee (ET 25/2017). Adult *Tg(vasa:EGFP)* zebrafish[60] were maintained under standard conditions at the Otago Zebrafish Facility, University of Otago[61]. Embryos were obtained through natural spawning and grown in 28.5 °C egg water (NaCl 5.0 mM, KCl 0.7 mM, CaCl 0.33 mM, $MgSO_4$ 0.33 mM). After the hatching period, larvae were transferred to the central system. Embryos, larvae, young and adult fish were euthanized by rapid cooling in ice cold water for 10 min[62] and then they were visualised with a LEICA M205 FA fluorescence microscope and a LEICA DFC490 CCD camera.

Preparation of embryonic cells for sorting. Fish of different developmental stages were dissociated by vigorous pipetting in 500 µl TrypLE^{TM} Express (ThermoFisher, 12604021). For 24-hpf and 48-hpf embryos, eggs were manually dechorionated using two tweezers, for post-hatching fish until 14 dpf whole fish were trypsinized. For older fish (>14 dpf) the gonadal region was dissected, and cells were dissociated. To stop the trypsin reaction, and stain cell nuclei, we added 20 µl of foetal calf serum plus 1:10,000 DAPI (ThermoFisher, 10091-148) per reaction. Disaggregated cells were separated from debris using a 40 µm nylon cell strainer (Biologix, 15-1040) and keep on ice prior sorting.

Fluorescence-activated cell sorting of zebrafish germ cells. Disaggregated cells were passed across a 488 argon laser to detect EGFP (BD Fortessa, BD Biosciences; BD FACSAria sorter, BD Biosciences). Forward scatter (FSC) and side scatter (SSC) parameters were used for observation of the cell distribution profile. We used relative FSC, SSC, and EGFP intensities to identify a germline subpopulation and these cells were gated and sorted. Cells were collected in 0.2 ml tubes containing 20 µl ddH_2O and stored at −80 °C or in 100 µl of PBS for fluorescence visualisation. For methylation analysis of embryos, larvae and juvenile fish between 1 and 28 days, 3 replicates for EGFP −ve and +ve were obtained for each time point (1, 2, 4, 7, 11, 14, 21, 25, 28 dpf), except for EGFP +ve cells at 28 dpf when 5 replicates were analysed ($n = 56$). To evaluate the gonad transformation period and sexual maturity (35, 40, 45, 50, 70 dfp), 3 replicates were obtained in a similar fashion for each sex ($n = 60$). As such, the total number of samples purified for high-throughput methylation analysis was 116. Additionally, EGFP +ve and −ve cells were sorted from 15 individuals between 35 and 40 days; samples which were used to verify fem-rDNA using quantitative PCR. The purity of sorted cells was assessed using raw images captures by an IncuCyte FLR imaging system (Essen Instruments).

DNA extraction. Total nucleic acids were purified using the Bio-On-Magnetic-Beads (BOMB) approach[63]. Briefly, a guanidine isothiocyanate lysis buffer was used to homogenise cells and then was combined with TE-diluted Sera-Mag Magnetic SpeedBeads (GE Healthcare, GEHE45152105050250) and isopropanol in a volumetric ratio of 2:3:4 (beads:lysate:isopropanol). Beads were captured with a neodymium magnet and washed once with isopropanol, twice with 70% ethanol and resuspended in milliQ water.

PBAT library preparation and sequencing. Bisulfite-converted genomic libraries were prepared using a modified post-bisulfite adaptor tagging (PBAT) method[64,65]. Bisulfite treatment was performed according to the EZ Methylation Direct Mag Prep kit (Zymo, D5044) instruction manual. To synthesise the first strand, we used converted DNA and 5′-biotinylated adaptor primers bearing seven random nucleotides at its 3′ end (BioP5N7, biotin- ACACTCTTTCCCTACACGACGCT CTTCCGATCTNNNNNNN). The first strand product was purified using streptavidin-coated magnetic beads (ThermoFisher, 11205D) and alkaline denaturation. Second strand DNA was synthesised using the immobilised first strand DNA and another adaptor primer also bearing seven random nucleotides at its 3′ end (P7N7, GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNN). Second strand DNA was eluted, amplified by 15 cycles of PCR and size selected by PEG-diluted SPRI beads. During PCR, sample-specific barcodes and sequences required for Illumina flow-cells binding were added to libraries using 1× HiFi HotStart Uracil + Mix (KAPA, KK2801 and 10 µM indexed Truseq-type oligos). Library integrity was assessed by agarose gel electrophoresis and sequenced on a 150 bp single-end run on Illumina MiSeq.

Bioinformatic analysis. The quality of the raw FASTQ files was evaluated using FastQC software (v0.11.4). Raw reads were trimmed using Trim Galore! v0.4.2, in a two-step process. First, adaptors were removed and 10 bp was hard-trimmed from the 5' end of all reads and low-quality base calls (Phred score < 20) were removed. Read mapping and base calling was performed using Bismark v0.19.0[66] with the option --pbat specified. Zebrafish genome version 11 (GRCz11) was used as reference. Global methylation in CG context was calculated as the proportion of total methylated cytosines in CG context over total cytosines in CG context using non-overlapping windows of 10 Mb in SeqMonk programme v1.43.0. The non-conversion rate during the bisulfite treatment was evaluated by calculating the proportion of non-CG methylation; by this measure, all libraries must have had a bisulfite conversion efficiency of at least 96.02% (Supplementary Data 1). A BSGenome data package was forged for using the latest UCSC zebrafish genome

version 11 (GRCz11) (BSGenome v1.46)[67] and used for all the analysis requiring this dependency in R (v3.4.4). To analyse differential DNA methylation levels in repetitive and non-repetitive sequences we used RepeatMasker annotations obtained from UCSC (v4.0.5 RepBase library: 2014013)[68].

Margin of error estimation for low-coverage WGBS. To evaluate the number of CGs required to accurately predict the genome-wide methylation status we used empirical bootstrap sampling[30] from previous high-coverage zebrafish methylation datasets (SRR800056, SRR800081)[15]. Briefly, we used fastq-tool v0.8 (https://homes.cs.washington.edu/~dcjones/fastq-tools/) to obtain 1000 random samples with replacement in regular intervals of CG calls from approximately 100 to 30,000. Each sample was processed as mentioned previously and the proportion of data falling within the 0.5–99.5 percentiles was calculated to generate a margin of error (99% confidence interval).

Ribosomal DNA bioinformatic analysis. To determine overrepresented regions among the genome, EGFP +ve replicates for each time point (21, 25, and 28 dpf) were merged and the number of CG calls per Mb was calculated and divided by the average number of calls in all the probes. To identify the amplified region, reads were aligned to complementary converted strands of chromosome 4 (CTOT and CTOB) using Bowtie2 v2.3.2[69] with the --very-sensitive option (-D 20 -R 3 -N 0 -L 20 -i S,1,0.50) to increase mapping sensitivity and accuracy. Manhattan plot and coverage plot were drawn using ggbio v1.26.1[70].

The genome coordinates of sex-linked SNPs previously published[38] were converted from Zv9 to GRCz11 using CrossMap v0.3.0[71]. Previous methyl-seq datasets were obtained from SRA and processed as mentioned above with the option --directional specified[15]. Number of reads and CG methylation for fem-rDNA were quantified for the region chr:77,549,891:77,567,278. For low coverage WGBS, methylation for fem-rDNA was calculated as the proportion of methylated C's in CG context over the total C's in CG context in samples with at least 10 calls for the region of interest. For deep sequenced datasets, fem-rDNA methylation was quantified as the proportion of methylated C's in CG context over the total number of C´s in CG context within the 17.3 Kb amplified region. Scatter plots were drawn using ggplot2 v3.0.0 in R v3.4.4.

Quantitative PCR. Quantitative PCR was performed using the SensiFAST™ SYBR® No-ROX Kit (Bioline, BIO-98020) and the LightCycler® 480 instrument (Roche). Specific PCR primers sequences for fem-rDNA and somatic rDNA are listed in Supplementary Table 1. Negative controls included EGFP −ve cells from females and males at 35 dpf, EGFP +ve cells from males at 35 dpf and embryonic cells at 24 hpf. The final volume in each reaction was 12 µl including 6 µl of SensiFAST™ SYBR® No-ROX mix and primers at a concentration of 900 nM. The reactions were incubated in white 96-well plates at 98 °C for 5 min, followed by 45 cycles of 98 °C for 20 s, 60 °C for 15 s, and 72 °C for 20 s. All reactions were run in duplicate. Data was analysed with the LightCycler® 480 software (Roche) determining the threshold cycle (Ct) by the second derivative max method. A baseline level of amplification was determined as the mean of Δ Ct (Ct fem-rDNA – Ct som-rDNA) for non-germline samples (EGFP −ve females and males) and the value obtained was used as control for sample normalisation (i.e., ΔΔ Ct method).

Statistics. Statistical analysis between groups was performed using Wilcoxon signed-rank tests. In all cases significance was set as $p < 0.05$.

Data availability

The accession number for the FastQ files and CG calls of the low coverage WGBS libraries reported in this paper is GEO: GSE122695. All other relevant data supporting the key findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request. The source data underlying Figs. 1a–f, 2a–h, 3a–c, 4a–b, 5a-b and Supplementary Figs. 1−5 are provided as a Source Data file. A reporting summary for this Article is available as a Supplementary Information file.

Code availability

The source code of the analysis is publicly available on Github at https://github.com/OscarOrt/Met_zebrafish_germline

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Author contributions

T.A.H. conceived and funded the study. T.A.H. and O.O.-R. designed the experiments, with contributions from N.J.G. O.O.-R. collected the samples and performed the laboratory work. O.O.-R. and T.A.H. performed bioinformatic analysis. R.C.D. aided with sequencing. O.O.-R. and T.A.H. wrote the manuscript. All authors contributed and approved the final manuscript.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-10894-7.

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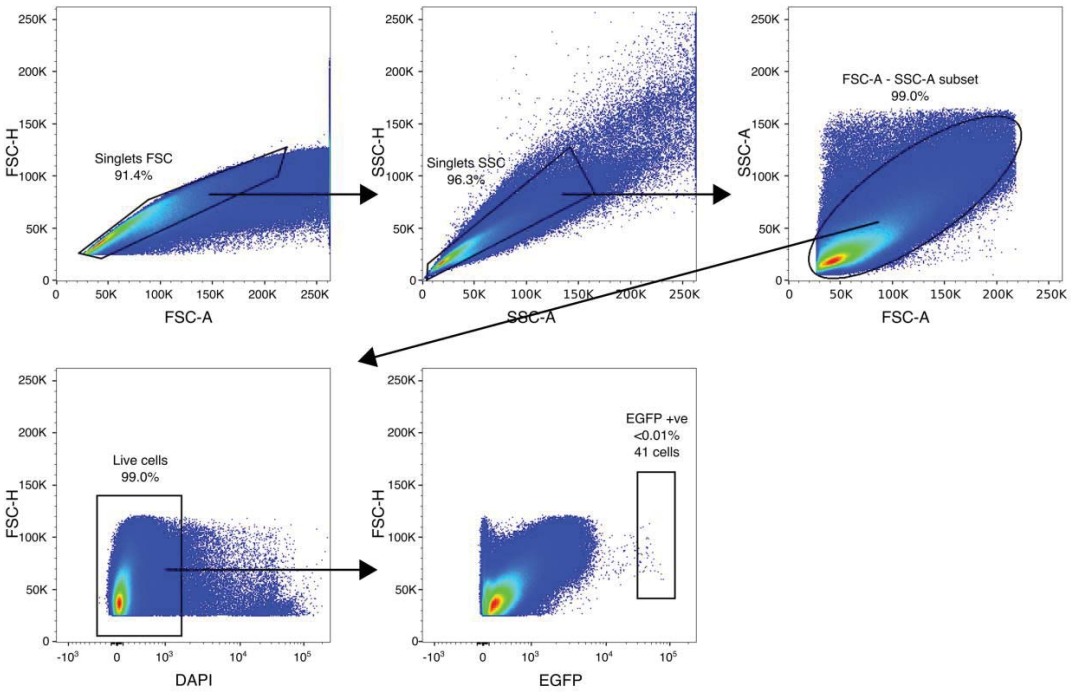
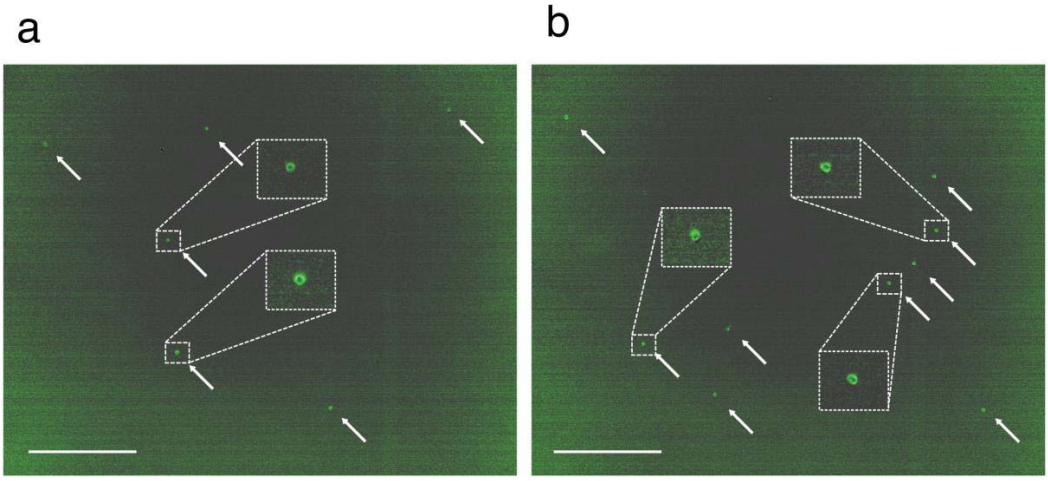
Supplementary information

Zebrafish preserve global germline DNA methylation while sex-linked rDNA is amplified and demethylated during feminisation, Ortega-Recalde *et al.*

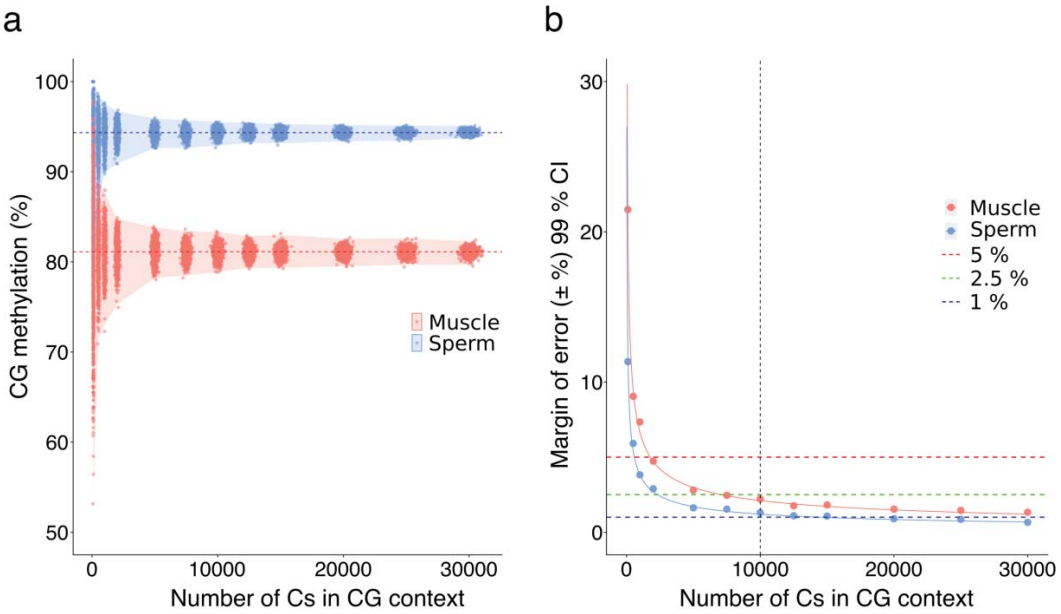
Supplementary Figures



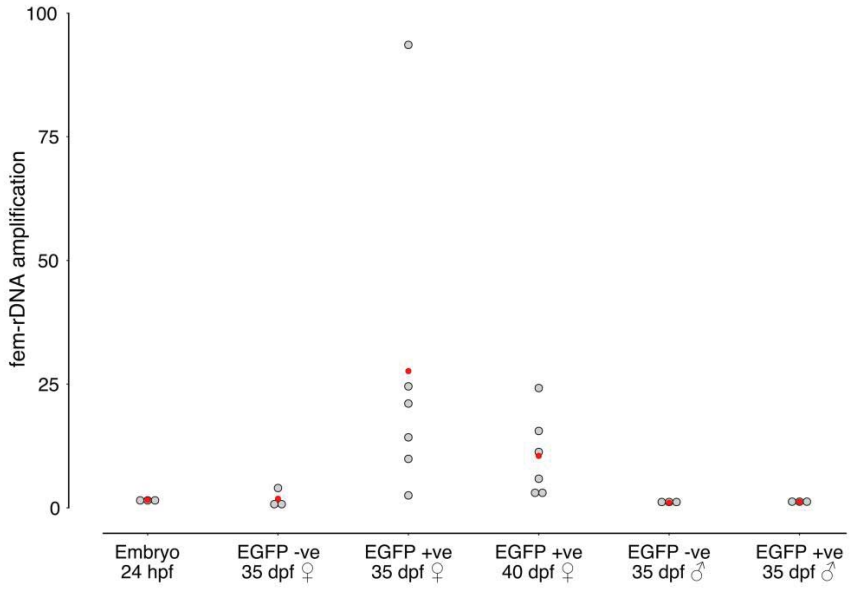
Supplementary Figure 1. *Gating strategy used for cell sorting.* Cells were first gated by forward and side scatters (FSC and SSC respectively) to exclude doublets. The live cell gate was then set to eliminate dead cells stained with DAPI. The gate boundaries for 'positive' and 'negative' cells were determined within the live cell gate by three criteria: i.) large EGFP intensity differences. ii.) cell clustering. iii.) EGFP expression in tissues containing non-germline cells. The same strategy was used to sort vasa:EGFP +ve cells for results presented on Fig. 1B, 1C, 2G and 2H. Blue dots indicate discrete data points (i.e. cellular events), whereas green, yellow and red colouring indicate increasing data density.



Supplementary Figure 2. *Germline isolation by FACS.* **a-b** Fluorescence microscopy of vasa:EGFP +ve sorted cells. Magnification of EGFP +ve labelled cells is shown inset (dashed lines). The proportion of EGFP +ve cells ranged from 93.8% to 100%. Scale bars are 400 µm.



Supplementary Figure 3. *Empirical bootstrap sampling of high coverage zebrafish methylation datasets.* **a** Percentage of methylation per number of Cs sampled for 1,000 replicates at each sample size. **b** Confidence Interval (CI) at different sample sizes. Margin of error ± 99% CI (*n* = 1,000).



Supplementary Figure 4. *Amplification of fem-rDNA determined by quantitative PCR.* A baseline level of amplification was determined as the mean of Δ Ct (Ct fem-rDNA – Ct som-rDNA) for non-germline samples (EGFP -ve females and males) and the value obtained was used as control for the ΔΔ Ct method (*n* = 3 non-germline samples, EGFP +ve males and embryos at 24 hpf, *n* = 6 EGFP +ve 35 and 40 dpf females). Arithmetic means are represented by red dots.

Supplementary Table

Supplementary Table 1. *List of oligonucleotides used in this study.*

Name	Sequence
BioP5N7	[Bio] - ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNN
P7N7	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNN
Zf_fem_rDNA_1F	ACACACATTGATCATCGACCT
Zf_fem_rDNA_1R	TAGCAAGGCGACCCTCAGA
Zf_som_rDNA_1F	GATGCCCTTAGCTGGGTGT
Zf_som_rDNA_1R	CCTAGCTGCGGTATTCAGCG

Description of Additional Supplementary Files

File name: Supplementary Data 1

Description: *Low coverage bisulfite sequencing of zebrafish germinal cells and control somatic tissues.* The table lists the general sequencing statistics as well as the number of cytosine calls at either CG dinucleotides ('CG') or in other sequence contexts ('non-CG'), for germline cells or control cells, mapped against the Zebrafish genome assembly version 11 (GRCz11). Details of bioinformatic processing are provided in the Methods section. The frequency of non-CG methylation indicates the maximum rate of non-conversion during the bisulfite treatment step; by this measure, all libraries had a bisulfite conversion efficiency of at least 96.02%.

File name: Supplementary Data 2

Description: *Conversion sex-linked SNPs Zv9 to GRCz11.* Conversion of genome coordinates from published zebrafish sex-linked SNPs (Wilson *et al*, 2014) between Zv9 and GRCz11. Only results located on chromosome 4 are shown.

File name: Supplementary Data 3

Description: *Amplification and methylation of fem-rDNA.* Amplification and methylation of fem-rDNA. Total number of reads mapping to GRCz11 is indicated in column E (Total unique mapped reads), as is the number mapping to fem-rDNA, column F(chr4:77,549,891-77,567,278). CG calls originating from those reads are shown in columns I-J.

File name: Supplementary Data 4

Description: *fem-rDNA amplification and demethylation at different developmental stages.* Number of reads, CG calls and percentage of metylation for fem-rDNA at different developmental stages (Potok *et al*. 2013)

Supplementary files could be found at:

https://www.nature.com/articles/s41467-019-10894-7

Chapter 3

"DNA methylation in the vertebrate germline: balancing memory and amnesia"

Contribution

This chapter consists of a full accepted review manuscript published in *Essays in Biochemistry*. The paper examines the dynamics of DNA methylation memory in vertebrates supported by my findings in **Chapter 2** (Ortega-Recalde & Hore, 2019).

DNA methylation memory in the vertebrate germline: balancing memory and erasure. **Oscar Ortega-Recalde**[1] and Timothy A. Hore[1]. *Essays in Biochemistry*. EBC20190038. 2019. doi:10.1042/EBC20190038.

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As the first author, I wrote the first draft of the manuscript and figures, and along with my supervisor, Dr Timothy A. Hore, prepared the final draft.

In this manuscript, I introduced the main features of DNA methylation in vertebrates and contrasted current evidence for maintenance and erasure of epigenetic memory in mammals and non-mammalian vertebrates. I focused on non-mammalian models to further explore the importance of DNA methylation memory in vertebrates and proposed that divergent vertebrates possess different dynamics of global DNA methylation reprogramming, particularly in the germline. Additionally, I highlight the importance of DNA methylation in non-mammalian vertebrates as a conduit of transmission for epigenetic information between generations with a potentially important role in heredity and evolution.

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Review Article

DNA methylation in the vertebrate germline: balancing memory and erasure

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Cytosine methylation is a DNA modification that is critical for vertebrate development and provides a plastic yet stable information module in addition to the DNA code. DNA methylation memory establishment, maintenance and erasure is carefully balanced by molecular machinery highly conserved among vertebrates. In mammals, extensive erasure of epigenetic marks, including 5-methylcytosine (5mC), is a hallmark of early embryo and germline development. Conversely, global cytosine methylation patterns are preserved in at least some non-mammalian vertebrates over comparable developmental windows. The evolutionary mechanisms which drove this divergence are unknown, nevertheless a direct consequence of retaining epigenetic memory in the form of 5mC is the enhanced potential for transgenerational epigenetic inheritance (TEI). Given that DNA methylation dynamics remains underexplored in most vertebrate lineages, the extent of information transferred to offspring by epigenetic modification might be underestimated.

Introduction

Epigenetic information constitutes a set of stable modifications to DNA that does not affect its sequence. Among these modifications, DNA methylation has been studied the longest [1]. In jawed vertebrates, DNA methylation occurs predominantly at the C5 position of the cytosine base (5mC) when in the CG dinucleotide (CpG) context [2,3]. This unique palindromic arrangement allows 5mC in the CpG-context to be preserved following cell division, enabling DNA methylation to act as a stable memory module residing within the genome. Despite being mitotically stable, two genome-wide waves of methylation erasure occur during mammalian development. The first of these occurs immediately post-fertilization, whereas a second, more extensive erasure occurs in the germline, essentially blocking transgenerational inheritance of DNA methylation memory. Whereas two recent reviews have examined comprehensively the profiles and functions of DNA methylation in eukaryotes and mammals [4,5], the role of 5mC as a conduit for epigenetic memory transfer between vertebrate generations is less explored. Recent studies in fish suggest that 5mC information is unlikely to be erased in the early embryo and germline of at least some non-mammals. Such 5mC retention may be common in vertebrates and could have profound repercussions for our understanding of inheritance. This review summarizes the current knowledge regarding DNA methylation memory dynamics in vertebrates and discusses the implications of its preservation between generations.

Biochemical foundations of CpG methylation memory

DNA methylation was the first epigenetic mark to be discovered [6,7] and gene knockout studies have shown that it is essential for vertebrate development and cellular differentiation [8–11]. The mechanisms by which DNA methylation is written, maintained and erased have been intensively studied [12] (Figure 1). In vertebrates, DNA methylation is first established by members of the DNA methyltransferase 3 family (DNMT3) [13,14]. In mammals, these correspond to DNMT3A, DNMT3B and DNMT3L [15]. DNMT3A interacts with DNMT3L, a related mammal-specific homolog that lacks a catalytic domain, forming complexes that stabilize and enhance *de novo* methyltransferase activity [16].

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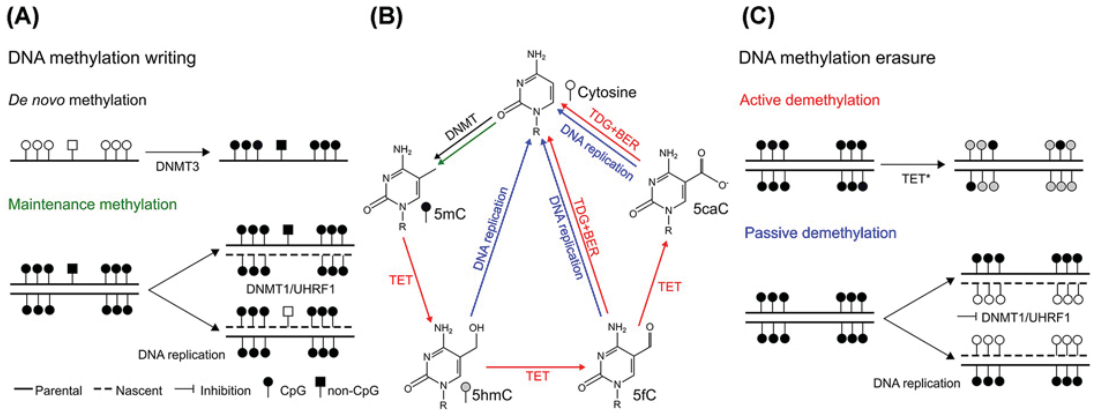

Figure 1. Biochemical foundations of DNA methylation in vertebrates

(**A**) DNA methylation is established *de novo* and maintained by DNMT enzymes. *De novo* methylation of cytosine (white-filled lollipops) to 5-methylcytosine (black-filled lollipops) is established by the DNA methyltransferase DNMT3 family. In jawed vertebrates, methylation occurs principally in palindromic CpG dinucleotides (circle lollipops). DNA methylation is faithfully inherited during cell division by the maintenance DNA methyltransferase DNMT1 recruited by UHRF1 (ubiquitin-like containing PHD and RING finger domains 1). Non-CpG methylation (square lollipops) is catalyzed by DNMT3, while DNMT1 is not associated with non-CpG methylation patterns. (**B**) DNA methylation writing, maintenance and erasure involves several biochemical pathways and intermediates. (**C**) DNA methylation erasure occurs through active or passive mechanisms. Passive demethylation is carried out by DNA synthesis without maintenance methylation, leading to progressive dilution after replication. Conversely, DNA methylation memory can be actively erased by iterative oxidation to 5-hydroxymethylcytosine (5hmC - gray-filled circle lollipop), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) catalyzed by ten eleven translocation (TET) enzymes, followed by progressive dilution of 5hmC, 5fC and 5caC after replication (active medication-passive removal) or excision of 5fC or 5caC mediated by thymine DNA glycosylase (TDG) coupled with base excision repair (BER) (active modification-active removal).

While the DNMT3 proteins are able to deposit methylation in a range of cytosine contexts, within jawed vertebrates, DNA methylation primarily occurs at CG dinucleotides [3,17]. The reason for this is that the maintenance methyltransferase machinery is able to recognize where a cytosine has been methylated, and then propagate this mark to the cognate cytosine on the other strand (i.e. complementary to the guanine on the original strand). Thus, even in the absence of signals creating DNA methylation *de novo*, epigenetic memory in the form of DNA methylation can be faithfully inherited over a lifetime by maintenance methyltransferases. In vertebrates, DNMT1 is the maintenance methyltransferase, on account of a strong preference for hemimethylated CG dinucleotides and targeting interactions with UHRF1 (ubiquitin-like containing PHD and RING finger domains 1) and PCNA (proliferating cell nuclear antigen) [15]. Divergent vertebrate models have revealed a complex and dynamic evolution of the DNMT family, including gene duplication and subfunctionalization [18–20]. Most striking among these is the large number of DNMT paralogs in teleost fish [21], some of which may be tasked with regulating distinct biological processes such as sex determination. Indeed, it was recently discovered that at least in the gonads of the sex-changing bluehead wrasse (*Thalassoma bifasciatum*), sex-specific DNMT paralogs switch their expression as the ovary spectacularly transforms into testis [22].

Erasure of DNA methylation marks can be achieved through passive and active mechanisms [23]. During passive demethylation, the activity of DNA maintenance machinery is inhibited leading to progressive loss of methylation marks upon replication. Alternatively, active demethylation of 5mC can be achieved through the activity of dedicated enzymes. Of these, the TET (ten-eleven translocation) enzymes are the most well understood—TET proteins oxidize iteratively 5mC to 5-hydroxymethylcytosine (5hmC) and other derivatives which can be replaced and ultimately removed, or diluted during replication [24].

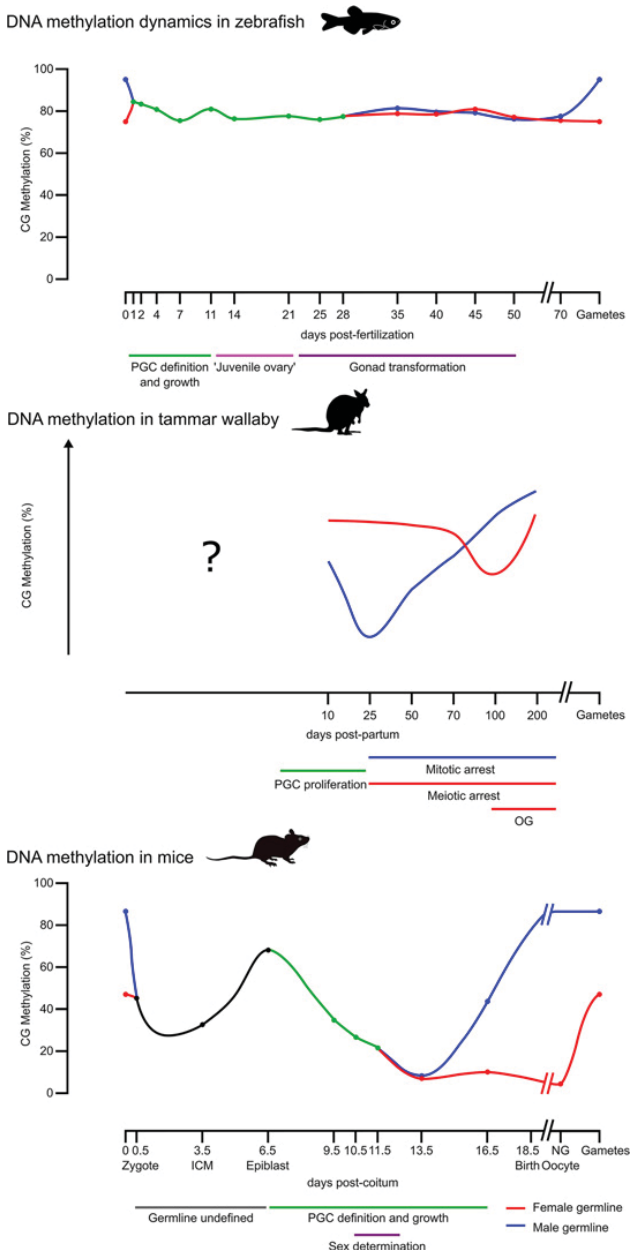

Figure 2. Dynamics of DNA methylation dynamics in zebrafish, tammar wallaby and mice

The zebrafish germline preserves global levels of methylation post-fertilization and during germline specification and development. Soon after fertilization, the maternal methylome is reprogrammed to match the paternal methylome. Germplasm inherited from the egg is mitotically segregated until 512 cells (2.75 hpf) when it is deposited in four PGCs which generate the germline. Methylation levels are maintained though germline development and sex-specific patterns are acquired during late gametogenesis. The tammar wallaby undergoes at least one major wave of germline demethylation. PGCs are demethylated post-natally when they have colonized the gonad. Whereas for males, demethylation occurs between 10 days post-partum (dpp) and 25 dpp, for females occurs principally between 70 and 120 dpp. The mouse germline undergoes two major waves of demethylation. The first in the early embryo, starting just after fertilization until blastocyst stage (embryonic day 3.5 E3.5). A second major wave specific for PGCs occurs between E6.5 and E13.5, after they are specified by extracellular signals in the epiblast. Methylation is afterward re-established in a sex-specific manner. Abbreviations: OG, oocyte growth; NG Oocyte, non-growing oocyte.

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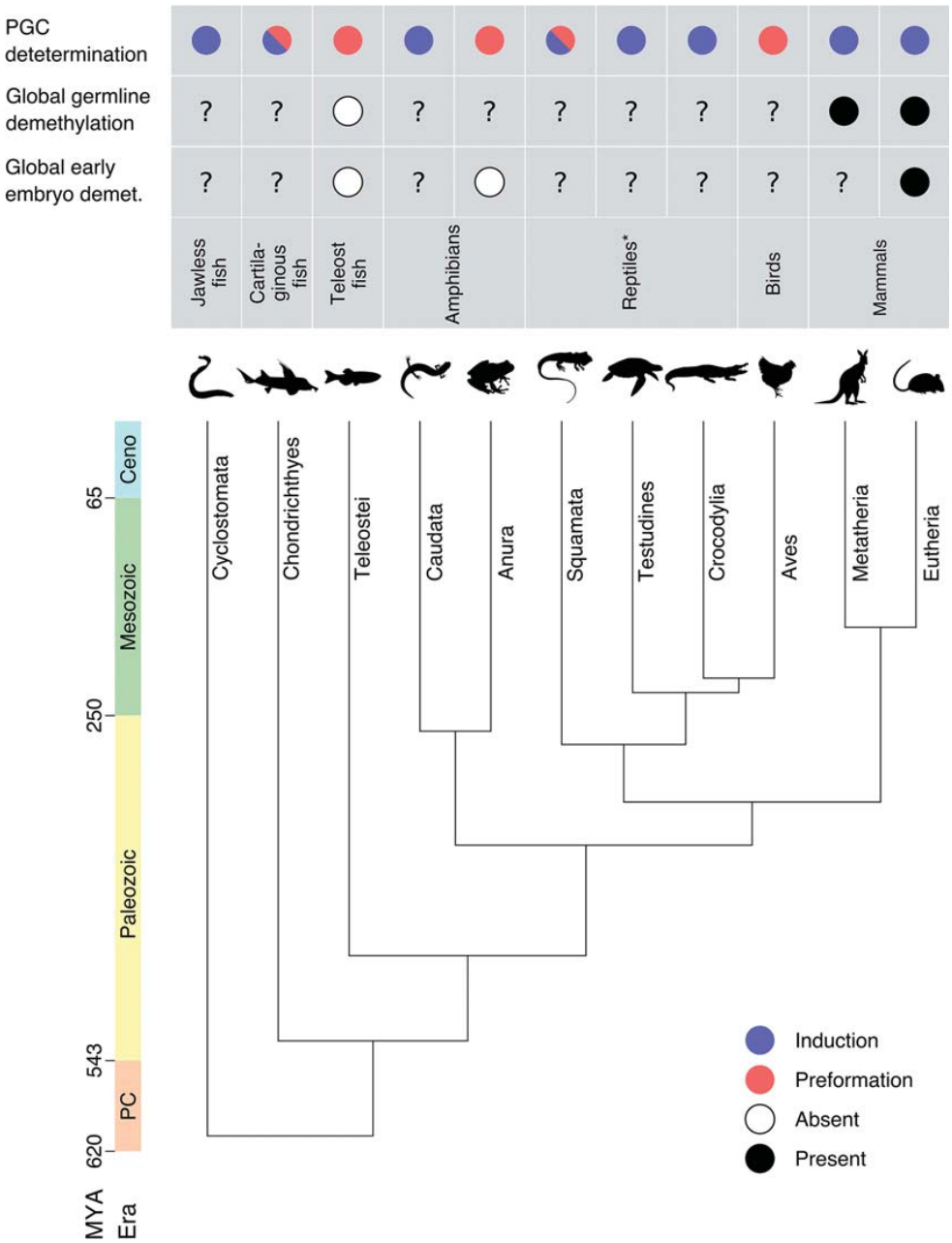

Figure 3. Germline specification and DNA methylation erasure across vertebrates

DNA methylation dynamics is unknown in most vertebrate lineages. Nevertheless, it seems possible that global demethylation might be related to germline specification strategy. Species which derive their germline by induction from epiblast cells (blue circles) may require extensive erasure of DNA methylation memory (e.g. mammals), whereas species with a preformed germline (red circles) do not require extensive DNA methylation reprogramming (e.g. zebrafish). The mammalian genome is globally demethylated in the early embryo, conversely DNA methylation is preserved in some mammalian species (e.g. zebrafish, *Xenopus*), suggesting although extensive DNA methylation erasure may facilitate reprogramming in vertebrates is not a conserved feature of pluripotency in vertebrates. Alternative explanations to explain global DNA methylation erasure in mammals may include activation of the genome post-fertilization that is dependent on ERV-demethylation, and erasure of genomic imprinting between generations. Abbreviations: Ceno, Cenozoic; MYA, million years ago; PC precambrian; Reptiles*, non-avian reptiles.

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Making and erasing DNA methylation memory in the mammalian germline

DNA methylation has been extensively studied in mammals, particularly mice and humans. For both species, the beginning of life coincides with major erasure of DNA methylation. This initial reprogramming event occurs in two waves, the first where the paternal genome is actively demethylated and the second where passive demethylation takes place on both the maternal and paternal genomes. In mice, this erasure continues until the blastocyst stage (embryonic day 3.5, E3.5) when global methylation levels reach ∼30% [25,26]. At this stage, global demethylation is tightly linked to the acquisition of pluripotency [27–29]. Recently, oscillations in DNA methylation during exit from pluripotency have been associated with the emergence of epigenetic heterogeneity in the early embryo [30]. Early human embryos follow a similar dynamic [31,32]. The first wave of demethylation occurs within the first 12 h post-fertilization (hpf) and is independent of replication [33]. Active demethylation occurs principally in the paternal genome and is much faster than mice, being complete at the two-celled stage. Then two additional phases of demethylation, the first between the late zygote and the four-celled stage embryo and the second between the eight-celled embryo and the inner cell mass/trophectoderm of the blastocyst, decrease methylation levels to ∼24%.

Substantial DNA methylation that is new to the embryo occurs at the transition from blastocyst to the post-implantation epiblast and it is targeted to repress lineage-specific genes [34]. At E6.5, mouse epiblast cells have recovered global methylation levels to ∼70%, nevertheless primordial germ cells (PGCs), which are induced at this period by cell signalling (approximately E6.25), experience a more profound and dramatic second wave of genome-wide demethylation [35,36]. The first stage of demethylation begins soon after PGC specification when Prdm1 and Prdm14 repress the expression of *de novo* DNA methyltransferases and Uhrf1 [37,38]. Repression of *de novo* and maintenance DNA methylation machinery leads to passive dilution of 5mC [39]. Then, a second stage involving active demethylation principally induced by TET takes place in a locus-specific manner [40,41]. Methylation marks in imprinting control regions and promoters of genes involved in meiosis and gamete generation are erased on this stage. Epigenetic reprogramming of PGCs, along with proper timing of these two stages, are required for proper germline differentiation [42,43]. At the end of the second wave (E13.5) methylation levels are approximately 14 and 7% for males and females, respectively, and re-methylation should take place in a sex-specific manner to acquire mature gametic patterns. PGC specification in humans occurs around the second and third week of development and at week 5, PGC methylation levels are lower than somatic cells [31,32,44]. By week 8 DNA methylation in PGCs reach a minimum of ∼4.5%. Although differences in the PGC specification mechanism exist between human and mouse, in both species the germline is reprogrammed from differentiated epiblast cells (termed as the 'induced' or broken germline) [45].

Zygotic DNA demethylation has been described for other eutherian mammals such as cattle, sheep, rabbits and rats [46–49]. While some studies show contradictory results [50,51], which could be due to differences in quantification methods, epigenetic reprogramming in the early embryo seems to be conserved in eutherians, and single-base resolution studies would be useful to detect interspecific differences. Post-fertilization demethylation has not been studied in marsupials; however, methylation reprogramming in PGCs has been found in the marsupial tammar wallaby (*Macropus eugenii*) [52,53]. Interestingly, this occurs in PGCs post-natally, when they are well-established in the gonad. The existence of basic common mechanisms to reprogram PGCs between eutherian mammals and marsupial suggests erasure of DNA methylation marks is conserved in the mammalian germline.

Increasing evidence suggests that at least some biological information associated with DNA can be transmitted between generations that is not encoded by DNA sequence (for example, DNA methylation) [54,55]. According to the number of generations to which this information is inherited, these effects can be classified as 'intergenerational' or 'transgenerational' epigenetic inheritance. Intergenerational epigenetic inheritance (IEI) is restricted to direct effects on parents. In mammals, for example, an environmental stimulus can affect the mother (F0), her embryos/fetuses (F1) and the developing germline (F2). For males, direct effects are limited to sperm (F1). Transgenerational epigenetic inheritance (TEI), on the other hand, extends beyond the second generation in males (F2) and the third generation (F3) in females. Importantly, intergenerational and transgenerational effects may share common mechanisms. A consequence of the extensive erasure of DNA methylation in mammals is the limited potential to transmit acquired DNA methylation between generations. Indeed, although residual sex-specific methylation patterns exist in the mammalian embryo transiently (reviewed [5]), long-term intergenerational transfer of epigenetic memory appears to be restricted to imprinted genes and particular classes of repeat sequences [36,40]. For imprinted genes, methylation status is preserved in the post-fertilized embryo and removed in the germline during PGC reprogramming. Oocytes and zygote factors, such as Dppa3 (also known as stella or PGC7) and NLPRs, and Zfp57 respectively,

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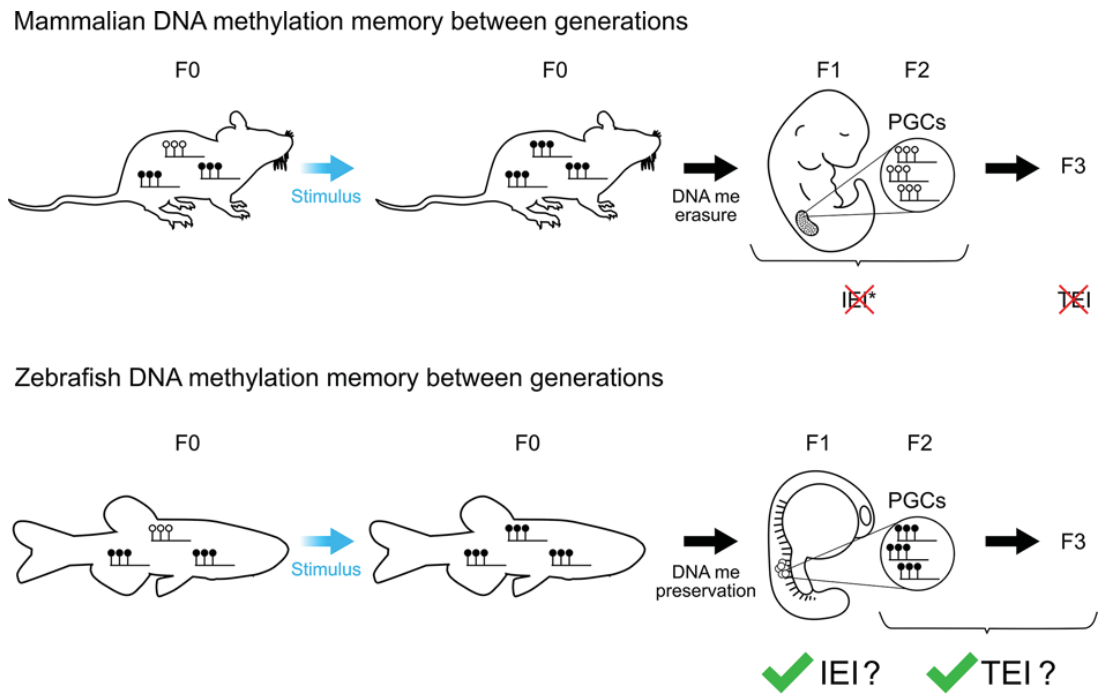

Figure 4. DNA methylation memory between generations

Extensive erasure of DNA methylation in mammals precludes IEI and TEI between generations, with the notable exception of imprinted regions and particular repeat sequences (e.g. IAP), meaning embryos inherit a largely unmodified genome. IEI in mammals include direct effects in embryos (F1) and the developing germline (F2) during gestation. TEI refers to transmission of biological information not ascribed to direct effects in parents. In contrast, preservation of global DNA methylation in non-mammalian erasure could enable IEI and TEI and mean 5mC acts as an additional memory module between generations. For species without parental influence during germline development, IEI is limited to F1.

appear to be critical in maintaining DNA methylation in mouse early embryos [56]. Among repeat sequences, endogenous retroviruses (ERVs) of the intracisternal A particle (IAP) class are the most prominent, being implicated in the Agouti viable yellow (*Avy*) and Axin fused (*AxinFu*) phenotypes [57,58]. In both cases, the phenotype resulting from each locus are correlated with methylation level of a long terminal repeat (LTR) promoter within the IAP, and are inherited as 'metastable epialleles' to offspring [59,60]. Despite the initial possibility that IAPs act as general reservoirs of transgenerational epigenetic memory, a comprehensive study showed methylation variability of IAP is mainly lost and re-established between generations and very few act as promoters [61]. Moreover, when the entire genome (i.e. repeats and non-repeats) is examined following significant dietary intervention, preservation of differentially methylated regions is very rare [62], even if specifically timed to maximize disruption of DNA remethylation in PGCs [63].

DNA methylation memory in non-mammalian vertebrates: unexplored paradigm

DNA methylation is not well understood in non-mammalian vertebrates and several lines of evidence suggest its potential for inheritance is fundamentally different for mammals. One of the most striking examples of this can be found in the half-smooth tongue sole (*Cynoglossus semilaevis*) [64]. For this species, sex reversal induced by exposure to thermal stress is associated with widespread changes in the methylome, including chromosome W silencing. Pseudomales (ZWm) are fertile and produce viable offspring when they mate with normal females (ZWf). Interestingly, offspring exhibit an extremely high sex-reversal rate even in the absence of environmental stimuli and the methylome of these individuals resembles the paternal DNA methylation pattern. These results suggest that DNA methylation marks are preserved between generations for at least this fish species.

Another example of epigenetic inheritance between generations in fish comes from transgene methylation and silencing in zebrafish (*Danio rerio*). The Gal4/UAS:GFP is an epigenetically regulated fluorescent reporter consisting of a Gal4-VP16 gene/enhancer trap vector, a multicopy upstream activator sequence (UAS) and the green fluorescent protein (GFP) gene, which can become methylated and silenced in zebrafish [65]. Interestingly, once this transgene is silenced, it rarely reactivates during subsequent generations unless crossed into *dnmt1* deficient lines. This implies that if global demethylation exists in zebrafish, it does not act upon this transgene otherwise it should get activated at each generation. Indeed, as detailed below, recent bisulfite sequencing experiments in the early embryo and germline of zebrafish show that DNA methylation does not undergo global erasure as it occurs in mammals.

Post-fertilization methylation in the non-mammalian embryo

Unlike mammals, studies in zebrafish and *Xenopus* early embryos suggest the genome does not undergo genome-wide DNA demethylation. This was first predicted using Southern blots for repetitive sequences [66,67] and has since been supported for the entire genome using bisulfite sequencing [68–70]. Interestingly, in zebrafish, oocyte DNA methylation patterns are replaced with those characteristics of the soma post-fertilization, however, there is no evidence for global DNA methylation erasure as it occurs in mammals [69,70]. Accordingly, 5hmC as a marker of global DNA demethylation is not detectable at significant levels in the zebrafish zygote and early cleavage stages [71]. A key mediator of maternal reprogramming appears to be variant 'placeholder' histones which mark DNA hypomethylated regions in sperm and during the transcriptionally quiescent cleavage phase [72]. For *Xenopus*, the genome is heavily methylated in blastula and gastrula stages even at the promoters of genes highly expressed during these times. This indicates temporal uncoupling between methylation and transcriptional repression in the early embryo [68].

Importantly, although global erasure of DNA methylation marks is absent and demethylation by TET proteins has not been detected in pluripotent cells for those species, TET-dependent demethylation at enhancers occurs during the vertebrate phylotypic period [70,73,74]. Thus, although global hypomethylation is apparently not a conserved aspect of pluripotency in vertebrates, localized reprogramming is critical for development and proper cell differentiation [74–76].

Despite bisulfite sequencing providing convincing evidence that global DNA demethylation does not occur in the early embryo of zebrafish or *Xenopus*, two recent studies have claimed DNA hypomethylation is a feature of post-fertilization development in some fish species [77,78]. For medaka (*Oryzias latipes*), Wang and Bhandari [78] reported that soon after fertilization, the hypermethylated paternal methylome is erased during the first cell cycle and remains hypomethylated until 16-celled stage, after which DNA methylation levels are progressively restored until the gastrula stage. Accordingly, levels of 5hmC peak at approximately two and four-celled stages, and *tet2* and *tet3* are expressed in sperm whereas *tet1* is highly expressed during cleavage and blastula stage. For mangrove killifish (*Kryptolebias marmoratus*), a self-fertilizing hermaphrodite fish, Fellous et al. [77] reported a decrease in global methylation levels after fertilization, with the lowest point at gastrulation (15.8%) followed by remethylation until the liver formation stage (90 hpf, ∼70%). Tet expression showed an irregular expression pattern, not consistent with active demethylation.

Despite the intriguing nature of these results, it is important to point out significant technical limitations and their relationship to unique aspects of fish development. Most significantly, fish generally develop without ongoing maternal care, therefore in contrast with mammals, substantial nutrition and resources must be provided for the embryo by mother in the form of yolk, organelles and cytoplasmic proteins. Accordingly, fish oocytes have a staggering number of mitochondria compared with mammals (e.g. zebrafish have 190-fold greater mitochondria compared with mice) [79,80]. In addition, to allow for increased ribosome production, ribosomal DNA is known to be extrachromosomally amplified to numbers greatly exceeding chromosomal copies. The consequence of this is that in zebrafish, mature oocytes contain on average 19 million mitochondrial DNA molecules and ∼1000 copies of oocyte-specific extrachromosomal ribosomal DNA [80,81]. Thus, the total mass of non-nuclear DNA in this situation greatly exceeds genomic DNA. Given that both mitochondrial and extrachromosomal circular rDNA is known to be demethylated [69,70,82,83], much or all of the demethylation signals reported could relate to non-genomic DNA. Indeed, rapid replication of genomic DNA during cleavage and early embryogenesis restores regular stoichiometry between chromosomal and non-chromosomal DNA, potentially explaining the 'rebound' in methylation by gastrulation, observed by both studies. While it remains possible that genomic methylation erasure exists in these species, the true level of genomic methylation erasure will remain obscure until more sophisticated 5mC quantification methods are used.

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DNA methylation memory in the germline of non-mammalian vertebrates

Like the zebrafish post-fertilization embryo, two recent studies have identified global DNA methylation is largely preserved during germline differentiation and development in zebrafish [84,85]. Skvortsova et al. [85] analyzed four time points ranging from 4 to 36 hpf, matching the developmental period when PGC DNA methylation reprogramming occurs in mice. Genome-wide DNA demethylation was absent during the time points assessed, and the paternal methylome patterns characteristic of early embryo were preserved. Reflecting prior observations of transcriptional uncoupling in the *Xenopus*, the authors identified disruption of DNA methylation silencing in the germline. Our group [84] used a low-coverage bisulfite-sequencing strategy to assess a broader range of time spanning 24 hpf to 70 days post-fertilization including early PGC development, gonadal primordium, 'juvenile ovary', gonad transformation and sexually mature stages. Global methylation levels in PGCs were consistently similar to somatic cells at any of the stages evaluated, irrespective of the sex of the embryo. This suggests that the re-establishment of sex-specific differences in global methylation occurs during very late stages of gametogenesis. Although the possibility of DNA methylation erasure at the final stages of gametogenesis cannot be formally excluded, both studies support the proposal that the methylome is globally preserved during germline development (Figure 2).

Attempts have been made to assess DNA methylation dynamics in the germline of chicken; however, the presence of genome-wide methylation erasure is still unclear. Kress et al. [86] examined cultured PGCs using dot-blot assays and did not find any DNA methylation loss compared with other developmental stages. He et al. [87] found chicken PGCs at E5 are actually hypermethylated in relation to embryonic stem cells at stage X and spermatogonial stem cells (E19). In contrast, Yu et al. [88] using dot-blot analysis reported progressive demethylation of chicken PGCs starting at E3.5 until E6.0, and Rengaraj et al. [89] performed immunofluorescence that showed some migrating PGCs do not stain for DNA methylation, whereas (unquantified) numbers of other PGCs show normal levels of methylation. In summary, the existence of chicken PGC reprogramming and its extent is currently uninterpretable from current datasets, however, as for zebrafish, use of fully quantitative and single base pair technologies such as bisulfite sequencing may soon clarify the situation.

Evolutionary causes and consequences of DNA methylation erasure in the germline

Global DNA demethylation is a process that risks cell integrity—hypomethylation of transposable elements is associated with genome instability and oncogenic processes [90,91]. It is therefore perhaps not surprising that some organisms (such as zebrafish) may evolve to avoid this completely. Nevertheless, it is not clear why dramatic erasure of DNA methylation is such a prominent feature of the mammalian embryo post-fertilization, and that even more extensive erasure of methylation can be found in mammalian PGCs. We briefly outline some potential explanations.

The mammalian genome is unique among vertebrates for becoming activated before significant numbers of cell divisions have taken place; embryonic gene transcription occurs by the two- to eight-celled stage in mammals so far tested (reviewed in [92]), a process mediated the eutherian-specific DUX family of transcription factors [93–95]. Interestingly, a major target of the DUX transcription factors is ERV-derived sequences that act as transcriptional start sites for many genes active at early stages development [96,97]. It is conceivable that DNA methylation erasure in the early mammalian embryo potentiates genome activation by allowing ERV activation [98]. In contrast, the embryos of non-mammals undergo many rounds of cleavage before the embryonic genome and become activated by an entirely different set of transcription factors including Pou5f1, Nanog and SoxB1 [99,100], and perhaps, therefore, do not require genome-wide demethylation. Further testing of reprogramming in species holding critical phylogenetic positions (such as marsupials, which also lack DUX transcription factors), will help to test this hypothesis.

The reason why genome-wide demethylation has evolved or persisted in the PGCs of mammals, despite at least one non-mammalian vertebrate not requiring it, is also currently unclear. Genomic imprinting is a unique feature of mammalian genomes whereby parent-specific methylation marks are inherited between generations, and consequently drive mono-allelic parent-specific gene expression in offspring [101]. In order for genomic imprinting to persist, imprints from the previous generation must be erased during germline development and replaced with sex-specific marks—a process that overlaps with PGC methylation erasure. While it would seem unlikely that the entire genome should undergo epigenetic erasure *carte blanche* to allow imprint reprogramming at only a few loci, this nevertheless could provide a convenient explanation for epigenetic reprogramming at this time.

One further explanation could be related to germline specification strategy. Mammals derive their germline from epiblast cells of the soma on the basis of signals emanating from extraembryonic tissue [102]. This extraordinary cellular transformation may require erasure of epigenetic memory, thus allowing the newly formed genome to 'forget' its embryonic origins. In reptiles, fish, birds and anuran amphibians the germline is 'preformed', meaning that maternally inherited cytoplasmic determinants are responsible for the specification of the germline [45,103] (Figure 3). Under this scenario, extensive DNA demethylation is likely not required; the germline does not need reprogramming from another distinct cell type. Again, the study of animals holding key phylogenetic positions will test this hypothesis. For example, if this proposal is correct, we might expect the continuously defined germline of chicken to preserve DNA methylation in PGCs, but that salamanders and axolotls show epigenetic erasure on account of their germline defined by induction [104].

Notwithstanding the reasons why differences in DNA methylation memory or erasure have evolved in various vertebrate groups, it is worth considering the consequences of whether a species undergoes epigenetic reprogramming or not. As discussed, epigenetic erasure is a strong barrier for IEI and TEI in mammals [105,106]. In those species where epigenetic erasure does not occur, increased opportunity for epigenetic inheritance between generations may exist (Figure 4). As mentioned, the half-smooth tongue sole provides an intriguing example of how DNA methylation could impact upon fundamental biological processes such as sex determination. One further recent example of how epigenetic inheritance could regulate phenotype between generations comes from the damsel fish (*Acanthochromis polyacanthus*), which acclimates to increased water temperatures by altering the expression of multiple genes involved with aerobic capacity. Importantly, those fish with parents who were also subjected to high temperatures were found to acclimate better, implying some form of intergenerational instruction [107]. Genes involved with the aerobic capacity response show expression plasticity that is associated with corresponding changes in DNA methylation [108]. While the authors did not test inheritance stability of these altered DNA methylation marks (by placing progeny back at normal temperatures), these experiments nonetheless suggest that life-experience in one generation can be passed on to the next, potentially via DNA methylation. Further experimentation of this kind could ultimately force us to reformulate our understanding of what information is held within genome as it is passed from one generation to the next.

Summary

- 5-mC is a stable epigenetic mark that acts as a memory module.
- In eutherian mammals, two genome-wide waves of DNA demethylation robustly preclude IEI and TEI.
- Some vertebrate species (e.g. zebrafish) preserve DNA methylation memory through germline development, leaving open the possibility for increased IEI and TEI.
- While the mechanisms vastly different DNA methylation dynamics emerge during evolution are unknown, preservation of epigenetic memory through generations has important repercussions in heredity and evolution.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Abbreviations

DNMT1, DNA methyltransferase 1; DNMT3, DNA methyltransferase 3; dpf, days post-fertilization; dpp, days post-partum; Dppa3, developmental pluripotency-associated 3; DUX, double homeobox; E, embryonic day; ERV, endogenous retrovirus; GFP, green fluorescent protein; hpf, h post-fertilization; IAP, intracisternal A particle; IEI, intergenerational epigenetic inheritance;

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LTR, long terminal repeat; MYA, million years ago; NLPR, NLR family pyrin domain containing proteins; PCNA, proliferating cell nuclear antigen; PGC, primordial germ cell; PGC7, primordial germ cell protein 7; Pou5f1, POU domain, class 5, transcription factor, 1; Prdm, proline-rich domain containing, with ZNF domain; SoxB1, SRY-box transcription factors B1; TEI, transgenerational epigenetic inheritance; TET, ten eleven translocation; UAS, upstream activator sequence; UHRF1, ubiquitin-like containing PHD and RING finger domains 1; Zfp57, zinc finger protein 57; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine.

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Chapter 4

"Stress, novel sex genes, and epigenetic reprogramming orchestrate socially controlled sex change"

Contribution

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Stress, novel sex genes, and epigenetic reprogramming orchestrate socially controlled sex change. Erica V. Todd[1*], **Oscar Ortega-Recalde**[1*], Hui Liu[1*], Melissa S. Lamm[2], Kim M. Rutherford[1], Hugh Cross[1], Michael A. Black[3], Olga Kardailsky[1], Jennifer A. Marshall Graves[4], Timothy A. Hore[1‡], John R. Godwin[2‡] and Neil J. Gemmell[1‡]. *Science Advances*. Vol. 5, no 7, eaaw7006, 10 Jul 2019, doi: 10.1126/sciadv.aaw7006.

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As co-first author, my contribution to the paper was conducting bioinformatics experiments, analysing the data, preparing the figures and helping write the manuscript. In this study, we applied transcriptomic and methylome approaches to dissect the molecular pathways involved in sex change in the wild coral reef fish bluehead wrasse. Specifically, I performed principal component analysis (PCA) of transcriptomic and methylome data, conducted gene set enrichment, simulated the effect of mixed RNA datasets, analysed differential expression and WGBS data, and correlated transcriptomic and methylome data. This international collaborative project is the only genome-wide methylation study to date which examines sex change in sequential hermaphrodites.

Our results show that female-to-male sex change involves a cascade of collapsing feminising genes. In addition, stress axis activation and repression of gonadal aromatase (enzyme converting androgens to oestrogens) were identified as hallmarks of early transformation. Interestingly, we found gonad transition involves epigenetic reprogramming and an intermediate state with altered

epigenetic machinery, which resemble early developmental cells of mammals. The relevance of this study for epigenetics is that it provides a detailed molecular picture of cell commitment and reprogramming during gonad transformation, highlighting the important role of epigenetic memory and epigenetic reprogramming in sex change.

ORGANISMAL BIOLOGY

Stress, novel sex genes, and epigenetic reprogramming orchestrate socially controlled sex change

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Bluehead wrasses undergo dramatic, socially cued female-to-male sex change. We apply transcriptomic and methylome approaches in this wild coral reef fish to identify the primary trigger and subsequent molecular cascade of gonadal metamorphosis. Our data suggest that the environmental stimulus is exerted via the stress axis and that repression of the aromatase gene (encoding the enzyme converting androgens to estrogens) triggers a cascaded collapse of feminizing gene expression and identifies notable sex-specific gene neofunctionalization. Furthermore, sex change involves distinct epigenetic reprogramming and an intermediate state with altered epigenetic machinery expression akin to the early developmental cells of mammals. These findings reveal at a molecular level how a normally committed developmental process remains plastic and is reversed to completely alter organ structures.

INTRODUCTION

In most organisms, a fundamental dichotomy is established in early embryonic development; individuals become either female or male and maintain these fates throughout life. However, some plant and animal species exhibit remarkably diverse and plastic sexual developmental patterns (*1*, *2*), and some even retain the ability to change sex in adulthood (*3*). Such functional sex change is widespread in marine fishes, appearing in 27 families (*4*). Among the most outstanding, and well-studied, example of sex change is the bluehead wrasse (*Thalassoma bifasciatum*), a small coral reef fish that undergoes rapid and complete female-to-male sex reversal in response to a social cue (*5*).

While the sex change process and its evolutionary advantages are well known (*3*, *6*), there remain long-standing questions about how environmental influences initiate these dramatic changes in sexual identity and what molecular processes orchestrate this transformation (*7*, *8*).

Across vertebrates, antagonism between core male- and female-promoting gene networks is now recognized as crucial to the establishment and maintenance of gonadal fate (*9*). Discoveries in mice that loss of the feminizing FOXL2 (Forkhead Box L2) transcription factor is sufficient to induce the transdifferentiation of mature ovaries into testes (*10*), and that testicular cells will become ovarian cells if the masculinizing transcription factor DMRT1 (Doublesex and Mab-3 Related Transcription Factor 1) is lost (*11*), suggest that gonadal bipotentiality is retained into adulthood and presents a mechanism through which female-male and male-female gonadal sex reversal may be controlled in fish. A key target of both factors is the gene encoding aromatase (the enzyme responsible for estrogen production), the expression of which is known to be environmentally sensitive and when inhibited, results in female-male sex reversal (*12*).

Epigenetic processes are suspected to be key mediators and effectors of such environmentally induced sex reversal. Temperature-sensitive sex reversal involves global methylation modification in the half-smooth tongue sole (*13*), with sex-specific methylation states of major sex-pathway genes inverted following sex reversal. Similarly, sex reversal in the dragon lizard (*14*), and temperature-dependent sex determination in red-eared slider turtles (*15*), appears to involve temperature-sensitive expression changes in epigenetic regulator genes of the Jumonji family, namely, *kdm6b* and *jarid2*. Therefore, a change in methylation state may facilitate reprogramming, and subsequent canalization, of sexual fate at a cellular level (*9*).

Sex reversal in response to social cues, as seen in the bluehead wrasse, is an especially striking example of phenotypic and sexual plasticity. Most bluehead wrasses begin their reproductive life as females but routinely reverse sex in the absence of a socially dominant male (*16*). Removal of the dominant terminal-phase (TP) male from its territory induces rapid and complete sex change of the largest female (*5*). Alternatively, bluehead wrasses may mature first as non-aggressive initial-phase (IP) males that mimic the female phenotype, and can also become TP males under the same social stimulus (*16*). Transitioning individuals exhibit behavioral changes within hours, and in females, complete transformation of mature ovaries to functional testes can occur in 8 to 10 days (Fig. 1). The absence of differentiated male sexual tissue in the ovaries of sex-changing wrasses implies that some form of cellular reprogramming underlies sex change in these species (*17*), either by a direct cellular transition (i.e., transdifferentiation) or via a dedifferentiated intermediate stage, rather than by alterations in the proportion of spermatogenic and oogenic cell populations as in the bisexual gonad or ovotestis of many other sex-changing fishes [e.g., anemone fish and bidirectional sex-changing gobies (*7*)].

Here, we applied transcriptomic and nucleotide-level methylation approaches to identify the primary trigger and subsequent molecular cascade that orchestrates gonad remodeling in the bluehead wrasse. Our results provide a detailed molecular picture of female-to-male sex reversal in a vertebrate and underscore the role of epigenetics and pluripotency in sex determination.

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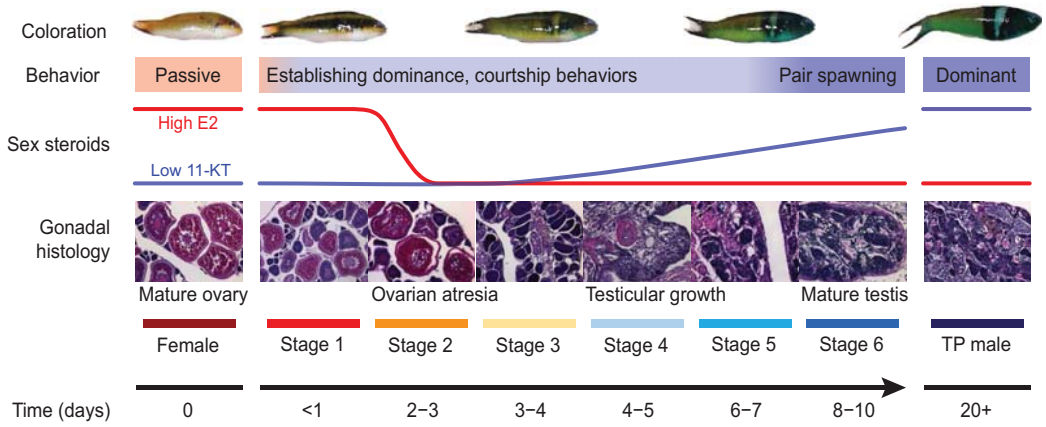

Fig. 1. Sex change in the bluehead wrasse. Schematic of sex change summarizing changes in external coloration, behavior, serum steroid levels, and gonadal histology across time. Within hours of removing TP males, the largest female displays aggression and male courtship behaviors, and adopts darker spawning coloration, but still has healthy ovaries (stage 1). Transitioning females establish dominance within 1 to 2 days, after which serum estrogen [17β-estradiol (E2)] levels collapse and ovarian atresia is observable (stage 2). Ovarian atresia is advanced by 3 to 4 days (stage 3). Testicular tissues are observed by days 4 to 5, and serum 11-ketotestosterone (11-KT) begins to rise (stage 4) before spermatogenesis begins by days 6 to 7 (stage 5). Within 8 to 10 days, transitioning fish are producing mature sperm and successfully reproducing with females (stage 6). Full TP male coloration develops within ~20 days. Gonadal stages are classified according to (*17*). Hormonal changes are predicted on the basis of patterns in the congener *Thalassoma duperrey* (*17*). For detailed descriptions of behavioral and morphological changes, see (*5*, *18*). Photo credit: J. Godwin, North Carolina State University (fish images); H. Liu, University of Otago (histology images).

RESULTS

We induced wild female bluehead wrasses to change sex in the field by removing dominant TP males from established social groups on patch reefs off the Florida coast. We collected a time series of brain and gonadal samples across the entire sex change process, assigning animals to six successive stages based on behaviors observed at the time of capture (*18*) and gonadal histology (Fig. 1) (*17*). As controls, we collected six small females that experienced the social manipulation but showed no signs of sex change (control females) and eight dominant TP males.

Transcriptome-wide gene expression patterns across sex change

We quantified transcriptome-wide gene expression across sex change using RNA sequencing (RNA-seq) and the de novo assembled transcriptome from our previous study (*19*) as a reference. Expression patterns across samples were visualized using principal components analysis (PCA). We observed very little variation in global gene expression in the brain (Fig. 2A and fig. S1), consistent with other studies in teleosts also showing limited sex bias in brain transcriptomic profiles (*20*, *21*).

In contrast, striking variation in expression was observed in the gonad, and samples strongly clustered by sex change stage (Fig. 2B). Samples at stage 5 (ongoing spermatogenesis) formed two distinct clusters in the PCA and were subdivided into stages 5a and 5b in further analyses. Notably, gonad samples were clearly organized, first, by sexual development from female to male (PC1) and, second, by developmental commitment (PC2) (Fig. 2B). This suggested that intermediate phases of sex change may represent unique transitional cell types rather than simply different proportions of differentiated male and female tissues. To validate this computationally, we generated "mixed" samples by combining subsampled reads from control male and female libraries in different ratios before rerunning the PCA (fig. S2A). As expected, the mixed samples are separated primarily along PC1, lying between stage 4 gonads (when male cells are first identifiable histologically) and fully differentiated TP male testes. This pattern supports the assertion that gonadal sex change involves unique transitional cell types rather than a proportional change in sexually differentiated cells.

Transitional stages are also characterized by unique expression states and functional categories. To investigate those transcripts contributing most to each extreme of PC1 (female and male) and PC2 (differentiated and transitionary), we used component loadings to select the top 500 transcripts driving each axis direction (Fig. 2C). This resulted in an extensive overlap between the "male" and "differentiated" regions of the PCA. Visualizing expression of transcripts unique to each group (Fig. 2D) confirmed that the extremes of PC1 are characterized by transcripts that are female biased and down-regulated or male biased and up-regulated across sex change, whereas PC2 is driven by transcripts with the highest expression in either transitionary stages or sexually differentiated gonads. Gene Ontology (GO) terms (Fig. 2E and data S1) associate transcripts of the "female" and "male" regions with oocyte- and sperm-specific processes, respectively, and sex-specific transcription factor activity. Unique GO terms were associated with the "transitionary" transcripts and include innate immunity, protein catabolism, cell proliferation, and adhesion processes, reflecting dynamic disassembly and rebuilding of the gonad at mid-sex change. "Transitionary" transcripts were also enriched for cholesterol homeostasis, which is a known signal of apoptotic events (*22*) and is affected by levels of cholesterol-derived hormones (*23*). Estrogen, androgen, and cortisol are all expected to undergo turnover in transitioning gonads (Fig. 1) (*7*, *24*), consistent with the expression patterns that we observe for the relevant regulatory genes (Figs. 3 and 4). The "differentiated" region was associated with protein kinase activity, β-catenin signaling, and metabolic and developmental processes.

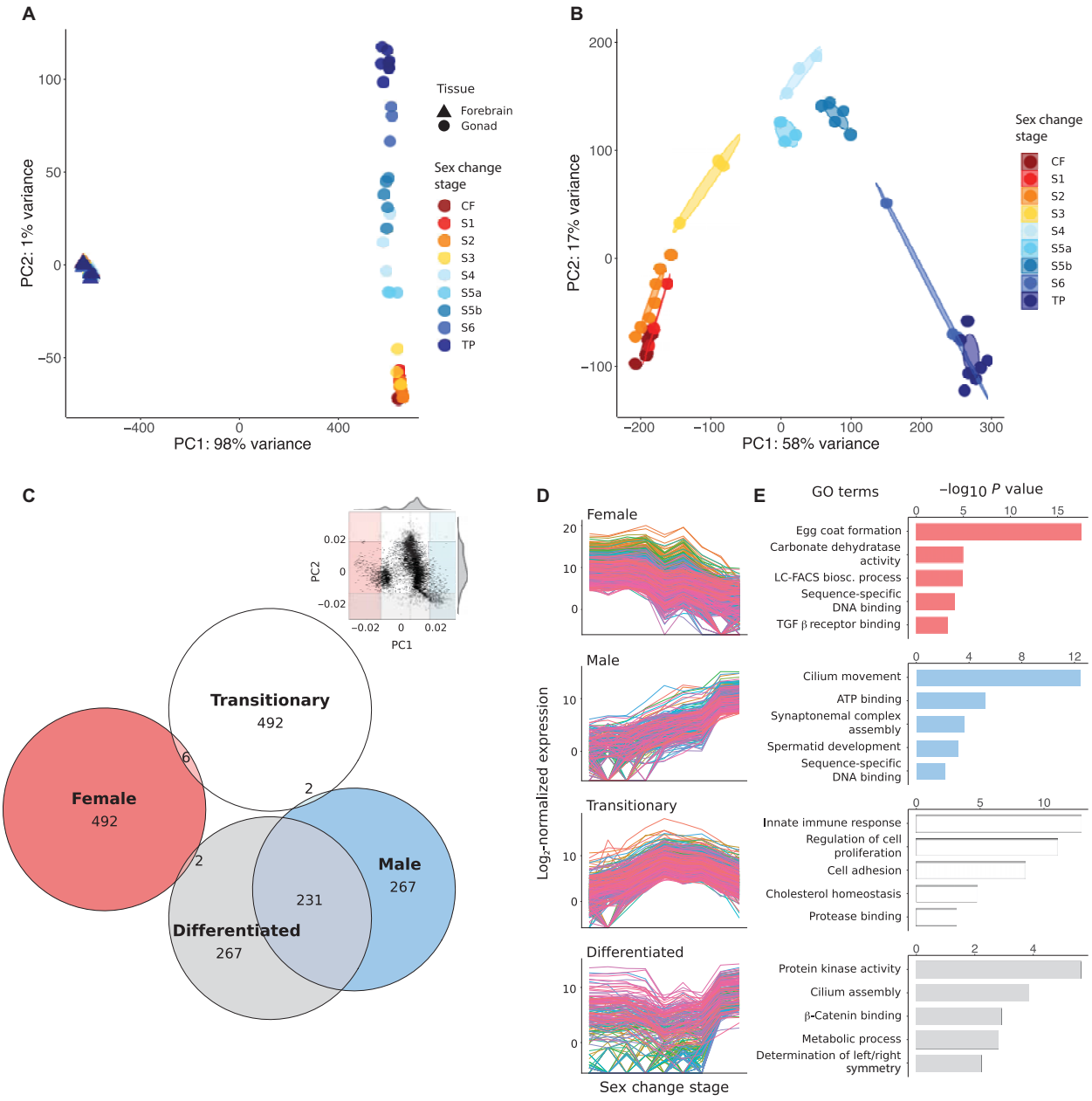

Fig. 2. Global gene expression changes during sex change. (**A**) PCA showing close clustering of brain samples but separation of gonad samples by sex change stage. (**B**) PCA (10,000 most variable transcripts) of gonad samples. The transition from ovary to testis is captured along PC1 (58% variance), whereas PC2 (17% variance) delineates fully differentiated gonads of control females (bottom left) and TP males (bottom right) from those of transitioning fish. (**C**) Inset: Component loadings were used to identify transcripts contributing most to PC1 and PC2. Shaded sections define 5th and 95th percentiles defining four spatial regions: "Female" (left), "Male" (right), "Differentiated" (bottom), and "Transitionary" (top). The Euler diagram shows numbers of transcripts uniquely assigned to each region. (**D**) Expression patterns across sex change of transcripts uniquely assigned to each of the four spatial regions, showing four distinct patterns of expression: Female (declining), Male (increasing), Transitionary (highest mid-sex change), and Differentiated (lowest mid-sex change) and (**E**) representative GO terms for these transcripts. LC-FACS bios. process, long-chain fatty-acyl–coenzyme A biosynthetic process; TGF β receptor binding, transforming growth factor–β receptor binding; CF, control female; S1 to S6, stages 1 to 6; TP, TP male; ATP, adenosine 5′-triphosphate.

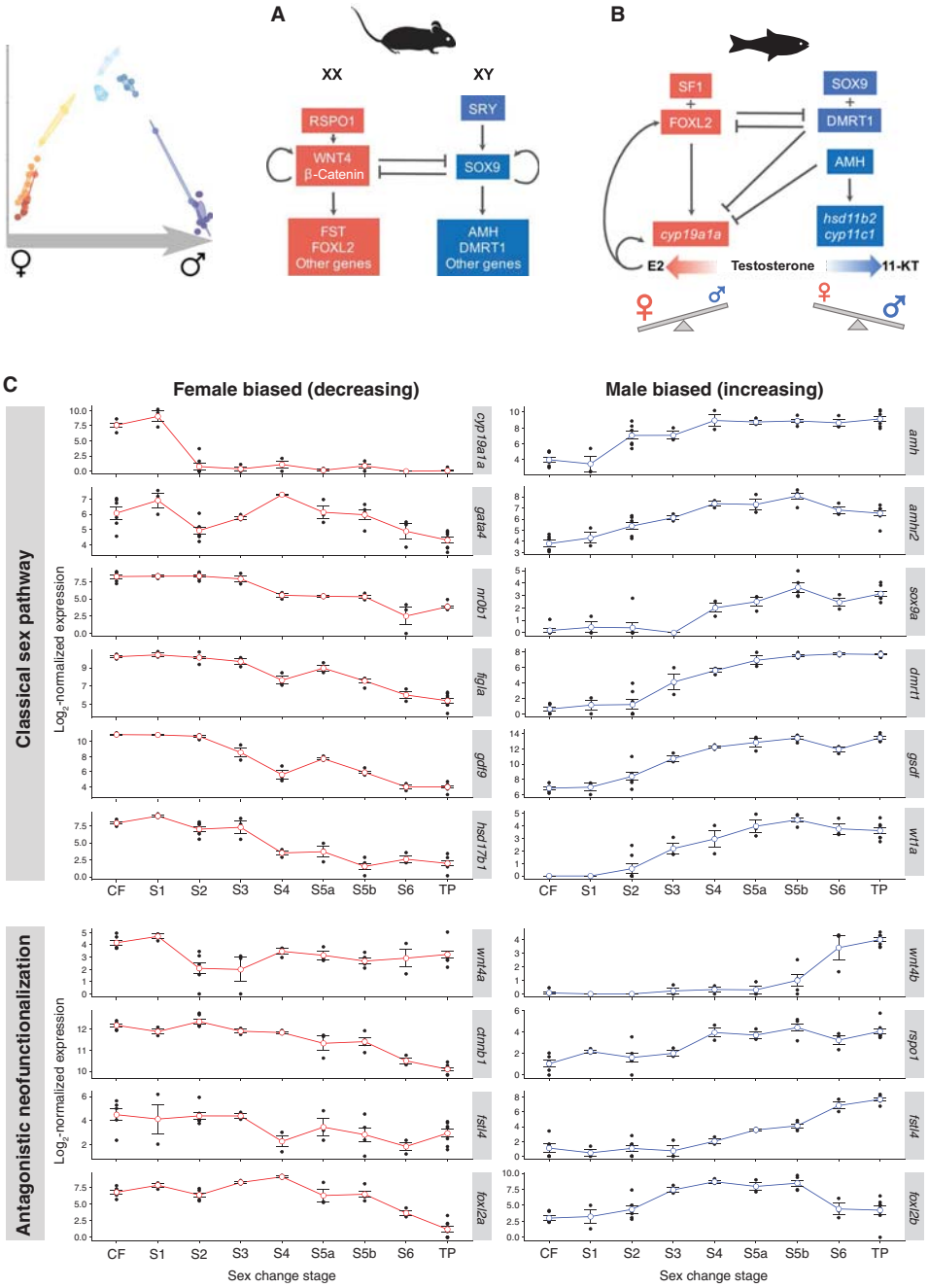

Fig. 3. Sex change involves transition from female- to male-specific expression and gene neofunctionalization. (**A**) Mammalian model of sex determination and development. In males, SRY (sex-determining region Y) activates SOX9 (SRY-related HMG box 9) to initiate male development while blocking expression of feminization genes that would, in turn, antagonize masculinizing expression (*9*). (**B**) Teleost model of sexual development. Diverse factors determine sex in fishes, yet conserved downstream effectors act in feminizing and masculinizing pathways to promote female or male development, respectively, while antagonizing the opposing sexual pathway. Testosterone is a prohormone and is converted to estrogen (E2) by gonadal aromatase (encoded by *cyp19a1a*) to promote ovarian function or to 11-KT by the products of *hsd11b2* and *cyp11c1* to promote testicular function. (**C**) Normalized expression of classical sex-pathway genes across sex change in bluehead wrasse gonads. At stage 2, *cyp19a1a* is sharply down-regulated, after which feminizing expression collapses as female fish transition to males. Up-regulation of masculinizing gene expression begins with *amh* and its receptor *amhr2*. Classically feminizing genes within the R-spondin/Wnt/β-catenin signaling pathway (*wnt4a/b*, *rspo1*, and two transcripts annotated as *fstl4* are shown as examples), and also *foxl2*, are duplicated in the bluehead wrasse with orthologs showing testis-specific expression.

Specific gene expression changes

Few transcripts were differentially expressed in forebrain samples, with the greatest differential expression seen among females and males (fig. S3A and data S2). Gonadal sex change involves transition from a female-biased expression landscape to one that is male biased. This transition is not linear and is punctuated by large numbers of differentially expressed transcripts between stages 3 (advanced ovarian atresia) and 4 (early testicular development) and between stages 5 (early spermatogenesis) and 6 (spermiation) (fig. S3B). Classical ovary-specific genes [*nr0b1* (*dax1*), *figla*, and *gdf9*] became progressively down-regulated as sex change progressed (Fig. 3).

Testis-specific genes became activated from mid-sex change. This began with *amh* (anti-Müllerian hormone) and its receptor *amh2*, which were the first male-pathway genes to show increased expression, at stage 2 and before the appearance of male tissues in the gonad, followed by other classical male-promoting genes (e.g., *dmrt1*, *sox9*, and *gsdf*) (Fig. 3). Stages characterized by active testicular growth and spermatogenesis (stage 4 onward) displayed increasing expression of *cyp11c1* and *hsd11b2*, consistent with their function to convert testosterone to the more potent teleost androgen 11-ketotestosterone (11-KT) (Fig. 4).

To identify the most upstream effectors of sex change, we focused on expression changes in the earliest phases. Notably, *cyp19a1a* and *cyp19a1b* were sharply downregulated at early sex change, in gonad (Fig. 3) and brain (fig. S3C), respectively, and remained at low levels thereafter. These genes encode the brain- and gonad-specific forms of aromatase, which converts testosterone to estradiol. The balance of estrogen [17β-estradiol (E2)] versus androgen (11-KT) production is known to control sexual fate in teleost fish (*2*), and aromatase inhibitors can effectively induce female-to-male sex reversal (*12*). Our data provide whole-transcriptome evidence that aromatase down-regulation immediately precedes a cascaded collapse in feminizing gene expression and is an early switch initiating sex change in both the brain and gonad under natural conditions.

Neofunctionalization of ovary-promoting genes and new genetic pathways are implicated in ovary-testis transformation

Many paralogous genes, arising from an ancient teleost-specific whole-genome duplication (*25*), show divergent sex-specific expression patterns during gonadal sex change (Fig. 3C). These include critical female-pathway genes, such as *foxl2* and genes in the

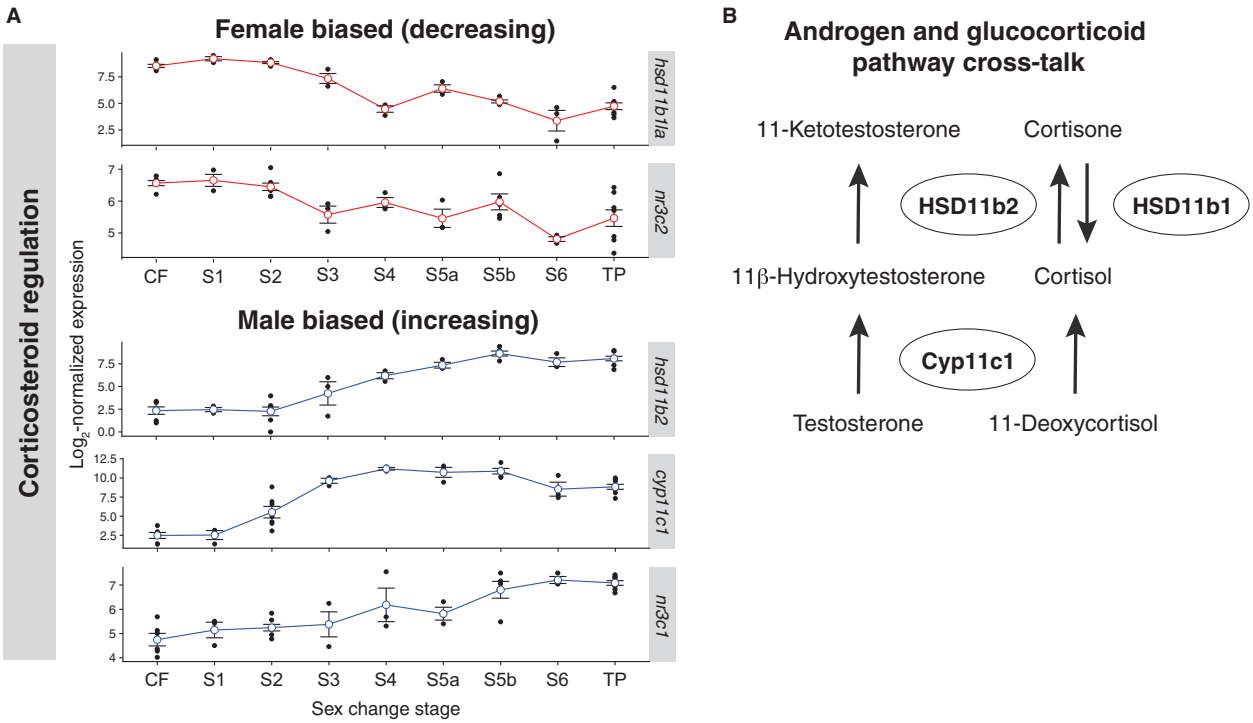

Fig. 4. Dynamic sex-specific expression of androgenic and glucocorticoid factors. (**A**) Normalized gonadal expression of androgen synthesis and cortisol pathway genes across sex change. Up-regulation of *cyp11c1* at stage 2 implies increased local cortisol production. Then, changes in expression of 11β-HSD enzymes at stage 3 suggest a shift from cortisone-cortisol regeneration (*hsd11b1a* down-regulated) to cortisol-cortisone inactivation (*hsd11b2* up-regulated). Concurrent up-regulation of *hsd11b2* would also promote 11-KT synthesis, the most potent teleost androgen. Genes encoding the glucocorticoid (*nr3c1*) and mineralocorticoid (*nr3c2*) receptors show opposing sex-specific expression patterns that also imply highest cortisol activity at early sex change. These expression patterns suggest a window of high cortisol activity at stage 2 that is concurrent with arrested aromatase expression and ovarian atresia, followed by the stimulation of 11-KT production by stage 3. (**B**) Schematic showing cross-talk between the androgen synthesis and glucocorticoid pathways. The 11β-hydroxylase enzyme (Cyp11c1, homologous to mammalian CYP11B) and two 11β-hydroxysteroid dehydrogenases (HSD11b1 and HSD11b2) are together responsible for 11-KT production and the interconversion of cortisol. Cortisol is produced via the action of Cyp11c1, whereas 11β-HSD enzymes control its interconversion with inactive cortisone, thus mediating the stress response. Cortisol itself stimulates 11β-HSD expression, resulting in the production of both 11-KT and inactive cortisone.

Rspo1/Wnt/β-catenin signaling pathway known to regulate ovarian differentiation in mammals (*26*). *Wnt4* (wingless-type MMTV integration site family, member 4), which activates *Ctnnb1* (β-catenin) and *Fst* (follistatin) to maintain mammalian ovarian development (Fig. 3A) (*27*), is duplicated in the bluehead wrasse: *wnt4a* was down-regulated early along with *cyp19a1a*, consistent with a conserved feminizing role, while its paralog *wnt4b* was sharply up-regulated in late sex change and is expressed only in mature testes (Fig. 3C). Furthermore, *rspo1* (R-spondin–1), which stimulates *Wnt4* in mammals (Fig. 3A), also showed increasing expression during testicular construction, and multiple follistatin-like genes (e.g., *fstl4*) showed opposing sex-specific expression patterns (Fig. 3C). Neofunctionalization of duplicated sex-pathway genes, by introducing important functional gene diversity, may underpin the notable sexual plasticity of teleost fish.

Another unexpected result from our analysis was the progressive up-regulation of genes involved in the Janus kinase–signal transducers and activators of transcription (JAK-STAT) signaling pathway, from stage 2 to 5 (table S1). JAK-STAT signaling plays an important role in determination and maintenance of male sexual fate in *Drosophila melanogaster* (*28*) but has not previously been implicated in vertebrate sexual development.

Cortisol pathways show dynamic expression across sex change

Cortisol, a glucocorticoid that controls stress-induced responses in all vertebrates, may be an important mediator of environmental sex determination (*29*). We therefore determined whether cortisol-pathway genes show altered expression during early sex change stages. We find that the 11β-hydroxylase gene *cyp11c1*, responsible for cortisol production, is up-regulated in bluehead wrasse gonads from stage 2. Furthermore, two genes encoding 11β-hydroxysteroid dehydrogenases (11β-HSD), *hsd11b2* and *hsd11b1-like*, show differential expression across sex change but in opposite directions (Fig. 4A). The products of these genes mediate the stress response by respectively producing cortisol from inactive cortisone and vice versa, and their expression patterns imply a shift from cortisol production to inactivation from stage 3. We also observed opposite expression changes in genes encoding the glucocorticoid and mineralocorticoid receptors, *nr3c1* and *nr3c2* (nuclear receptor subfamily 3 group C members 1 and 2), respectively; *nr3c2* matched *hsd11b1la* expression changes, while *nr3c1* followed *hsd11b2* (Fig. 4A). Together, high expression of *cyp11c1*, *hsd11b1la*, and *nr3c2* at stage 2 (Fig. 4A) implies a window of high cortisol activity at the beginning of sex change, coincident with aromatase silencing and early ovarian atresia. Our data therefore suggest that dynamic cortisol production and signaling at early sex change may be a key factor triggering female-to-male transition in the bluehead wrasse.

Ovary-to-testis transformation involves extensive epigenetic reprogramming

Epigenetic marks such as DNA methylation and trimethylation of histone H3 lysine 27 (H3K27me3) are required for the specification and maintenance of cellular identity (*30*, *31*) and are increasingly recognized as key mediators of sexual differentiation (*15*, *32*). To explore the role of epigenetic marks in bluehead wrasse sex change, we investigated the expression profiles of genes encoding their regulatory machinery. We found that *ezh2, suz12,* and *eed* and their cofactor *jarid2*, components of the Polycomb repressive complex 2 (PRC2) responsible for creating H3K27me3 in vertebrates (*33*), were highly expressed in differentiated male and female gonads but displayed reduced expression during intermediate stages (Fig. 5A and 5D). This parallels similar findings for mammalian naïve pluripotent stem cells (*34*) and primordial germ cells (PGCs) (*35*) (the bipotential germline progenitors of egg and sperm), where H3K27me3 is also low but increases in the differentiated cells that they give rise to. The histone variant *h2afz*, which also features in pluripotent stem cells and PGCs, showed its highest expression at stage 3, dropping sharply at stage 4 when testicular development is first observed histologically, before recovering again (Fig. 5D). This dynamic expression pattern is interesting, given that H2A.Z colocalizes with Polycomb group (PcG) proteins at silenced developmental genes in mammalian embryonic stem cells but is dynamically redistributed during lineage commitment to potentially arbitrate developmental cell fate transitions (*36*). Writers and erasers of histone acetylation (acetyltransferases and deacetylases, respectively) were also expressed dynamically (fig. S4), further indicating that active chromatin modification accompanies gonadal sex change.

In mammals, global reprogramming of DNA methylation is a feature of both naïve pluripotent stem cells and PGCs, a process that is driven by down-regulation of de novo DNA methyltransferase 3 (DNMT3) genes, deactivation of maintenance DNA methyltransferase activity (DNMT1), and overexpression of the ten-eleven translocation (TET) family of DNA demethylation enzymes (*30*). Here, we found not only reduced de novo methylation machinery at intermediate stages of sex change but also wholesale replacement of sex-specific DNMT3 orthologs during the transition from ovary to testis (Fig. 5A and 5B); *dnmt3bb.3* (ortholog of mammalian *Dnmt3b*) showed female-specific expression that declined during sex change and was replaced by male-specific expression of *dnmt3aa*, *dnmt3ab*, *dnmt3ba*, and *dnmt3bb.1* (orthologs of mammalian *Dnmt3a* and *Dnmt3b*). Expression of TET genes was highest at mid-late sex change (Fig. 5A and 5C). Together, this indicates that like H3K27 modification, there is an intense period of DNA methylation reprogramming in the gonad at intermediate stages of sex change. Such distinct sex-specific expression of DNA methyltransferase genes and other epigenetic modifiers was also recently observed in gonadal transcriptomes of other sex-changing fishes (*37*) and may be important for sexual plasticity.

Sex change involves genome-wide methylation reprogramming

To characterize the epigenetic effect of DNA methylation machinery replacement during sex change, we performed low-coverage bisulfite sequencing of DNA derived from the gonads of control females, TP males, and transitioning individuals. We found that ovary and testis showed significantly different cytosine-guanine (CG) methylation levels (70.7 and 82.1%, respectively; $P < 0.001$), with gonads undergoing masculinization progressively accumulating methylation (Fig. 6A). To confirm that this effect was specific to the gonad, we also tested brain tissue and found that CG methylation was relatively low (64.1%) and not sexually dimorphic.

To characterize where global methylation changes were occurring during sex change, we undertook deep sequencing of selected female, intermediate, and TP male libraries and compared this to a draft de novo assembly of the bluehead wrasse genome (Supplementary Materials and Methods). When 2-kb running

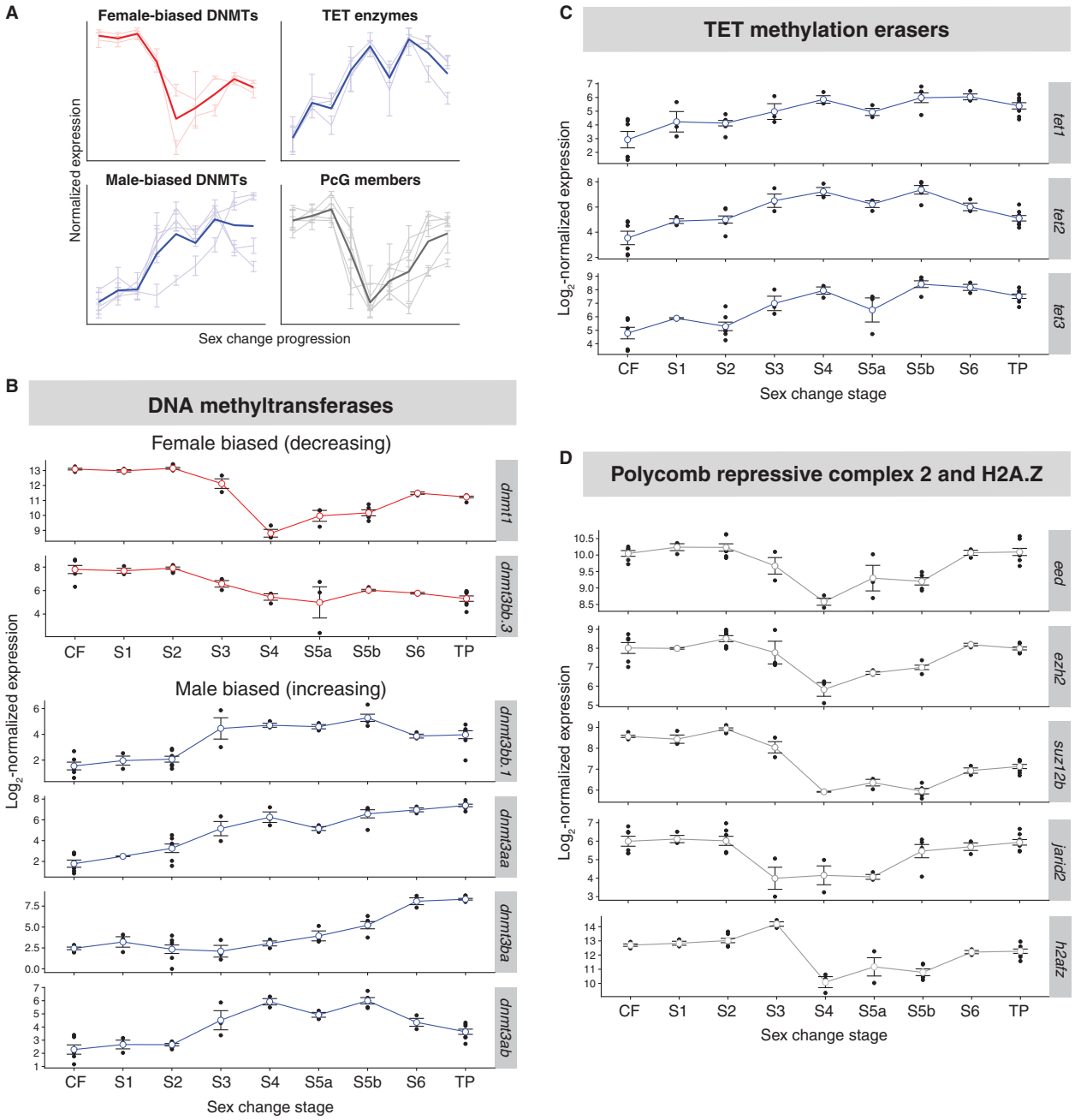

Fig. 5. Epigenetic factors orchestrate sex change. (**A**) Schematic summary of sex-specific expression patterns for epigenetic factors across sex change. (**B**) Normalized gonadal expression of DNA methyltransferase genes, showing a turnover in sex-specific expression across sex change. The maintenance methyltransferase ortholog *dnmt1* is female biased and has lowest expression at mid-sex change, whereas most methyltransferase *dnmt3* orthologs, responsible for de novo methylation, show increasing expression toward maleness. DNMTs, DNA methyltransferases. (**C**) Ten-eleven translocation (TET) methylcytosine dioxygenases, which demethylate 5-methylcytosines, show the highest expression during late sex change. (**D**) Genes encoding core proteins (*eed*, *ezh2*, and *suz12b*) and cofactors (*jarid2*) of the chromatin remodeling PRC2 are suppressed at mid-sex change. The variant histone H2A.Z gene (*h2afz*) shows a similar expression pattern.

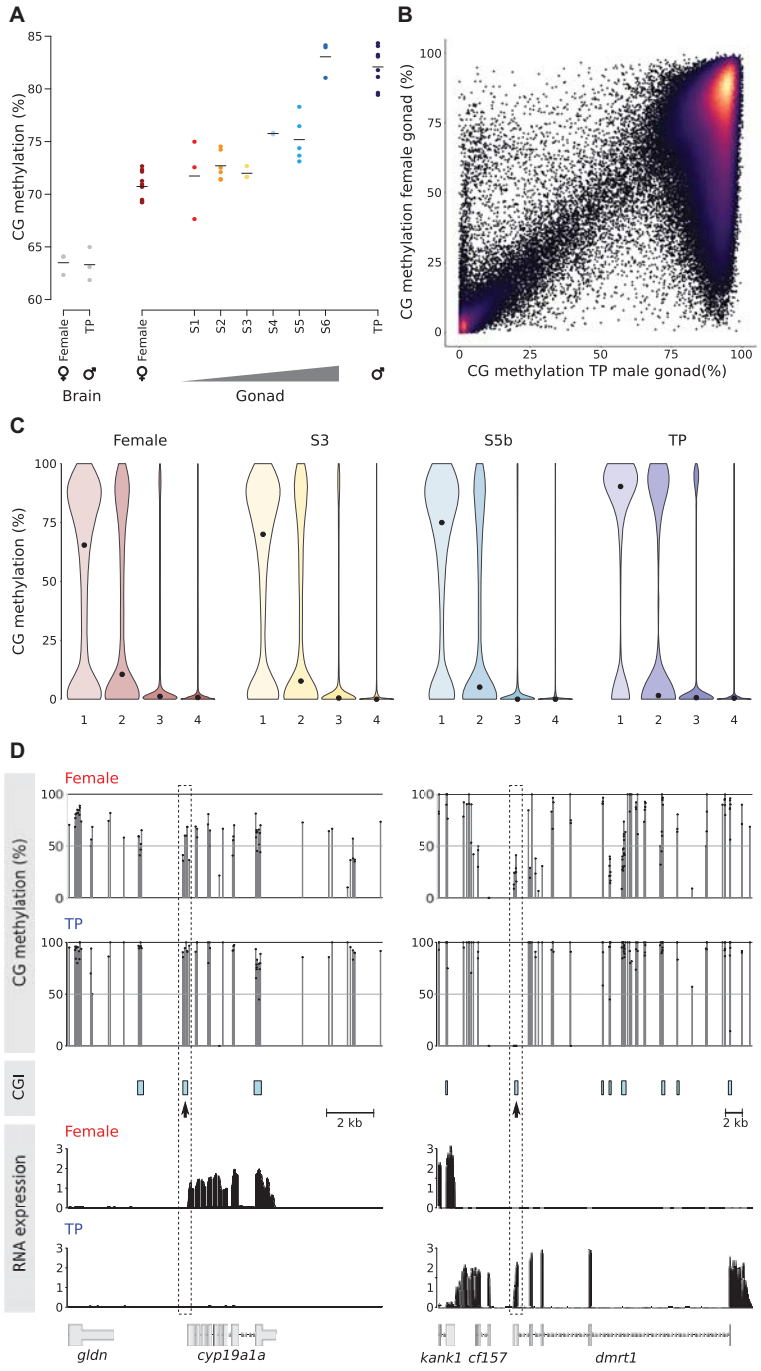

Fig. 6. Global methylation changes and relationship between methylation and gene expression during sex change. (**A**) Global CG methylation levels during sex change examined by low-coverage sequencing. The horizontal bar indicates the mean. Gray dots, brain samples; colored dots, gonadal samples. (**B**) Comparison of methylation (2-kb running windows) between female and TP male gonadal methylomes. Only probes with >100 calls were included for the analysis. (**C**) Violin plot showing distribution of methylation at transcription start sites (TSS) of genes classified into quartiles according to expression level (highest, 4). Each violin is scaled to the same maximum width (total area is not constant between violins) to demonstrate distributions for each quartile. Black dots denote the median. (**D**) Relationship between CpG methylation and RNA expression for *cyp19a1a* and *dmrt1*. CG methylation track shows methylation levels for dinucleotides with >10 calls; CGIs were predicted according to the Gardiner-Garden and Frommer criteria. Changes in TSS methylation containing CGIs (dashed box) are negatively correlated with gene expression for both genes (bottom).

windows were analyzed genome-wide, a notable number of low- and intermediately methylated regions in females became fully methylated in TP males (Fig. 6B). However, when partitioned in a PCA, methylomes are organized, first, by stage of sexual development from female to male (PC1) and, second, by developmental commitment (PC2) (fig. S2B), a pattern notably similar to that of the RNA-seq data (Fig. 2B). Therefore, transitionary gonads are not molecularly intermediate between differentiated ovary and testis but are distinct both in terms of global gene expression and DNA methylation.

When targeted to transcription start sites (TSS), particularly those with enriched levels of CG dinucleotides [known as CpG islands (CGIs)], DNA methylation is associated with gene silencing throughout all jawed vertebrates (*38*). To explore what effect the global increase in DNA methylation has on gene expression in bluehead wrasse during sex change, we binned genes into quartiles according to their expression levels and asked what levels of DNA methylation existed in their TSS (Fig. 6C). We found that DNA methylation and gene silencing were coupled in a similar fashion throughout sex change, meaning that despite the major increase in global DNA methylation during transition, DNA methylation has the capacity to enforce gene silencing at all stages of reprogramming.

The methylation patterns of key genes involved in the sex change process provides evidence for the role of DNA methylation reprogramming in gonadal transformation. A CGI linked to the aromatase (*cyp19a1a*) transcriptional start site (TSS) was hypermethylated as gene silencing progressed during sex change (Fig. 6D). Reciprocally, as the *dmrt1* gene became up-regulated in transitioning fish, a promoter-linked CGI was progressively demethylated.

DISCUSSION

Transcriptomic and methylome analyses across sex change in the iconic bluehead wrasse have identified the triggers of socially induced sex change and enactors of gonadal metamorphosis. Our results suggest that the environmental stimulus is exerted via stress, that the subsequent steps involve repression of aromatase, and that distinctive epigenetic reprogramming is associated with reengineering ovaries into testes (Fig. 7). Importantly, this does not occur by direct transdifferentiation but involves an intermediate state with altered epigenetic machinery expression that is reminiscent of mammalian naïve pluripotent stem cells and PGCs.

We observed dynamic expression of the cortisol pathway that mediates stress. Removing the dominant TP male from a social group may represent an important social stress for large females, who must now compete for the reproductively privileged position of dominant TP male. Within minutes, a transitioning female displays aggression and courtship behaviors typical of the dominant male, along with a rapid temporary color display (a bluish coloring of the head and darkening of pectoral fin tips) that is used to establish dominance (Fig. 1). Serum cortisol levels spike during early female-male sex change in bluebanded goby (*24*) and male-female sex change in anemonefish (*39*), as well as following social rank changes in *Astatotilapia* (*40*), indicating that a stress response accompanies sexual metamorphosis and social transitions. Activation of stress pathways may also serve to meet the increased energy needs required for sex change and for maintaining social dominance (*24*, *40*). Stress responses also vary by social rank (*24*), and an intriguing question is whether IP individuals exhibit a rank-dependent sensitivity to stress that may affect who undergoes sex change.

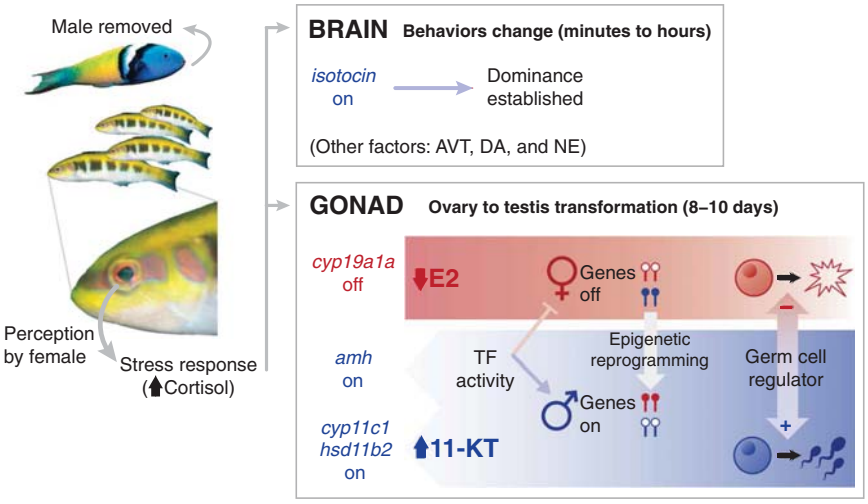

Fig. 7. Mechanistic hypothesis of socially cued sex change. Perception of a social cue (absence of a dominant male) promotes sex change in the largest female of a social group via raised cortisol. In the brain, cortisol increases *isotocin* expression to promote male-typical behaviors that rapidly establish social dominance. Other neuroendocrine factors may play a role [e.g., arginine vasotocin (AVT), dopamine (DA), and norepinephrine (NE)], although expression changes were not seen for their encoding genes. In the gonad, cortisol promotes transition from ovary (red) to testis (blue) via three pathways: (i) down-regulates aromatase (*cyp19a1a*) expression causing estrogen (E2) production to cease and feminizing expression to decline, causing ovarian atresia; (ii) up-regulates *amh* expression, which, as a transcription factor (TF) and a germ cell regulator, can suppress feminizing genes and promote oocyte apoptosis while promoting masculinizing expression and spermatogonial recruitment; and (iii) up-regulates androgenic genes *cyp11c1* and *hsd11b2* to increase 11-KT production to support testicular development. Epigenetic reprogramming, via changes in sexually dimorphic DNA methylation (represented by lollipops; open, unmethylated and filled, methylated), rewrites cellular memory of sexual fate and canalizes sex-specific expression. Photo credit: E. D'Alessandro, Oregon State University (male fish); R. Fenner, wetwebmedia.com (female fish).

The stress response in sex-changing females is expected to elicit rapid signaling changes of brain neurotransmitters, such as arginine vasotocin, gonadotropin-releasing hormone, and norepinephrine, which have been suggested to control behavioral sex change in social wrasses (*8*). We observed no significant early expression changes in genes encoding these neuropeptides or their precursors in the brain, but this may reflect the limitations of transcriptomic studies performed at coarse anatomical scales (i.e., whole brain or forebrain) in detecting neural signals that may be subtle (*20*, *21*) or highly localized (*41*). However, we did observe up-regulation of isotocin (*it*) (fig. S3C), the homolog of mammalian oxytocin, at stage 1. In fish, *it* is linked to territoriality and aggression (*20*), is up-regulated in response to cortisol (*42*), and so may facilitate early dominance establishment in transitioning females (Fig. 7).

Cortisol can then initiate gonadal sex change, and our data implicate pathways that promote stress-induced masculinization of genetic females in artificial settings (Fig. 7): (i) suppression of aromatase expression via glucocorticoid response elements in the *cyp19a1a* promoter, (ii) up-regulation of *amh* expression to induce germ cell apoptosis and promote maleness, and (iii) cross-talk with the androgen synthesis pathway via increased *cyp11c1* and *hsd11b2* expression (dual roles in 11-KT synthesis and cortisol metabolism) (*29*, *43*). Our finding of opposing expression patterns for genes encoding glucocorticoid and mineralocorticoid receptors (*nr3c1* and *nr3c2*) and the enzymes that control cortisol production (*cyp11c1* and *hsd11b1la*) and inactivation (*hsd11b2*) implies highest cortisol production in early sex change (stage 2) (Fig. 4), consistent with observations in fishes and other vertebrates undergoing natural or temperature-induced sex reversal (*14*, *24*, *39*). Therefore, in vertebrates where the environment exerts an influence on sex, cortisol might critically link environmental stimuli with sexual fate by initiating a shift in steroidogenesis.

Following the sharp down-regulation of aromatase and estrogen production, we observed a steady collapse of the female network before a male-promoting network was progressively established. Although previous studies on protogynous wrasses (*44*) and protandrous anemone fish (*45*) are equivocal about the importance of the Foxl2 and Dmrt1 transcription factors in the sex change process, our data show that they are important later and are not triggers of sex change.

Neofunctionalization, where a gene homolog acquires a novel function following gene duplication (*25*), is also readily evident in our data. Important sex-pathway genes are duplicated in the bluehead wrasse and appear to have acquired new, sometimes unexpected roles. While duplicated copies of male-promoting genes (e.g., *sox9*) appear to have retained male-specific functions, many duplicated female-promoting genes have homologs showing a complete reversal in sex-specific expression (e.g., *foxl2*, *wnt4*, and *fstl*) (Fig. 3C). In particular, key components of the Rspo1/Wnt/β-catenin signaling pathway, which regulates ovarian fate in mammals, are duplicated with one homolog showing up-regulated expression during mid-to-late sex change when testicular structures are forming or have formed. This expression pattern suggests that these duplicates have acquired new roles associated with male sexual fate, notably, testicular differentiation. This flexibility in roles suggests that a less conserved female genetic network operates in bluehead wrasses and potentially other teleosts. Gene neofunctionalization following duplication allows for diversification of a standard genetic network and may be one factor contributing to the sexual plasticity that is characteristic of fishes.

The use of neofunctionalized paralogs in sex change was not restricted to hormonal and signaling pathways; we also found duplicated epigenetic machinery in bluehead wrasses that exhibited female- or male-specific expression. Global DNA methylation was remodeled, as expression of female-specific DNMTs was replaced with male-specific expression. The peak in TET expression seen at this time indicates remodeling of DNA methylation that is typical of mammalian PGCs and both naïve and classically grown pluripotent stem cells (*30*). The same appeared to be true for histone-modifying machinery; the PRC2 complex is associated with differentiation in mammals and showed high expression in cells belonging to committed sexual phenotypes but was deactivated in the gonads of transitional fish. Likewise, dynamic expression of histone demethylases, acetyltransferases, and variant histones further suggest active chromatin modification during gonadal sex change. Differential intron retention within Jumonji chromatin modifier genes has been associated with environmental sex reversal in a dragon lizard (*14*), yet we did not find consistent evidence of intron retention for Jumonji family genes in the bluehead wrasse. Our study shows comprehensive replacement of epigenetic machinery during sex change in a vertebrate and that this is analogous to epigenetic reprogramming in germline and pluripotent cells of mammals. We hypothesize that sex-specific changes in the expression of epigenetic machinery is central to plasticity of sexual phenotype seen in sex-changing fishes, where potentially a prevailing form of epigenetic memory is removed and later replaced to canalize the terminal male phenotype. Experimental inhibition of, for example, DNMTs or PRC2 activity, will be important in testing these hypotheses.

Sexual fate has long been assumed to be canalized and stable throughout life, as it generally is for mammals and birds. However, manipulation of genes in the sex determining pathway of mammalian models revealed that gonadal sex requires active maintenance via antagonistic genetic signaling to suppress pathways of the opposite sex into adulthood (*10*, *11*). Thus, sexual fate may be inherently plastic in all vertebrates, not just in sex-changing fish.

In summary, this study reveals how environmental factors trigger gonadal sex change via a genetic cascade that reengineers ovaries into testes through an epigenetically distinct intermediate state. These findings enhance our understanding of how tissue reprogramming is controlled at the most fundamental level, and our model (Fig. 7) raises novel hypotheses about the mechanisms involved. These results also shed light on the evolution of sex determination and differentiation mechanisms in vertebrates, identifying common regulatory factors in environmentally sensitive sex determination and raising the interesting possibility that network plasticity via neofunctionalization of gene duplicates contributes to the remarkable sexual plasticity seen across teleost fishes.

MATERIALS AND METHODS

Experimental design

Female bluehead wrasses were induced to change sex by removing dominant TP males from established social groups on patch reefs off the coast of Key Largo, FL in May 2012 and May to July 2014 (*5*). On each reef, IP males and females larger than the 45-mm standard length were captured and sexed by examination of the sexually dimorphic genital papilla and extrusion of gametes by gentle abdominal pressure. Females were tagged and returned to home reefs. Between 0 and 15 IP males were captured from experimental reefs and relocated

to distant reefs to prevent competition with females for social dominance. Within 2 days following tagging and IP male removal, TP males were removed to allow females to compete for dominance and undergo sex change. Females exhibiting TP male–typical behaviors (*18*) were captured at increasing time points to produce a time series of samples across the sex-change process. Sequential removal of sex changers also served to initiate sex change in the next female (*5*). Tagged females showing no signs of behavioral or gonadal sex change (verified by histology) were captured as controls on a range of days following TP male/sex changer removal to control for varying degrees of social upheaval. Control TP males used in reported analyses were captured from unmanipulated reefs and served as a reference. All samples were collected around the daily spawning period.

Fish were euthanized with an overdose of MS-222 (Tricaine methanesulfonate) (Sigma-Aldrich) within 2 min of capture, and the brain and gonads were dissected immediately. The brain and one gonadal lobe were preserved in RNAlater (Life Technologies Inc.) on ice, followed by storage at −20°C overnight and then −80°C until RNA extraction. The second gonadal lobe was fixed for histological analysis in 4% paraformaldehyde/1× phosphate-buffered saline (PBS) overnight at 4°C, followed by storage in 1× PBS before fixation in paraffin for histological sectioning with hematoxylin and eosin staining [Histology Laboratory, College of Veterinary Medicine, North Carolina State University (NCSU)]. Experiments were approved by the Institutional Animal Care and Use Committee at NCSU.

Gonadal sections were examined under a light microscope to determine sex change status. In total, 41 samples were partitioned into successive stages (Fig. 1) based on gonadal histology (*17*) and behaviors observed at the time of capture (*18*). Females showing no signs of behavioral or gonadal sex change (healthy ovaries with mature follicles and intact zona pellucida) served as control females. Behavioral and histological characteristics of sex change stages are summarized in Fig. 1. Sex changers at stage 5 (ongoing spermatogenesis) were further divided into stages 5a and 5b to reflect their divergent global gene expression patterns (Fig. 2B). Sample sizes for each stage were as follows: six control females (CF), three stage 1 (S1), seven stage 2 (S2), three stage 3 (S3), three stage 4 (S4), three stage 5a (S5a), five stage 5b (S5b), three stage 6 (S6), and eight TP males.

RNA-seq and whole-genome bisulfite sequencing

Brain and gonadal tissues were homogenized using TissueLyser II (Qiagen), and total RNAs were extracted with TriZol (Life Technologies), using chloroform (forebrain and midbrain) or bromochloropropane as the phase separation reagent. RNA samples were column-purified with either a NucleoSpin RNA XS kit (MACHEREY-NAGEL) after deoxyribonuclease (DNase) treatment (TURBO DNA-free Kit, Ambion) (2012 samples), or a Total RNA Purification kit (Norgen Biotek) with on-column DNase digestion (RNase-free DNase I Kit, Norgen Biotek) (2014 samples). Only the forebrain and midbrain were used for RNA extraction (removing the hindbrain containing corpus cerebelli, pons, and medulla), as these contain regions belonging to the social behavior network and the mesolimbic reward system, two neural circuits that are involved in the regulation of social decision-making (*41*) and likely to be key integrators and drivers of socially induced sex change.

Total RNA concentration was measured with a Qubit 2.0 Fluorometer (Life Technologies). RNA integrity was assessed with an Agilent 2100 Bioanalyzer. Sex-changing gonads consistently showed RNA profiles with a strong peak of low–molecular weight RNA, which possibly corresponds to massive 5*S* ribosomal RNA (rRNA) expression in atretic ovaries and masks the 18*S* and 28*S* rRNA peaks used for calculating RNA integrity numbers (RINs) in teleost ovaries and intersex gonads (*46*). Therefore, RIN values could not serve as useful measures of RNA integrity in sex-changing gonads of bluehead wrasses, although only samples with visibly intact rRNA peaks were used.

Library preparation and RNA-seq were performed by the Otago Genomics and Bioinformatics Facility at the University of Otago under contract to New Zealand Genomics Limited. Samples were prepared as individual Illumina TruSeq Stranded mRNA libraries. The 2012 and 2014 samples were sequenced, with multiplexing, on separate occasions. For samples collected in 2012 (three control females, three TP males, and six intersex fish), 100–base pair (bp) paired-end (PE) reads were generated over four lanes of an Illumina HiSeq 2000. For the 2014 samples (three control females, five TP males, and 21 intersex fish), 125-bp PE reads were generated over 1.5 lanes of an Illumina HiSeq 2500. Sequencing results including short read archive (SRA) accessions are summarized in data S3.

Genomic DNA for whole-genome bisulfite sequencing (WGBS) was extracted from the TRIzol-homogenized samples used for RNA-seq following the manufacturer's instructions, with postextraction cleanup by magnetic beads (*47*). DNA was also extracted from the brain and gonad of additional animals collected in Belize (2013) and Florida (2016) using a dual RNA/DNA Purification kit (Norgen) or a lithium chloride protocol (*48*).

WGBS was undertaken using a post-bisulfite adapter tagging (PBAT) method adapted from (*49*). Briefly, bisulfite treatment was performed with an EZ Methylation Direct Mag Prep kit (Zymo Research) following the manufacturer's instructions. Bisulfite treatment was performed before adapter tagging, enabling simultaneous DNA fragmentation and conversion of unmethylated cytosines. Sequencing adapters were then added by complementary strand synthesis using random heptamer priming, and last, unique molecular barcodes and sequences necessary for binding to Illumina flow cells were added to libraries by polymerase chain reaction.

Initially, we measured global DNA methylation by performing low-coverage sequencing of 6 brain (female and TP male) and 38 gonad samples (all stages) using a single-end MiSeq 100-bp protocol (Illumina) until the desired depth (at least 10,000 mapped CG calls) was attained (*38*). Subsequently, we performed deep sequencing on a subset of 17 gonad samples on a HiSeq 2500 instrument (Illumina) using a rapid run mode, to obtain full methylomes and nucleotide-level methylation data for replicate females, TP males, and sex changers (stage 2 to 5). Detailed sequencing and CpG quantification results are provided in data S4.

Statistical analysis

Expression quantification

Read quality was assessed in FastQC v0.11.5 (www.bioinformatics.babraham.ac.uk/projects/fastqc). Raw reads were trimmed of sequencing adaptors and low-quality bases (PHRED < 5) using Cutadapt v1.16 (*50*). Expression estimates for each sample were obtained using the "align_and_estimate_abundance.pl" script within the Trinity package v2.6.6 (*51*): For each library, trimmed reads were mapped against our published bluehead wrasse transcriptome assembly (*19*) using Bowtie2 v2.3.2 (*52*), and transcript abundances were estimated using RSEM v1.3.0 (*53*) with default settings. Transcript-level count

matrices were generated for brain and gonad samples separately using Trinity's "abundance_estimates_to_matrix.pl" script and the scaledTPM method.

PCA and gene set enrichment

PCA (centered and unscaled) was used to visualize transcriptome-wide expression variation within groups and among samples, following normalization by variance stabilizing transformation in DESeq2 v1.20.0 (*54*) in R v3.5.0 (*55*). The top 10,000 transcripts with greatest variance across samples were used, and confidence ellipses around barycenters were plotted using the stat_conf_ellipse() function in ggpubr v0.1.8. For gonad only, to identify the 500 transcripts contributing most to each principal component, component loadings (defined as eigenvectors scaled by the square root of the respective eigenvalues) were represented as coordinates in a Cartesian plane. Given the bimodal and skewed distribution of the values, percentiles rather than SDs were used as thresholds. Thresholds were defined as the 5th and 95th percentiles and divided the plane into four spatial regions: "Female" and "Male" represented the extremes of PC1; sexually "Differentiated" and "Transitionary" represented the extremes of PC2. Among regions, unique and shared transcripts were represented in a Euler diagram using the R package eulerr v4.1.0. To validate the results of our method, we visualized normalized expression across sex change for unique transcripts from each spatial region.

To evaluate whether intermediate stages of sex change represented a unique transitional phase, and not a simple replacement from one differentiated population to another, we replicated the above PCA analysis but included simulated mixed datasets of male and female reads. We used fastq-tools v0.8 (http://homes.cs.washington.edu/~dcjones/fastq-tools/) to randomly subsample reads from three control female and three TP male libraries, adjusting the number of initial reads to represent different proportions in the simulated datasets (male:female, 5:95, 10:90, 20:80, 40:60, 60:40, and 80:20). Transcript expression for simulated samples was quantified as above, and the original PCA results were used as a training dataset to predict principal component scores for the simulated data.

To identify the functional categories of genes uniquely associated with each spatial region, GO term enrichment analysis was performed using TopGo v2.32.0 in R and zebrafish genome annotations downloaded from Bioconductor (org.Dr.eg.db v3.6.0). Significant GO terms ($P < 0.01$) for "biological processes" and "molecular function" were identified using Fisher's exact test with the "weight01" algorithm. To reduce GO term redundancy and summarize the results, GO terms and *P* values weighted on the basis of the scores of neighboring GO terms were used as input for REViGO (*56*), using SimRel as a semantic similarity measure, medium allowed similarity (0.7), and the *Danio rerio* GO database.

Differential expression and enrichment analyses

Differential expression analyses were performed for brain and gonad separately using a generalized linear model (GLM) framework in DESeq2. Differentially expressed transcripts were called using the Wald test in pairwise comparisons between sex change stages and control females and between neighboring stages, after fitting a single GLM to estimate size factors and dispersion across all samples per tissue. False discovery rate (FDR) was controlled at 5% to account for multiple testing, and an adjusted significance value (FDR-P) of <0.05 was used to define significant differential expression. For gonadal samples, only transcripts with a $\log_2$ fold change of >1 were considered. No fold change cutoff was applied in analyses of brain samples because of the relatively subtle expression differences observed.

Transcripts showing differential expression at each sex-change stage (compared to control females) and in comparisons between neighboring stages were searched against the Ensembl zebrafish protein database (Danio_rerio.GRCz10) (BLASTX, E-value cutoff: 10-10). Matched zebrafish protein IDs were converted to unique Ensembl zebrafish gene IDs in BioMart (*57*) and used for gene pathway overrepresentation analysis in DAVID v6.8 (*58*).

WGBS analysis and correlation with RNA-seq

Raw WGBS sequences were processed in TrimGalore! v0.4.5 (www.bioinformatics.babraham.ac.uk/projects/trim_galore/); sequencing adapters were removed, and 10 bp was trimmed from the 5′ end of reads to account for sequence biases associated with PBAT library construction, followed by removal of low-quality base calls (PHRED score < 20). Read mapping and base calling were performed in Bismark v0.19.0 (*59*) specifying the option --pbat. Our draft bluehead wrasse genome assembly (Supplementary Materials and Methods) was used as a reference, obtaining an average of 51.47% mapping efficiency (SD ± 3.07). BAM files were deduplicated, and reports containing CG methylation were generated. The bisulfite treatment nonconversion rate was evaluated with the frequency of non-CG methylation, with all libraries having a conversion efficiency of at least 98.94% (data S4).

CG methylation calls were analyzed in SeqMonk v1.42.0 (www.bioinformatics.babraham.ac.uk/projects/seqmonk/). Genome scaffolds were grouped into 23 pseudochromosomes, and tracks were built using in-house annotations (Supplementary Materials and Methods). Among-sample variation was examined with PCA, using 10-kb probes generated in nonoverlapping windows with a minimum of 100 CG calls. To compare among stages, the PCA was rerun with individual samples merged and using 2-kb windows with a minimum of 100 CpG calls.

To examine methylation across TSS, the longest transcript per gene was used. TSS were defined as 200 bp centered on the first nucleotide of an annotated mRNA, and a minimum of five methylation calls were applied as a threshold for inclusion. To evaluate coupling between TSS methylation and gene expression, trimmed RNA-seq reads were mapped to the reference genome using HTseq v0.9.1 (*60*) and imported into SeqMonk, specifying a minimum mapping quality of 60 to select only uniquely aligned reads. The SeqMonk RNA-seq quantitation pipeline was used to generate raw counts across exons of protein coding genes. Counts were corrected by transcript length and DNA contamination, and transcripts were divided into quartiles according to expression level.

To analyze methylation at individual CGs, probes of two consecutive nucleotides with a minimum of 10 methylation calls were generated and the percentage methylation measured as number of methylated calls/total calls. CGIs were identified using published methodology (*61*). For the regions of interest, 200-bp windows moving at 1-bp intervals were considered CGIs if the Obs/Exp value was greater than 0.6 and the GC content exceeded 60%.

Scatter plots and violin plots were drawn using ggplot2 v3.0.0 in R (*62*). Histograms and genome annotations were generated using Gviz v1.24.0 (*63*).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/7/eaaw7006/DC1

Supplementary Materials and Methods

Fig. S1. Lack of sex-biased variation in global gene expression in the bluehead wrasse forebrain.

Fig. S2. Intermediate gonads are molecularly distinct from ovary and testis.

Fig. S3. Differential transcript expression in forebrain and gonad of bluehead wrasses across sex change.
Fig. S4. Writers and erasers of histone acetylation are dynamically expressed across sex change.
Table S1. Genes enriched in the JAK-STAT signaling pathway are up-regulated across sex change.
Data S1. GO enrichment detailed results.
Data S2. Differential expression statistical results.
Data S3. RNA-seq metadata for bluehead wrasse brain and gonad samples.
Data S4. WGBS metadata for bluehead wrasse gonads.
References (*64*–*76*)

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Supplementary Materials for

Stress, novel sex genes, and epigenetic reprogramming orchestrate socially controlled sex change

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Supplementary Materials and Methods

Bluehead wrasse draft genome assembly, scaffolding and annotation

To provide a genomic reference for our methylation analyses, we constructed the first genome assembly for the bluehead wrasse. Ovary tissue of a single adult female (sex verified by histological analysis), from which methylome data was also derived, was used to provide DNA for sequencing. Genomic DNA was isolated using a lithium-chloride protocol (*48*), including RNAse digestion, followed by column purification through a DNA Clean & Concentrator kit (Zymo Research). Two TruSeq PCR-free libraries (350 and 550 bp inserts) and two Nextera mate-pair libraries (5 and 8 kb inserts) were constructed and sequenced together on 2 lanes of an Illumina Hi-Seq Rapid V2 (2x250 bp PE) at the Otago Genomics and Bioinformatics Facility at the University of Otago. Sequencing yielded a total of 1.84×10^{11} bases of data: 271.1, 182.4, 139.9 and 143.6 million paired reads from the 350 bp and 550 bp TruSeq, and 5 kb and 6 kb Nextera Mate Pair libraries, respectively. Based on the unassembled data, genome size was estimated at 0.76 Gb using SGA preqc (*64*).

Separate assemblies were performed for each TrueSeq library using the DISCOVAR de novo assembler (*65*) with default parameters. Based on the higher number of input reads and post-assembly statistics, the 350 bp insert assembly was used as a substrate for scaffolding. Prior to scaffolding, reads were trimmed of sequencing adapters and low-quality bases in Trimmomatic v0.35 (*66*), using the parameters 'TRAILING:26' and 'MINLEN:20'.

Scaffolding was performed in SSPACE v3.0 (*67*) using trimmed data from all four sequencing libraries. First, reads from both TruSeq libraries were mapped to the DISCOVAR contigs in

BWA (*68*). Mate Pair libraries were pre-processed with NextClip v1.3.1 (*69*) and all category A, B, and C mapping files were used for scaffolding. Resulting bam files were sorted by sequence name and converted to tab-delimited SSPACE files using the in-built script 'sam_bam2tab.pl'. SSPACE was run several times for parameter optimization, with the following parameters used for final scaffolding: 'minimum number of links to consider read pair (-k)', 3; 'maximum ratio between best two contig pairs (-a)', 0.7; 'minimum overlap between contigs to merge (-n)', 15; and contig extension: 'minimum number of reads needed to call a base during extension (-o)', 10; 'minimum number of overlapping bases during overhang consensus buildup (-m)', 50; 'minimal base ratio to accept overhang consensus base (-r)', 0.8.

GapFiller v1-11 (*70*) was used to close gaps in scaffolds. Three iterations were run, using data from all four libraries and the following parameters: 'minimum number of overlapping bases (-m)', 50; 'minimum number of reads to call a base (-o)', 2; 'minimal base ratio to accept overhang consensus (-r)', 0.7; 'minimum overlap to merge two sequences (-n)', 10; 'number of nucleotides trimmed at sequence edges of the gap (-t)', 10.

Annotation was performed following the Trinotate version 3.1.1 (*71*) and PASA version 2.2 (*72*) pipelines. To first improve gene models from previous transcriptome annotations, and determine gene positions across the genome, transcripts from our published transcriptome assembly for bluehead wrasse (*19*) were aligned to the genome assembly using GMAP version 2018-03-11 (*73*) and BLAT version 3.5 (*74*), and these results were then fed into PASA. The program seqclean version x86_64 (https://sourceforge.net/projects/seqclean/files/) was used to validate the transcript sequences and trim unwanted sequences (e.g., vectors, adaptors, polyA tails).

Using both the cleaned and original transcripts, several rounds of PASA were performed, incorporating the genome alignments, and our previous transcriptome annotations (*19*).

The Trinotate pipeline was used to annotate the mapped assemblies from the PASA output. First, TransDecoder version 5.0.2 (https://github.com/TransDecoder) was used to predict coding regions. Predicted peptide sequences and transcripts were then used as queries to search multiple protein and nucleotide databases. The protein database SwissProt was queried using BLAST (*74*), using *blastp* for peptide sequences and *blastx* for transcripts. Additionally, protein databases of the Zebrafish and Tilapia genomes were searched. The program HMMER 3.1b2 (http://hmmer.org/) was used to identify protein domains in the peptide sequences using the Pfam database. Search results were consolidated into a Trinotate sqlite database to produce an annotation report.

Custom Python and R scripts were used to extract annotations from the Trinotate report and link these to the mapped location of transcripts on the genome (https://github.com/hughcross/bluehead_methylome_bioinformatics). A custom Python script was also used to create separate mapping files (gff3 format) for the three gene references used (sprot, zebrafish, tilapia), using the best match annotation as the gene description. Mapping files were used to visualize gene annotations in SeqMonk.

The scaffolded assembly was more complete and more contiguous than the DISCOVAR assembly and was used in methylation analyses. The scaffolded genome included 379,332 scaffolds, with a scaffold and contig N50 of 15.6 and 12.5 kb, respectively, and total length of

1095.9 Mb. According to a BUSCO analysis (*75*), this assembly is relatively complete (96.7% complete, 1.4% fragmented orthologues), but with a duplication level of 13.2%. Although the large number of scaffolds indicates a fragmented assembly, over 91% of the total genome length is represented within large scaffolds (29,971 over 1 kb in length; 10,270 over 10 kb in length). Therefore, although scaffolds less than 1 kb in length were numerous (349,361; 92.1%) these comprise less than 9% of the genome length and, from initial surveys, largely represent repetitive regions. Furthermore, almost no genes mapped to these small scaffolds. Therefore, only scaffolds larger than 1 kb were used for bisulfite mapping and methylation analyses.

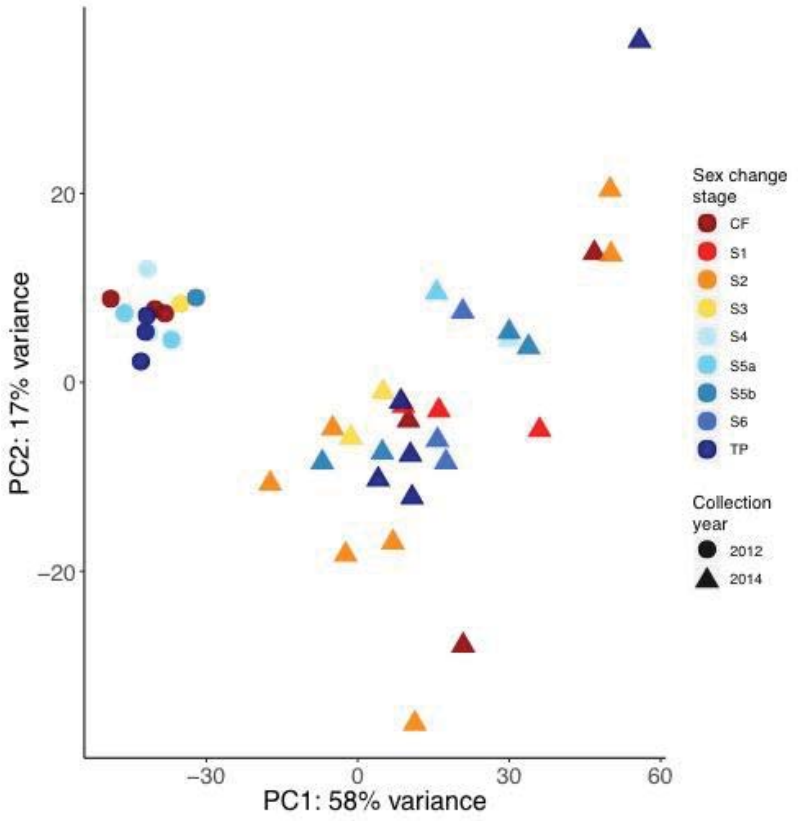

Fig. S1. Lack of sex-biased variation in global gene expression in the bluehead wrasse forebrain. Principal component analysis (PCA) of forebrain samples across sex change (10,000 most variable transcripts) reveals samples do not cluster by sex or transitional stage. Variation across collection years is evident, and samples collected in 2012 and 2014 were sequenced separately (Materials and Methods). CF, control female; S1-6, stage 1 to 6; TP, terminal phase male.

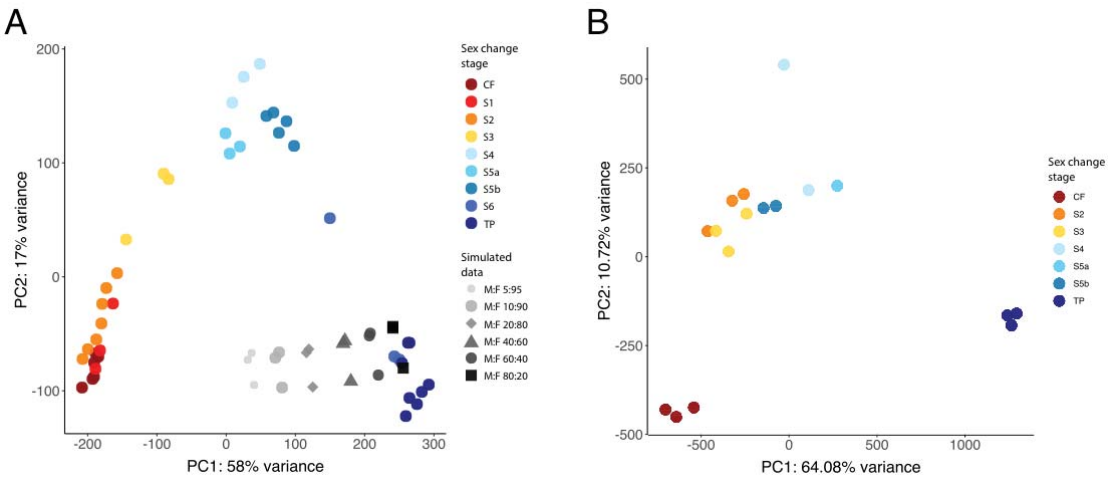

Fig. S2. Intermediate gonads are molecularly distinct from ovary and testis. (A) Principal component analysis (PCA) of gonadal gene expression (10,000 most variable transcripts) across sex change, plus simulated 'mixed' samples (grey) containing female (F) and male (M) reads combined in varying ratios (subsampled from three CF ovary and three TP testis libraries). The transition from ovary to testis is captured along PC1 (58% variance), whereas PC2 (17% variance) delineates fully differentiated gonads of control females (bottom left) and TP males (bottom right) from those of transitioning fish. Distribution of simulated 'mixed' samples along PC1 represents the expected location of intermediate samples should sex change occur via simple proportional change from female to male cells. (B) PCA of gonadal methylomes across sex change. Samples were combined by sex change stage and only probes of 10 kb with more than 100 CpG calls were included. CF, control female; S1-6, stage 1 to 6; TP, terminal phase male.

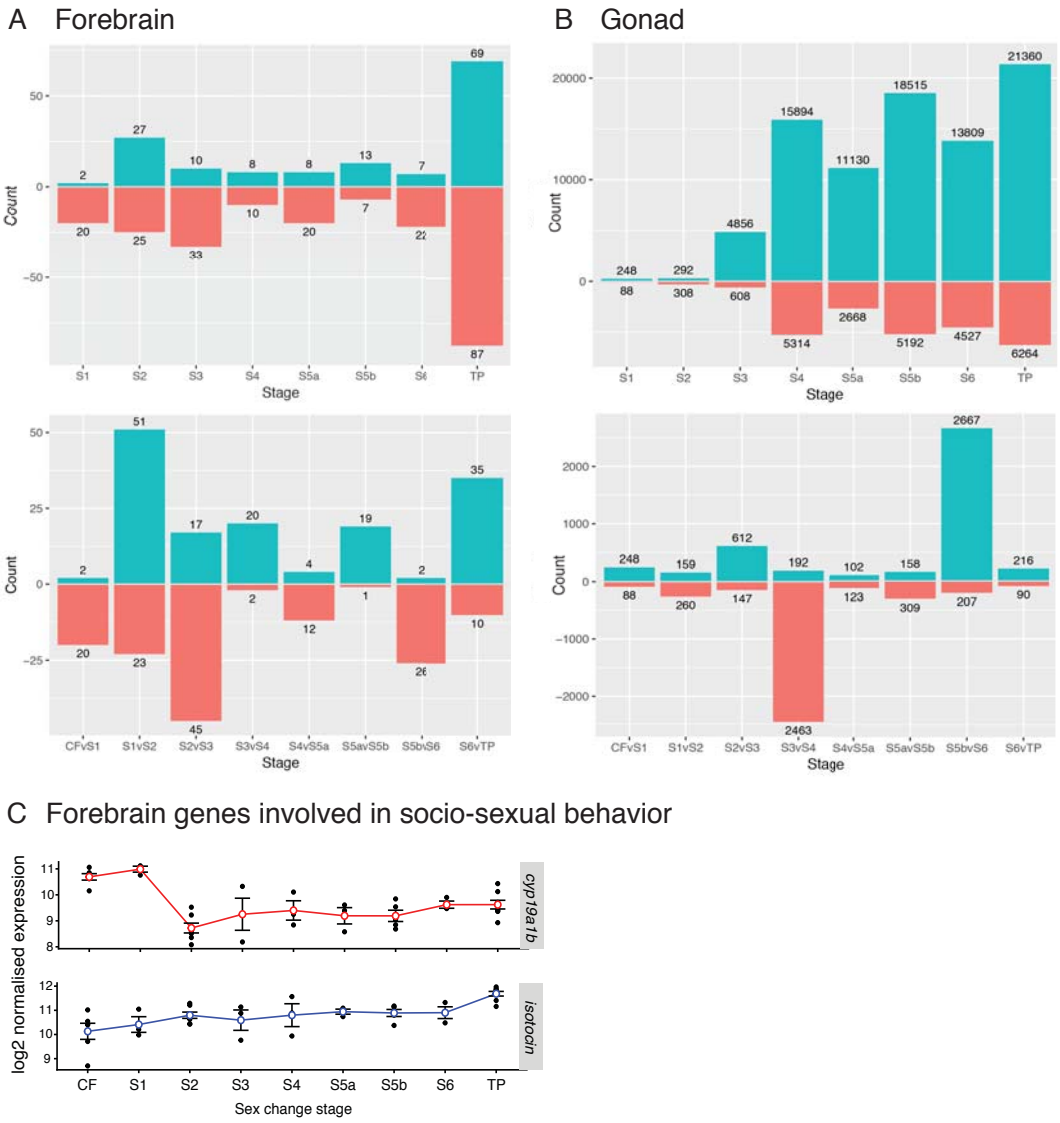

Fig. S3. Differential transcript expression in forebrain and gonad of bluehead wrasses across sex change. For (A) forebrain and (B) gonad, numbers of differentially expressed transcripts are plotted for pairwise comparisons between control females and sex-change stages (top) and between neighboring stages (bottom). Cut-off, gonad: adjusted p-value <0.05, fold-change >2. Cut-off, brain: adjusted p-value <0.05. Up/down-regulation refers to the second stage in each comparison. C) Normalized forebrain expression of selected key genes involved in teleost socio-sexual behavior. Brain aromatase *cyp19a1b* shows female biased expression and is sharply downregulated from stage 2, whereas *isotocin* expression is male biased and increases steadily across sex change. CF, control female; S1-6, stage 1 to 6; TP, terminal phase male.

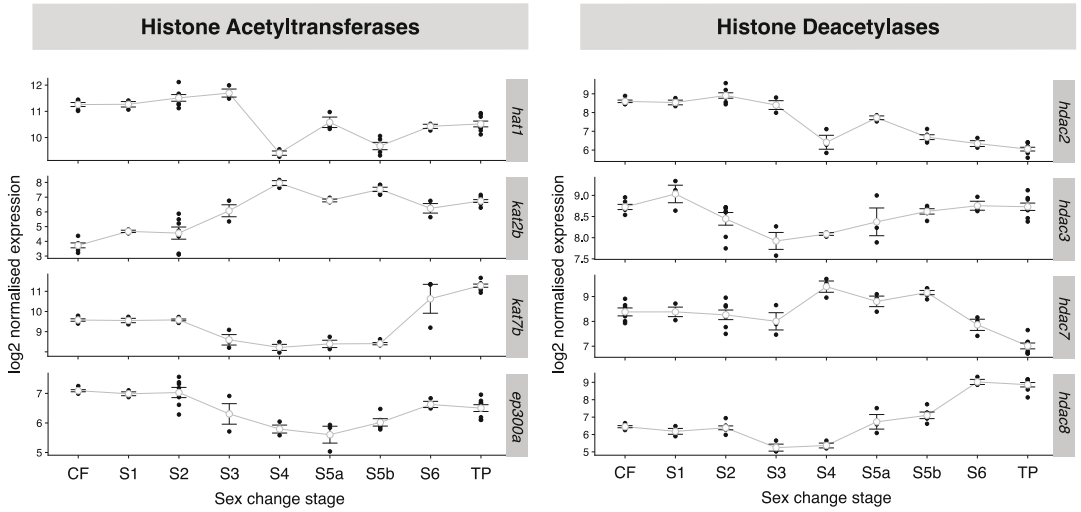

Fig. S4. Writers and erasers of histone acetylation are dynamically expressed across sex change. Normalized gonadal expression of genes encoding histone acetyltransferases (writers) and deacetylases (erasers) across sex change: *hat1* (histone acetyltransferase 1), *kat2b* (K(lysine) acetyltransferase 2b), *kat7* (K(lysine) acetyltransferase 7), *ep300a* (E1A binding protein p300a acetyltransferase), *hdac2* (histone deacetylase 2), *hdac3* (histone deacetylase 3), *hdac7* (histone deacetylase 7), *hdac8* (histone deacetylase 8).

Table S1. Genes enriched in the JAK-STAT signaling pathway are up-regulated across sex change. Genes upregulated in sex-changers (stage S2 to S6) against control females (CF) and enriched in the Jak-STAT signaling pathway. *Indicates significant enrichment (p-value <0.05) in a DAVID functional enrichment analysis.

Comparison	CF v S2	CF v S3*	CF v S4*	CF v S5a*	CF v S5b*	CF v S6
Number of genes	2	20	41	32	37	27
Fold enrichment	na	1.9	1.7	1.7	1.5	1.4
P-value	na	7.60E-03	1.90E-04	2.20E-03	7.80E-03	8.10E-02
Upregulated genes enriched in Jak-STAT signaling pathway			*akt3b*	*akt3b*	*akt3b*	*akt3b*
						bcl2l1
		ccnd2a	*ccnd2a*	*ccnd2a*	*ccnd2a*	*ccnd2a*
	cish	*cish*	*cish*	*cish*	*cish*	
		cntfr	*cntfr*	*cntfr*	*cntfr*	
			crfb4	*crfb4*	*crfb4*	*crfb4*
		csf2rb	*csf2rb*	*csf2rb*	*csf2rb*	*csf2rb*
		csf3r	*csf3r*	*csf3r*	*csf3r*	*csf3r*
		epor	*epor*	*epor*	*epor*	*epor*
			ghra	*ghra*	*ghra*	
			grb2b			
			ifngr1l		*ifngr1l*	
			il10		*il10*	
		il11ra	*il11ra*	*il11ra*	*il11ra*	*il11ra*
			il13ra1		*il13ra1*	
		il13ra2	*il13ra2*	*il13ra2*	*il13ra2*	*il13ra2*
		il21r.1	*il21r.1*	*il21r.1*	*il21r.1*	*il21r.1*
			il22ra2			
		il2rga	*il2rga*	*il2rga*	*il2rga*	*il2rga*
			il6	*il6*		
		il6st	*il6st*	*il6st*	*il6st*	

			il7r	*il7r*	*il7r*	
			irf9	*irf9*	*irf9*	
		jak1	*jak1*	*jak1*	*jak1*	*jak1*
			jak2b		*jak2b*	
		lifra	*lifra*	*lifra*	*lifra*	*lifra*
		m17	*m17*	*m17*	*m17*	*m17*
						pik3ca
			pik3cd	*pik3cd*	*pik3cd*	*pik3cd*
			pik3cg			*pik3cg*
			pik3r1	*pik3r1*	*pik3r1*	
			pik3r2	*pik3r2*	*pik3r2*	*pik3r2*
		pik3r5				
			pik3r3b	*pik3r3b*	*pik3r3b*	*pik3r3b*
			pik3r5	*pik3r5*	*pik3r5*	*pik3r5*
			pim1	*pim1*	*pim1*	
						pim2
					ptpn11b	*ptpn11b*
		ptpn6	*ptpn6*	*ptpn6*	*ptpn6*	*ptpn6*
		si:dkey-13m1.2	*si:dkey-13m1.2*	*si:dkey-13m1.2*	*si:dkey-13m1.2*	*si:dkey-13m1.2*
		socs1a	*socs1a*	*socs1a*	*socs1a*	*socs1a*
		socs3b	*socs3b*	*socs3b*	*socs3b*	
			socs7		*socs7*	
			stam2			*stam2*
	stat1b	*stat1b*	*stat1b*	*stat1b*	*stat1b*	*stat1b*
			stat4	*stat4*	*stat4*	

Additional data files:

Data S1. GO enrichment detailed results. Gene Ontology (GO) enrichment was performed on transcripts found uniquely assigned to each of four spatial regions identified in the PCA of gonadal gene expression: 'Female', 'Male', 'Transitionary' and 'Differentiated' (Fig. 2B). For each region, enriched GO terms and associated gene lists are provided in separate tabs.

Data S2. Differential expression statistical results. Pairwise comparisons of (A) forebrain of control females against sex change stages, (B) forebrain among neighboring sex change stages, (C) gonad of control females against sex change stages, and (D) gonad among neighboring sex change stages of bluehead wrasse. Statistical results are reported in separate tabs for each pairwise comparison, and for upregulated (up, blue tabs) versus downregulated (down, pink tabs) transcripts. Up/down-regulation refers to the second stage in each comparison. In each tab, transcripts with statistically significant differential expression are colored. Cut-off, gonad: adjusted p-value <0.05, fold-change >2. Cut-off, forebrain: adjusted p-value <0.05. CF, control female; S1-S6, sex change stages 1 through 6; TP, Terminal Phase male. Column headers: contigs, contig name; baseMean, mean normalized count value; log2FoldChange, effect size estimate; lfcSE, log2 fold change standard error; stat, Wald statistic; pvalue, uncorrected p-value; padj, FDR adjusted p-value; Zfish_name, Ensembl zebrafish gene annotation; Zfish_description, Ensembl zebrafish gene description; Sprot_name, SWISS-PROT protein annotation; Sprot_source, SWISS-PROT annotation source species. NA, not available.

Data S3. RNA-seq metadata for bluehead wrasse brain and gonad samples. The table lists the number of trimmed reads and average quality 'Q' value obtained for each RNA-seq library as used in downstream analyses, and the corresponding NCBI BioSample and SRA accession number/s. *RIN values for ovarian samples are not representative of RNA quality as the large quantity of small RNAs interferes with calculations. N/A not calculated. ^Number of read pairs after quality trimming and filtering, used in expression analyses.

Data S4. WGBS metadata for bluehead wrasse gonads. The table lists the number of cytosine calls at either symmetric CG dinucleotides ('CG') or in other sequence contexts ('non-CG'), mapped against the draft bluehead wrasse genome. Number of calls are following deduplication. The frequency of non-CG methylation indicates the maximum rate of non-conversion during the bisulfite treatment step; by this measure, all libraries had a bisulfite conversion efficiency of at least 98.94%. na, not applicable.

Supplementary material could be found at:

https://www.advances.sciencemag.org/cgi/content/full/5/7/eaaw7006/DC1

Chapter 5

"The Genetics and Epigenetics of Sex Change in Fish"

Contribution

This chapter consists of an accepted review manuscript to be published in *Annual Review of Animal Biosciences*. The paper examines the genetics and epigenetics of sex change in fish, supported by our findings in **Chapter 4** (Ortega-Recalde, Goikoetxea, et al., 2019).

The Genetics and Epigenetics of Sex Change in Fish. **Oscar Ortega-Recalde**[1]*, Alexander Goikoetxea[1]*, Timothy A. Hore[1], Erica V. Todd[1], and Neil J. Gemmell[1]. *Annual Review of Animal Biosciences*. Vol. 8, Feb 2020 (*Ahead publication*). doi: 10.1146/annurev-animal-021419-083634

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As co-first author, my contribution to the paper was leading the epigenetics section. I drafted this section in isolation of other co-authors, however, all authors (particularly Dr Hore) helped revise this manuscript during final preparation stages. Additionally, I created all the figures and participated in the revision of all other sections of the manuscript.

In this exhaustive review, we presented the latest advances in our understanding of the genetic and epigenetic mechanisms orchestrating sex change in fish. We highlight the importance of epigenetic modifications as critical links between environmental stimuli, the initiation and regulation of sex change, and the maintenance of sexual phenotype. Although stress response is proposed as one of the central players to initiate sex change, we recognize many questions remain unresolved. Finally, we proposed sex change as a powerful model to study cell commitment, cell reprogramming and the transduction of environmental cues to stable phenotypic changes by epigenetic factors.

Annual Review of Animal Biosciences

The Genetics and Epigenetics of Sex Change in Fish

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Keywords

epigenetics, phenotypic plasticity, sex change, sex reversal, sexual plasticity, sequential hermaphroditism

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),

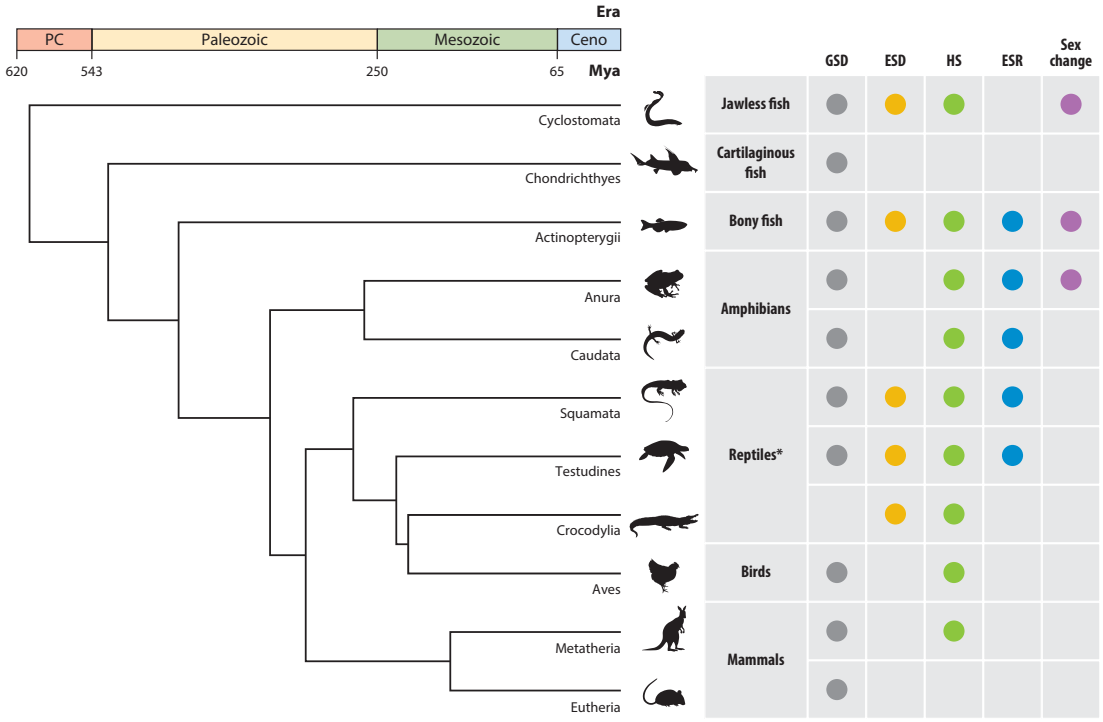


Figure 1

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).

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)

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?

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

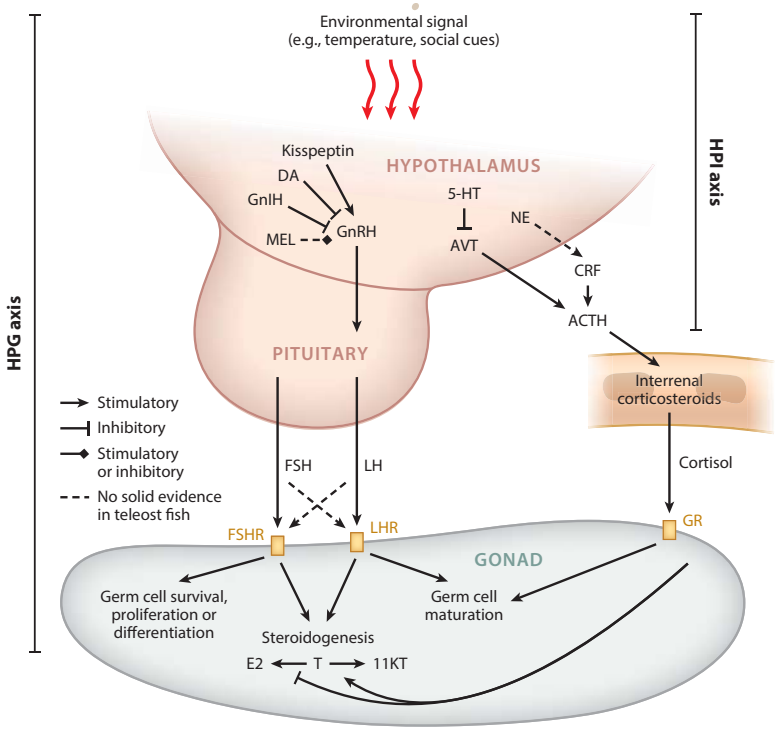


Figure 2

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 3, 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

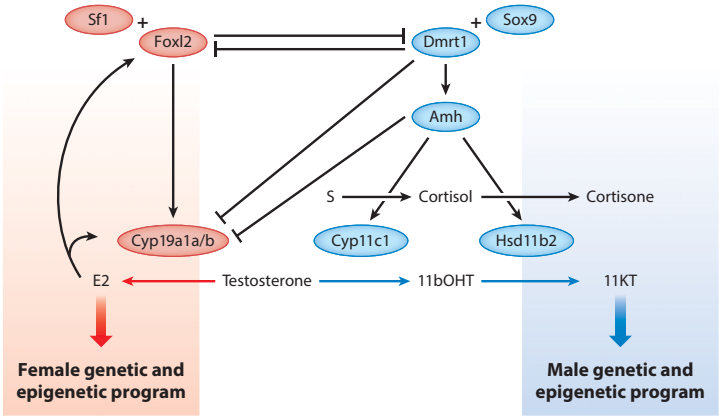


Figure 3

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 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-related HMG 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* [SRY (sex-determining region Y)-box 8], 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-ß 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.

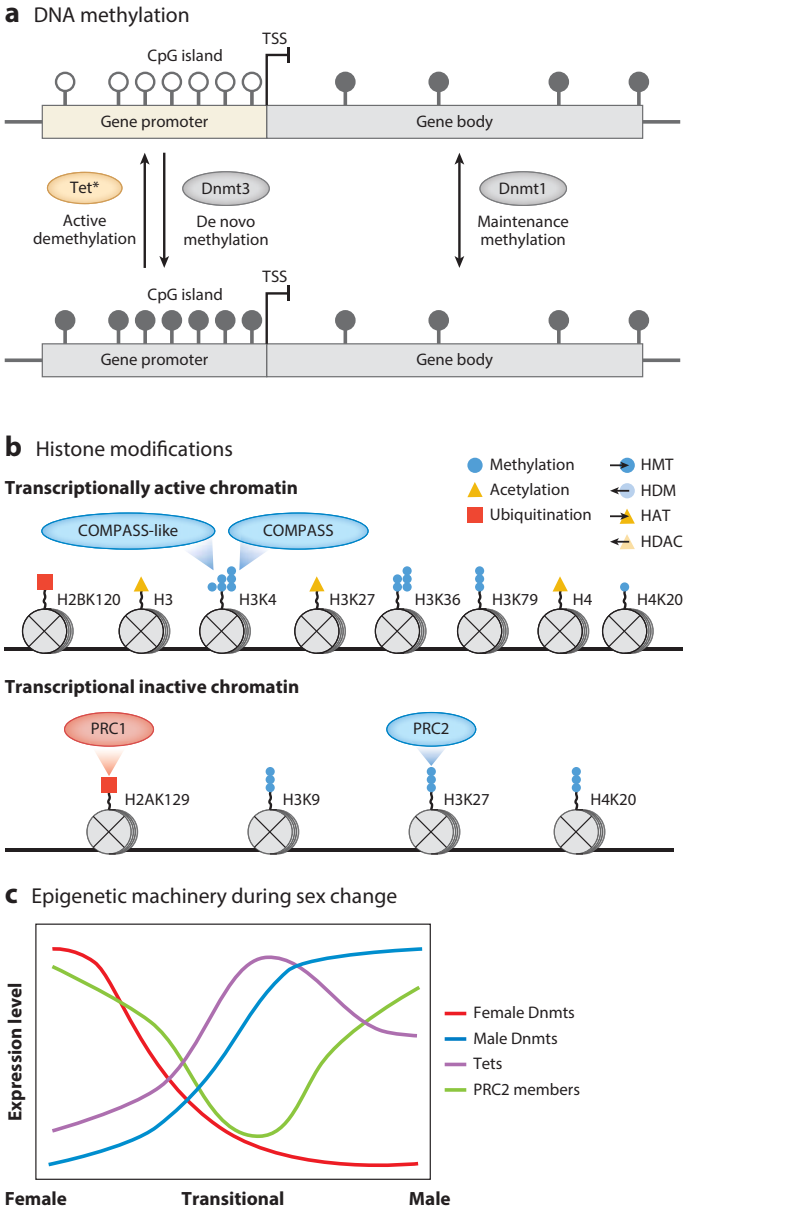
Work in other vertebrate systems continues to reveal genes with unexpected roles in sexual fate, and whose homologues in fish warrant investigation. Novel work on mice has also revealed a defining role for E3 ubiquitin ligase *znrf3* in the determination of sexual fate. Mutant male mice lacking *znrf3* 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



(*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).

preventing or favoring the binding and access of transcription factors (80, 81) or regulating 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"

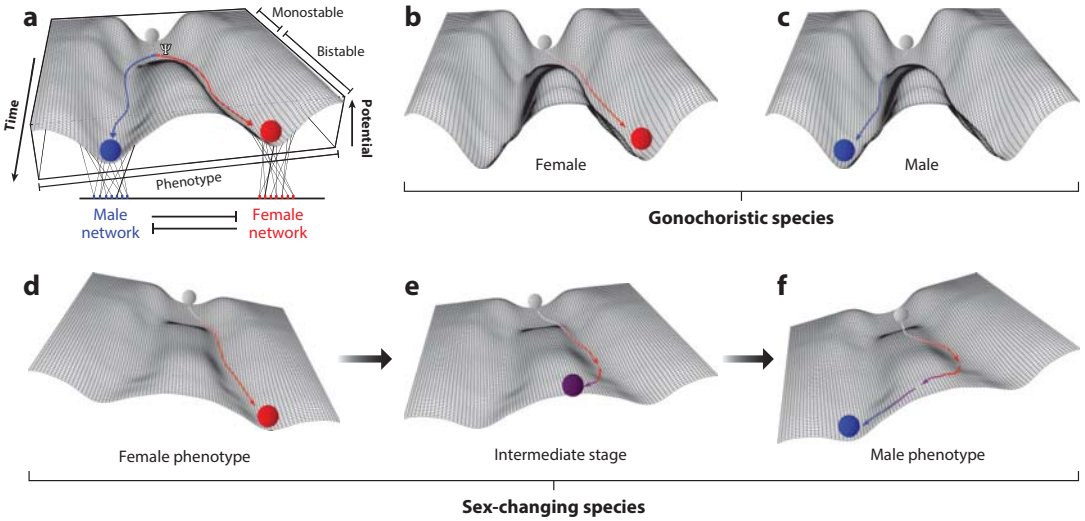


Figure 5

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.

(86). In gonochoristic species, the robustness of the sex program to environmental perturbations 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 to 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 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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Chapter 6

Discussion and conclusions

6.1 Discussion

The four manuscripts that constitute my thesis are connected by the common goal to examine the dynamics of epigenetic memory during major cellular transitions in vertebrates. One of the most significant cellular transitions in mammals during early development occurs when the germline is specified (and reprogrammed), thus separating it from ordinary somatic tissue. In mammals, another example of cellular transformation occurs when bipotential PGCs undergo feminisation or masculinisation following a sexual developmental program. Both cellular transitions are associated with a single dramatic erasure of DNA methylation, where less than 5% of CpGs remain methylated (Heather J. Lee et al., 2014). Although there are fundamental differences in germline specification and differentiation between mammals and other vertebrates, one might still hypothesize that these events are associated with a global DNA methylation erasure event. By extension, given the significant alteration in cellular identity, one might also assume that the transition of ovarian tissue to that of testis in fish, might also be associated with global DNA demethylation.

My work shows that global DNA methylation erasure is not a feature of primordial germ cell specification in zebrafish (**Chapter 2**) (Ortega-Recalde, Day, et al., 2019). Moreover, global erasure does not appear to be associated with differentiation of the zebrafish germline during gonad transformation (**Chapter 2**), and neither it is required for sex change in the gonads of bluehead wrasse as it transitions from female to male in adulthood (**Chapter 4**) (Ortega-Recalde, Day, et al., 2019; Todd et al., 2019).

In **Chapters 3 and 5** I have discussed the significance of these findings in isolation (Ortega-Recalde, Goikoetxea, et al., 2019; Ortega-Recalde & Hore, 2019), however, here I will review the major findings from each study and place them in the context of a single body of work. Furthermore, I will summarize the technical developments (in particular, low-coverage WGBS) which have driven this project forward and discuss the wider implications and limitations of my work that, due to space constraints, were not mentioned in previous chapters.

6.1.1 Low-coverage WGBS to examine epigenetic memory

In order to examine the dynamics of epigenetic memory in the germline and gonads of fish, I first needed to develop a technique that could robustly measure global levels of DNA methylation. Several methods allow DNA methylation quantitation, however, all have slightly different levels of accuracy and resolution (Kurdyukov & Bullock, 2016). Of these, WGBS is the most widely used, principally due to its flexibility and single-base resolution level. Despite being considered the gold standard for DNA methylation studies, the costs related to sequencing limits broader implementation, especially when a large number of samples are tested, or the amount of input is limited. To mitigate this

constraint, I simply undertook less sequencing per sample, meaning that more samples could be tested with each at a lower depth.

While this low-coverage WGBS procedure is intuitive in principle, when I embarked upon my project there was no formal definition as to how much sequencing was required in order to accurately represent global methylation in the CpG context. I used statistical sampling from deep-sequencing datasets to estimate the margin of error associated with predicting global CpG methylation over a range of low-coverage sequencing reads. These simulations clearly showed the existence of a minimum threshold (approximately 10,000 CpG calls), below which the margin of error increases exponentially (See **Chapter 2** Supplementary Figure 3). Initially, I developed this protocol to be used to quantify methylation in the zebrafish germline (**Chapter 2**) (Ortega-Recalde, Day, et al., 2019), although, other simulations for elephant shark (*Callorhinchus milii*) (**Appendix 1**) (Peat et al., 2017), bluehead wrasse (**Chapter 4**) (Todd et al., 2019) and mice (Ortega-Recalde O, *unpublished data*) have been performed.

Low-coverage WGBS provides several advantages over other methods for global quantitation of DNA methylation. One of the best features is that it is cost-effective and flexible; using sample indexing, multiple libraries can be pooled for sequencing. In my experience, more than 96 samples could be pooled in a single Illumina® MiSeq Reagent Kit v2, with each sample crossing the 10,000 CpG call threshold. Given that each of these runs cost approximately $500 NZD, sequencing costs per sample can be as low as $5 NZD. In contrast, performing deep-coverage sequencing can cost >$1000 NZD per sample, which is a considerable outlay when the quality of the library or samples taken is not known, or there are dozens of samples needing to be tested.

Another distinctive advantage of low-coverage WGBS is that, while only a survey of genomic methylation is undertaken, each read is still mapped back to the genome. This information is an important advantage compared to sequence-independent techniques such as HPLC, LC-MS and LUMO. Firstly, WGBS has the capacity to distinguish between CpG methylation and cytosine methylation in other contexts. The functional relevance of non-CpG methylation is currently unclear (Fuso, 2018; He & Ecker, 2015), however, because there is no known mechanism to maintain non-CpG methylation post-replication, it seems highly unlikely that it can transmit epigenetic memory. Significant quantities of non-CpG methylation can exist in the genome, particularly during early development (Arand et al., 2012; Ziller et al., 2011) Therefore, low-coverage WGBS provides a mechanism to separate non-CpG and CpG methylation, allowing the attainment of a more accurate representation of epigenetic memory in the genome.

Secondly, the mappability of low-coverage WGBS means that the origin of DNA can be confirmed. This outcome was particularly important for the results presented in **Chapter 2**, and discussed in **Chapter 3**; in the early embryo and germline of zebrafish and other species there is considerable amounts of mitochondrial and ribosomal DNA, which can outnumber chromosomal DNA (Brown & Dawid, 1968; Locati et al., 2017; May-Panloup, Chretien, Malthiery, & Reynier, 2007; Motta, Andreuccetti, & Filosa, 1991; Ortega-Recalde, Day, et al., 2019). HPLC, LC-MS, LUMO and other techniques measuring global methylation, in a manner that is unaware of the underlying sequence, fail to detect such bias.

Many different WGBS protocols exist, however, only some are adapted to work well with low DNA inputs. Many of these low input protocols are based upon

the PBAT system developed by Miura *et al.*, (2012) (Miura et al., 2012). Although a reduced number of cells negatively affect the efficiency of the library preparation, I was able to produce quality libraries with as few as 11 cells. When working with such limited material contamination is a constant concern. For each batch of PBAT samples, I included a negative control (no DNA), so that I could be sure there was no contamination in my buffers and reagents, giving rise to inaccurate results. No contamination was ever detected in my samples, although there have been occasions where this situation has arisen. Again, the mappability of PBAT data provides an extra level of security within the data; by sequencing contaminated libraries and controls it is possible to identify the source of contamination - in most cases, it appears to be human DNA (Hore TA, *unpublished observations*).

While my results show that low-coverage WGBS is a flexible and cost-efficient technique to study DNA methylation, it does have limitations and potential sources of bias. Some of these are inherent to the bisulfite-based technique, which can produce, for example, library preparation and sequencing bias (Aird et al., 2011; Olova et al., 2018). On the other hand, as mentioned before, 5mC and 5hmC are protected from deamination during bisulfite treatment (Huang et al., 2010). Therefore, these two epigenetic modifications cannot be distinguished in WGBS data. To circumvent this problem, alternative techniques, such as oxidative bisulfite sequencing and TET-assisted bisulfite sequencing, can be used (Booth et al., 2012; Yu et al., 2012). Finally, low-coverage sequencing does not allow gene-by-gene (i.e. locus-specific) analysis of methylation. Nevertheless, this last point can be easily counteracted by using subsequent deep-sequencing. For those libraries which meet quality control metrics and are of the most interest (perhaps because they appear to be representative of a group of samples), it is easy to return to the primary library and perform deep-sequencing. Indeed, this strategy

was employed in **Chapter 4**, where a combination of low-coverage and deep-coverage WGBS was used.

Despite these drawbacks, a broader implementation of the low-coverage WGBS method will make further study of global alterations in epigenetic memory more affordable, flexible, and reliable. Ultimately, this will help solve important biological questions in epigenetics and developmental biology.

6.1.2 DNA methylation memory dynamics in the vertebrate germline

The germline constitutes one of the most distinct, but also variable, cellular lineages in vertebrates. In mammals, germline specification is intrinsically associated with extensive epigenetic reprogramming, including massive loss of DNA methylation. In order to assess the dynamics of this epigenetic modification in divergent vertebrates, I examined DNA methylation in the zebrafish germline. In **Chapter 2**, I reported that this species preserves global DNA methylation during germline development (Ortega-Recalde, Day, et al., 2019).

A comprehensive assessment of germline cells ranging from 24 hpf to 70 dpf demonstrated that DNA methylation levels were not different in terms of global levels of modification to somatic cells (i.e. 72 – 85%). The stages evaluated included gonadal primordium, "juvenile ovary", gonad transformation, and sexual maturity. A detailed analysis of the samples showed that non-repetitive regions remained hypomethylated relative to repeated regions, nevertheless, none of those was hypomethylated relative to matched somatic samples. Furthermore, absence of extensive DNA methylation erasure was independently

documented by another group, who examined the zebrafish germline methylome during early stages (4 – 36 hpf) (Skvortsova et al., 2019).

Global erasure of DNA methylation in the mammalian germline is associated with germline specification and precedes sexual differentiation. My results show that this strategy is not common in all vertebrates (**Chapter 2**) (Ortega-Recalde, Day, et al., 2019). Instead, zebrafish may employ a combination of at least two factors. In the first place, additional molecular players (e.g. components of the germ plasm) can functionally replace the roles of germline DNA demethylation (e.g. reactivate the germline developmental program). Moreover, not global, but local and specific regulation of DNA methylation in key genes may play a role to control cell potency. Recent evidence shows that, at least during early stages, the activation of the germline developmental program relies on a small subset of genes, and the expression of those is uncoupled to the methylation status (Skvortsova et al., 2019). It remains to be tested if during later stages, local, but significant, demethylation occurs in critical germline genes and, if no DNA methylation occurs, whether other epigenetic mechanisms regulate the germline developmental program. Indeed, while beyond the scope of this thesis, from the zebrafish libraries created in this study, I have identified those of the most interest for deep-sequencing. Once deep-sequencing of the libraries is completed, there will be the opportunity to quantify the amount of germline reprogramming that occurs on a gene-by-gene basis.

One of the causal mechanisms to explain how those methylation dynamics evolved is the different mechanism of germline specification. This hypothesis is discussed in **Chapter 3**. It remains particularly intriguing that, whereas induction is considered the ancestral mechanism of germline specification, preformation

has evolved convergently in different lineages (Extavour & Akam, 2003; A. D. Johnson et al., 2003). Studies that examine the DNA methylation dynamics in early branching species and diverse lineages would determine if global DNA demethylation is more ancestral, and which is the distribution of both dynamics among vertebrates.

One of the limitations of my study was the fact that, among several epigenetic modifications, I only examined DNA methylation. Other epigenetic mechanisms have the potential to be information carriers between generations, for example, histone modifications and non-coding RNA (Bošković & Rando, 2018; Skvortsova et al., 2018). Histone modifications are widely reprogrammed during early embryogenesis in mammals, nevertheless recent studies have revealed the existence of intergenerational inheritance of H3K4me3 and H3K27me3 marks from the oocyte (Dahl et al., 2016; Inoue, Jiang, Lu, Suzuki, & Zhang, 2017; Zhang et al., 2016; Zheng et al., 2016). In vertebrates, nucleosomes in sperm are replaced by protamines with large degrees of variability, therefore less potential for transmission is expected. In mice, for example, just ~1% of the nucleosomes are retained, whereas zebrafish retains their entire nucleosome content (Brykczynska et al., 2010; S. F. Wu, Zhang, & Cairns, 2011). Paternal nucleosomes in zebrafish have an important role during DNA methylation reprogramming in the early embryo, providing an example of intergenerational inheritance of histone marks and the interplay between epigenetic mechanisms (Murphy, Wu, James, Wike, Cairns, et al., 2018). Histone modifications are also dynamic during PGC mammalian development and are considered critical for cell fate commitment and differentiation (Matsui & Mochizuki, 2014; Ng et al., 2013). Importantly, PGC histone modifications have been assessed exclusively on mammals, therefore, more studies are required to determine its dynamics in divergent vertebrate species.

Growing evidence supports the important role of non-coding RNA in germline development and transgenerational epigenetic memory. The most solid evidence comes from *C. elegans* and *D. melanogaster* where small RNAs have been associated with transcriptional silencing (Brennecke et al., 2008; Burton, Burkhart, & Kennedy, 2011; de Vanssay et al., 2012; Rechavi, Minevich, & Hobert, 2011; Vastenhouw et al., 2006). Interestingly, *C. elegans* lacks 5mC and 5mC levels in *D. melanogaster* are extremely low (Deshmukh, Ponnaluri, Dai, Pradhan, & Deobagkar, 2018; Wenzel, Palladino, & Jedrusik-Bode, 2011). For vertebrates, the evidence is less robust, nevertheless, it is increasing. In mice, recent studies show that RNA molecules are transported in the gametes and transmitted upon fertilization (Q. Chen et al., 2016; U. Sharma et al., 2016; Yang et al., 2016). Small-RNA, for example, seems to contribute to the intergenerational inheritance of metabolic traits, traumatic experiences, and exposure to chemical compounds (Q. Chen et al., 2016; Cropley et al., 2016; Katharina Gapp et al., 2014; Schuster, Skinner, & Yan, 2016). Perturbations in sperm long non-coding RNA, on the other hand, have been recently linked to intergenerational inheritance of phenotypic traits induced by traumatic experiences (K Gapp et al., 2018). Altogether, those studies suggest that epigenetic information in the germline can be transmitted by different molecular mechanisms and epigenetic memory is an important information module between generations.

An important question which requires further exploration is the impact of epimutations in the offspring and if they are stable between generations or actively erased during some stage of development. Whereas global DNA demethylation is absent in the zebrafish germline, local reprogramming cannot be discarded and may counteract deleterious epialleles. For *zebrafish and Xenopus tropicalis*, for example, widespread DNA demethylation of enhancers occurs during the phylotypic period (Bogdanović et al., 2016). Similar mechanisms can

restrict the number and impact of epimutations in the germline. Further studies using deep-sequencing and multiple generations may shed light on our understanding of intergenerational and transgenerational epigenetic inheritance.

6.1.3 A potential role for rDNA in sex determination

Interestingly, massive amplification and demethylation of oocyte-specific 45S rDNA were uncovered during zebrafish sex determination. This phenomenon is particularly striking because the rDNA cluster overlaps with a major sex-determining locus in natural zebrafish strains, is primarily female-specific and occurs in a critical period of gonad development. These results are significant because despite being the most intensively studied fish species, the primary trigger of sex determination in zebrafish is not known. It is likely that massive amplification of rDNA is required for the survival of peri-nucleolar oocytes, the key cells of sex determination. Interestingly, disruption of ribosomal biogenesis has shown to lead to p53-mediated apoptosis (Stedman et al., 2015). Thus, it is possible that the unique metabolic demands of peri-nucleolar oocytes regarding rDNA may represent an inflection point for apoptosis. On the other hand, growing evidence suggests ribosomes have the ability to regulate gene expression and cell commitment (Genuth & Barna, 2018; Kondrashov et al., 2011; Simsek et al., 2017). Therefore, amplification of oocyte-specific rDNA could affect the expression of genes critical for female development.

Testing if oocyte-specific 45S rDNA is the primary trigger for sex determination is particularly challenging because rDNA amplification is inextricably linked to oocyte development. Amplification of the cluster could be a consequence of the ongoing feminisation process and not the initiating signal. Thus, in future research, two approaches could be used to better explore this relationship.

The first approach is to evaluate the dynamics and regulation of oocyte-specific 45S rDNA using chemical treatment. Different environmental factors, such as temperature, rearing density, and chemical compounds, produce sex ratio bias in zebrafish (D. Santos et al., 2017). Hormone treatment with oestrogen and aromatase inhibitors produce female and male sex ratio bias respectively, especially when they are administered during sensitive periods (Luzio, Santos, Fontaínhas-Fernandes, Monteiro, & Coimbra, 2016; Takatsu et al., 2013). Similarly, treatment with 5-aza-2′-deoxycytidine (5-aza), a DNA methyltransferase inhibitor, has been described as a feminising agent (Ribas, Vanezis, Imués, & Piferrer, 2017). It is tempting to speculate that this effect is mediated by demethylation of oocyte-specific rDNA, however, demethylation of other genes first needs to be excluded as a potential confounding factor. Use of hormone treatment or demethylating agents during critical periods, and posterior correlation of the phenotypic data with amplification and methylation status of oocyte-specific 45S rDNA, may be useful to support or reject the role of oocyte-specific 45S rDNA in feminisation. Nevertheless, these experiments do not test causality.

A more targeted approach is to use reverse genetic/epigenetic techniques. An interesting hypothesis is that sex determination in zebrafish could be driven by epigenetic factors, specifically, methylation status of the oocyte-specific 45S rDNA cluster. In order to test causality, epigenetic editing provides a powerful platform to be exploited (Jurkowski, Ravichandran, & Stepper, 2015; Ravichandran, Jurkowska, & Jurkowski, 2018). Recently, a group working in close collaboration with the Hore laboratory has developed a chimeric methyltransferase dCas9-Dnmt3a-Dnmt3L (dCas9-DNMT3a3L), achieving efficient and widespread methylation in targeted genes (Stepper et al., 2017). dCas9-DNMT3a3L and dCas9-TET constructs could be used to silence or activate

oocyte-specific 45S rDNA, respectively. Tol2 fusion constructs containing guide RNAs targeting oocyte-specific 45S rDNA, and dCas9-DNMT3a3L, under control of *vasa* promoter sequence, would ensure specific and continual germline targeting. Although technically challenging, epigenetic engineering would provide invaluable insight to determine the role of rDNA demethylation in sex determination.

6.1.4 Epigenetic memory and sexual phenotype

Sex change in fish provides a fascinating window to explore the role of epigenetic memory in a naturally occurring major reprogramming event. As mentioned before, major developmental transitions in mammals are accompanied by extensive erasure of epigenetic marks. While global methylation erasure has not been reported in healthy mammalian adult tissue undergoing cellular transformation, there are no mammalian examples where post-embryonic cell plasticity is as extreme as in bluehead wrasse. To study this process, I examined the transcriptome and methylome of bluehead wrasse during female-to-male sex change. In the publication that constitutes **Chapter 4**, I reported that, whereas extensive DNA demethylation is not required to reprogram the sexual developmental program, sex change involves an epigenetic reprogramming event, which shares similarities and differences with mammals (Todd et al., 2019).

Transcriptomic analysis showed that masculinisation of the bluehead wrasse ovary is regulated by a subset of genetic and epigenetic factors. Early activation of the stress axis and repression of the aromatase gene seem to be the early triggers of the molecular cascade that orchestrate gonad remodelling. Associated with this repression is accumulation of methylation at the aromatase promoter

and at the sex-determining gene *Dmrt1*. These differences suggest that epigenetic changes on a gene-specific level could help the gonad transformation process (**Figure 5.6**). Principal component analysis of the methylation patterns showed that whereas the first component separates samples by sexual development, a second component can be attributed to developmental commitment, a pattern strikingly similar to transcriptomic data. Importantly, I found that during all the stages of transition, DNA methylation was correlated with gene silencing. This correlation means that despite the global shift in DNA methylation levels, DNA methylation principally represses gene expression during reprogramming, a pattern is shown in all jawed vertebrates (**Appendix 1**) (Peat et al., 2017).

Similar to other vertebrates, I found that global DNA methylation levels in bluehead wrasse testes were globally higher than in ovaries. Analysis by low-coverage WGBS demonstrated a progressive increase in the global levels of DNA methylation as sex change progressed. Importantly, none of the stages assessed showed evidence of global erasure of DNA methylation. Despite the absence of global demethylation, Tet expression peaked in intermediate-late stages, while Dnmt proteins were under-expressed during the same periods. Other epigenetic regulators, such as the components of the polycomb repressive complex 2, histone acetyltransferases and histone deacetylase were dynamically regulated during the transitional period. Remarkably, the expression pattern identified at those intermediate stages resembled dedifferentiated cell states in mammals (Hajkova et al., 2008; Marks et al., 2012), suggesting that pluripotency in vertebrates could be driven by a common network of epigenetic regulators.

Interestingly, naïve pluripotent cells in mammals are globally demethylated (Ficz et al., 2013; Habibi et al., 2013; Leitch et al., 2013). In contrast, there was no

evidence for a fully erased cell-type within the transitioning bluehead wrasse gonad. Like my work in zebrafish, this absence demonstrates that the strategies to regulate cell commitment and differentiation are dissimilar between mammals and other vertebrates. It would be interesting to evaluate if the same epigenetic dynamics (absence of global DNA demethylation) is followed in other postembryonic developmental processes, such as regeneration and whole-body metamorphosis. An interesting example in this regard would be antler regeneration in deer - the unique example of an annual epimorphic regenerative system occurring in mammals (C. Li, 2012). Preliminary low-coverage WGBS, based upon my protocols, has revealed that bulk CpG methylation is not erased in regenerating antlers, however, global methylation levels are reduced compared to ordinary somatic tissue (Bond D.M., Coates D. and Hore T.A., *unpublished observations*).

Preliminary analysis of the spotty wrasse (*Notolabrus celidotus*) methylome during sex change shows intriguing results regarding rDNA dynamics (Todd EV, Ortega-Recalde O, Gemmell N.J. and Hore T.A., *unpublished data*). This species is endemic to New Zealand and belongs to the same family as bluehead wrasse (Labridae) (G. P. Jones, 1980). Similar to other wrasses, the spotty wrasse is a protogynous hermaphrodite, where most individuals begin life as females, while some transition to become dominant territorial males defending a harem of roughly 20 females. Analysis by low-coverage WGBS showed that transitional stages do not undergo extensive DNA demethylation, however, there is amplification and demethylation of rDNA. The level of amplification is comparable to female zebrafish during gonad transformation, nevertheless, the role of this phenomenon during sex change is unclear. Remarkably, accumulation of 5S rRNA has been described during gonad development and sex change in other fish, including bluehead wrasse, thicklip gray mullets (*Chelon*

labrosus), and sharpsnout seabream (*Diplodus puntazzo*) (Diaz de Cerio, Rojo-Bartolomé, Bizarro, Ortiz-Zarragoitia, & Cancio, 2012; H. Liu et al., 2015; Manousaki et al., 2014). A recent study has even proposed rRNA profiling as a method to predict ovarian development and sexual phenotype, with promising results (Shen, Yao, Guo, Li, & Wang, 2017). Important differences in rRNA dynamics have been reported between seasonal breeders and non-seasonal breeders (Shen et al., 2017). Bluehead wrasse is a non-seasonal breeder, whereas spotty wrasse is a seasonal breeder, therefore it is possible that this different matting strategy may have affected our results. A detailed study taking this factor into consideration is in progress.

The results obtained for bluehead and spotty wrasse support the hypothesis that global DNA demethylation is not required to regulate sexual commitment, a difference also found in zebrafish during gonad transformation. In these cases, it is unclear how the specific remodelling occurs. It is possible that sex-specific DNA methylation machinery may play a role in this process. This concept is discussed in **Chapter 4** and **Chapter 5** for sex-changing species. For zebrafish, a recent study showed evidence of differential expression in epigenetic modifiers during gonadal commitment (S. L. J. Lee et al., 2017). Additionally, other epigenetic modifications may be critical for sex-change, as suggested by transcriptomic data. Interestingly, for male-to-female sex-changing species, such as barramundi (*Lates calcarifer*) and black porgy (*Acanthopagrus schlegelii*), DNA methylation in key sex genes (e.g. *cyp19a1a* and *dmrt1*) is suggested to play an important role during sex change (Domingos et al., 2018; G.-C. Wu et al., 2016). Whereas less studied those results suggest a central role of DNA methylation during sex change in protandrous species. Further studies in gonochoristic and hermaphroditic species, focused on epigenetic memory and its regulation, will shed more light on this topic.

Lastly, the importance of epigenetic memory in sex development and sex change in fish and vertebrates is widely discussed in **Chapter 5** and **Appendix 2**. Our observations in bluehead wrasse sex change, and literature revision allowed us to propose a model for sexual phenotype and sexual plasticity in vertebrates using Waddington's epigenetic landscape. Similarly, my results in zebrafish showed that an epigenetic signal may be essential to regulate the process of sexual development. The plastic nature of epigenetic memory, and its importance for gene regulation make it critical to understand how the environment, genotype, and phenotype are connected.

6.2 Conclusions

In this thesis I have examined the DNA methylation memory in two divergent vertebrate models during major developmental and reproductive transitions. From a technical standpoint, in doing this work, I have helped to consolidate a convenient and cost-effective platform to assess global DNA methylation levels. From a scholastic perspective, I discovered that the zebrafish germline preserves global levels of methylation during development. This work stands in stark contrast to the extensive erasure of DNA methylation in mammals and suggests an enhanced potential of DNA methylation to transmit epigenetic information between generations (Ortega-Recalde, Day, et al., 2019). In agreement with my results, related work supports the existence of different DNA methylation dynamics in non-mammalian vertebrates and its importance in transgenerational epigenetic memory (Shao et al., 2014; Skvortsova et al., 2019).

Unexpectedly, my research uncovered the surprising phenomenon of massive oocyte-specific rDNA amplification during gonad transformation in zebrafish, arising from the only loci linked to sex determination in zebrafish, and occurring

in the critical oocyte cells known to signal feminisation of the gonad. Although more experiments to examine its exact role during oocyte development and survival are required, my findings establish, for the first time, a potential link between rDNA and sex determination in any vertebrate.

Lastly, I discovered that sex change in fish involves an epigenetic reprogramming event that does not require global erasure of DNA methylation (Todd et al., 2019). Nevertheless, I uncovered extensive remodelling of global and specific DNA methylation patterns, and concomitantly, I found a shift in the epigenetic molecular machinery involved in this process. This work highlights the critical role of epigenetic memory to regulate sexual phenotype and its dynamic nature during sex change.

My results allow the expansion of our knowledge on the epigenetic memory of vertebrates. Large differences in the global DNA methylation dynamics between mammals and fish suggest that the regulation of this epigenetic mark is heterogeneous among vertebrates, and that those patterns can arise as a consequence of different selective pressures (Ortega-Recalde & Hore, 2019). Likewise, these different dynamics can exert an impact on adaptation and evolution. Even though my findings did not examine transgenerational epigenetic inheritance, they provide an important framework to further research in this area. In addition, my research provides new insight into the role of epigenetic memory in sex development and sex change. These observations are useful to understand how sexual phenotype is maintained and regulated in vertebrates (Ortega-Recalde, Goikoetxea, et al., 2019).

Despite the contrasting results found throughout my project, a common theme emerged from my work: the central role of DNA methylation as a dynamic memory module in vertebrates and its active regulation during different biological processes, such as germline and sexual development. Further research exploring other species and developmental processes will be not just exciting, but also useful to understand how this epigenetic mark regulates life on earth.

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Appendix 1

"The elephant shark methylome reveals conservation of epigenetic regulation across jawed vertebrates"

Contribution

This appendix consists of a full accepted research manuscript published in *F1000Resarch* (2017).

The elephant shark methylome reveals conservation of epigenetic regulation across jawed vertebrates. Julian R. Peat[1], **Oscar Ortega-Recalde**[1], Olga Kardailsky[1], Timothy A. Hore[1]. *F1000Research*. 20 Apr 2017, 6:526, doi: 10.12688/f1000research.11281.1

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My contribution to this paper was to design and perform a computational simulation of low-coverage WGBS in order to validate the technique and establish a threshold to accurately determine global levels of methylation.

In this publication, we described for the first time the methylome of the elephant shark, a cartilaginous fish that belongs to the oldest living group of jawed vertebrates. The elephant shark possesses the slowest evolving vertebrate genome documented, and as such, is a unique window to explore the evolution of DNA methylation in vertebrates. This paper reports that elephant shark methylome shares common patterns with higher vertebrates (e.g. mice, zebrafish). CGs are highly methylated across the genome rather than patterned in mosaic, as in invertebrates, and DNA methylation of transcription start sites is inversely correlated with gene expression. Thus, DNA methylation patterns and common regulatory functions of this modification can be traced to the earliest branches of jawed vertebrate phylogeny.

RESEARCH ARTICLE

The elephant shark methylome reveals conservation of epigenetic regulation across jawed vertebrates [version 1; referees: 2 approved]

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Abstract

Background: Methylation of CG dinucleotides constitutes a critical system of epigenetic memory in bony vertebrates, where it modulates gene expression and suppresses transposon activity. The genomes of studied vertebrates are pervasively hypermethylated, with the exception of regulatory elements such as transcription start sites (TSSs), where the presence of methylation is associated with gene silencing. This system is not found in the sparsely methylated genomes of invertebrates, and establishing how it arose during early vertebrate evolution is impeded by a paucity of epigenetic data from basal vertebrates.

Methods: We perform whole-genome bisulfite sequencing to generate the first genome-wide methylation profiles of a cartilaginous fish, the elephant shark *Callorhinchus milii*. Employing these to determine the elephant shark methylome structure and its relationship with expression, we compare this with higher vertebrates and an invertebrate chordate using published methylation and transcriptome data.

Results: Like higher vertebrates, the majority of elephant shark CG sites are highly methylated, and methylation is abundant across the genome rather than patterned in the mosaic configuration of invertebrates. This global hypermethylation includes transposable elements and the bodies of genes at all expression levels. Significantly, we document an inverse relationship between TSS methylation and expression in the elephant shark, supporting the presence of the repressive regulatory architecture shared by higher vertebrates.

Conclusions: Our demonstration that methylation patterns in a cartilaginous fish are characteristic of higher vertebrates imply the conservation of this epigenetic modification system across jawed vertebrates separated by 465 million years of evolution. In addition, these findings position the elephant shark as a valuable model to explore the evolutionary history and function of vertebrate methylation.

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Introduction

The methylation of DNA at cytosine bases constitutes an epigenetic regulatory system that is essential for the development of bony vertebrates[1–3]. Of particular significance is the modification of CG dinucleotides, whose symmetry allows methylation signals in this context to be perpetuated by maintenance methyltransferases following DNA replication[4]. CG methylation and the epigenetic memory encoded by it thus form a stable but flexible storage system for molecular information.

The methylomes of studied vertebrates – including bony fish, amphibians and mammals – exhibit similar global patterns in which the majority of CG sites are methylated in somatic tissues[5–9]. Regulatory elements such as promoters and enhancers are an important exception to this pervasive methylation landscape, particularly when associated with short CG-rich regions termed CpG islands. At the transcription start site (TSS), the presence of methylation is associated with transcriptional silencing, an effect achieved through the inhibition of transcription factor binding and the action of proteins that recognise methylated DNA and induce an inaccessible chromatin configuration[10,11]. The inverse relationship of TSS methylation with gene expression has been documented across a wide range of vertebrate taxa[5,8,12–16], indicating an evolutionarily important function. The molecular machinery that invokes an inactive state in response to methylation signals also appears to be conserved[10]. Differences in methylation at regulatory regions are linked to the definition of cell fate during developmental progression and the stable maintenance of this identity in differentiated tissues[5,17–19]. Indeed, widespread erasure of methylation marks in the cells of humans and mice plays a prominent role in the reprogramming of fate specification in both natural and experimental systems[17,20,21].

High levels of methylation outside the TSS of genes also serve an important function in vertebrate genomes. A substantial fraction of vertebrate genomes is composed of repetitive transposable elements (TEs), whose activity must be repressed to safeguard genome integrity[22,23]. These elements are ubiquitously methylated in vertebrate somatic tissues[8,9,16,24], and experiments performed in mammalian model systems has shown this to be critical for their transcriptional repression[25]. Hypermethylation of gene bodies is also a conserved feature of vertebrate genomes, and – unlike methylation at the TSS – this is compatible with active transcription in all species profiled to date[5,8,12,14–16,26–28]. Although the relationship of intragenic methylation with gene expression levels is complex and appears to vary across taxa and even cell-type[5,8,12–16,26,28,29], it has been shown to suppress spurious transcription[30] and regulate exon splicing[31,32] in mammalian systems.

The distribution and regulatory functions of methylation in vertebrates are unique amongst the metazoa, but the evolution of this system is poorly understood. In striking contrast to the pervasive hypermethylation that characterises vertebrates, invertebrate genomes are sparsely methylated and certain species such as the nematode *Caenorhabditis elegans* and fruit fly *Drosophila melanogaster* are apparently devoid of cytosine methylation[14,33–36]. Where present, the predominant pattern is a mosaic configuration, in which unmethylated regions are interspersed with hypermethylated sequences, the latter preferentially located in gene bodies and in loose positive association with transcription[14,33–35,37]. Significantly, invertebrates lack the inverse relationship between TSS methylation and expression that constitutes a key regulatory mechanism in vertebrates, and the low levels of methylation do not appear to act as a control against TE activity in their genomes[14,35,38–41].

Methylation in *Ciona intestinalis*, a sea squirt belonging to the subphylum tunicata, the chordate lineage most closely related to vertebrates[42] (Figure 1), typifies the invertebrate mosaic pattern[14,33,35]. The methylation system present in higher vertebrates can thus be inferred to have evolved at some point after the divergence of tunicates from vertebrate progenitors (~680 Mya[43]) and before the radiation of bony fish and tetrapods (~430 Mya[43]; Figure 1). Understanding the timing of this progression at greater resolution and the factors that stimulated its development is hindered by the absence of methylation data from basal vertebrate classes.

Here, we use whole-genome bisulfite sequencing to generate the first methylation profiles of a cartilaginous fish, the elephant shark *Callorhinchus milii*. Through detailed comparison with published methylation and expression datasets, we demonstrate that the elephant shark methylome is characteristic of vertebrates in its global hypermethylation – including at TEs and gene bodies – and, crucially, association with transcriptional silencing at the TSS. These findings indicate conservation of a complex methylation system across jawed vertebrates separated by 465 million years of evolution, and identify the elephant shark as an important model to examine the origins and function of methylation in vertebrates.

Methods

Tissue source and DNA extraction

Elephant shark tissue samples were sourced as by-product of deceased animals harvested from commercial fishing in the Otago coastal region. As such, no animal ethics permission was applicable in this circumstance. No animal experimentation or manipulation was undertaken as defined by the Animal Welfare Act (2009, New Zealand), or according to guidelines issued by the New Zealand National Animal Ethics Advisory Committee (NAEAC, Occasional Paper No 2, 2009, ISBN 978-0-478-33858-4).

DNA was purified using a modified magnetic bead approach[44]. Briefly, cells were first homogenised in "GITC" lysis buffer (4 M Guanidine thiocyanate, Sigma G6639; 50 mM Tris, Thermo 15568-025; 20 mM EDTA; Thermo 15575-020; 2% Sarkosyl, Sigma L9150-50G; 0.1% Antifoam, Sigma A8311-50ML), and this lysate mixture was then combined with TE-diluted Sera-Mag Magnetic SpeedBeads (GE Healthcare, GEHE45152105050250) and isopropanol in a volumetric ratio of 2:3:4, respectively. Following capture with a neodymium magnet, beads were washed once with isopropanol, twice with 70% ethanol and resuspended in filter-sterile milliQ water.

Preparation of WGBS-seq libraries

WGBS-seq was undertaken using a post-bisulfite adapter tagging (PBAT) method adapted from Peat *et al.*, 2014[45]. Briefly,

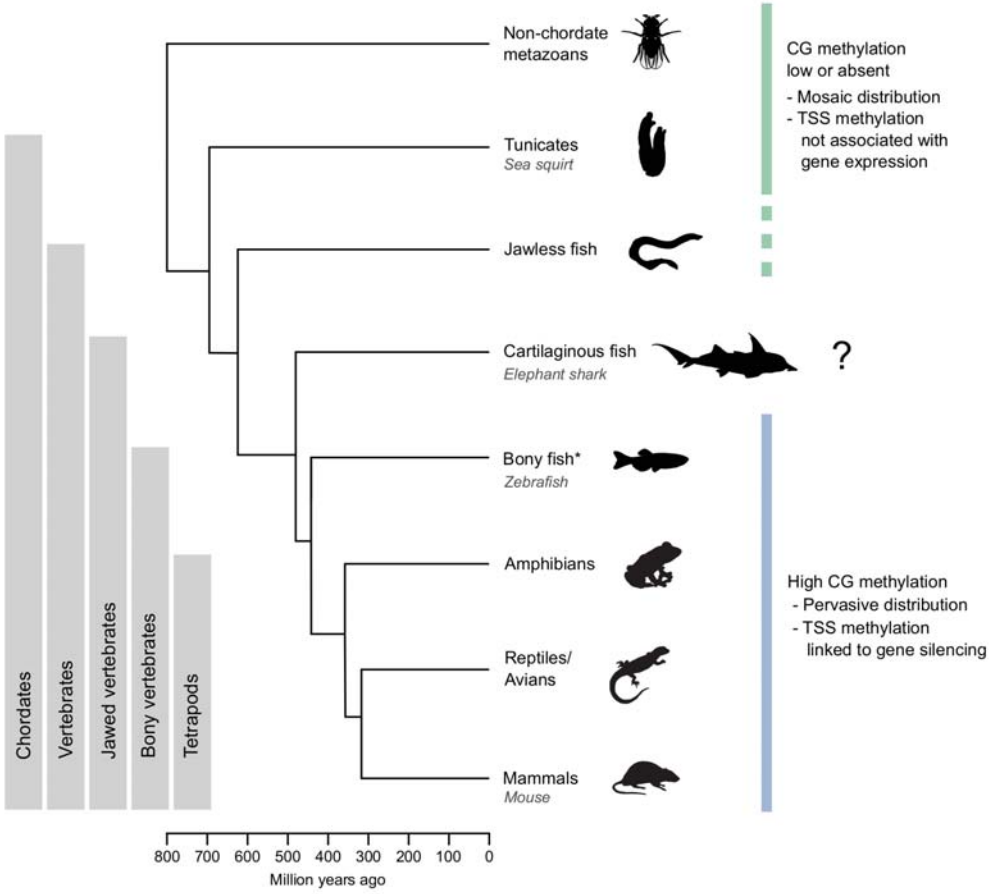

Figure 1. Phylogenetic tree showing major vertebrate groups, invertebrate outgroups and defining characteristics of their methylomes. The genomes of higher vertebrates are pervasively hypermethylated, with the exception of regulatory elements such as transcription start sites (TSSs), where the presence of methylation is associated with gene silencing (blue line). In contrast, invertebrate genomes are generally sparsely methylated in a mosaic pattern, and lack the inverse relationship between TSS methylation and expression that characterises vertebrates (green line). Certain invertebrate species appear to lack methylation altogether. Due to a paucity of data from basal vertebrate species, the evolutionary history of the CG methylation system present in higher vertebrates is unclear. Preprint methylation data from the sea lamprey *Petromyzon marinus* is not indicated here (see discussion). The names of organisms examined in this study are noted underneath the appropriate class. * The lobe-finned fish (sarcopterygii), as well as the cephalochordata (a basal chordate taxon), have been omitted for clarity. The following terms have been treated as equivalent: jawless fish and cyclostomata, jawed vertebrates and gnathostomata, cartilaginous fish and chondrichthyes, bony vertebrates and euteleostomi. Median divergence times from the TimeTree database[43] were used to construct the tree.

50–100 ng of purified DNA was subjected to bisulfite conversion using the Imprint DNA modification kit (Sigma, MOD50). Converted DNA underwent first strand synthesis with a biotin-labelled adapter sequence possessing seven random nucleotides at its 3' end (BioP5N7, biotin- ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNN). The product of first strand synthesis was captured using streptavidin-coated Dynabeads (Thermo, 11205D) and magnetic immobilisation. Double-stranded DNA was created using the immobilized first-strand as a template and an additional adapter that also possesses seven random nucleotides at its 3' end (P7N7, GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNN). Unique molecular barcodes and sequences necessary for binding to Illumina flow-cells were added to libraries by PCR using 1X HiFi HotStart Uracil+ Mix (KAPA, KK2801 and 10 µM indexed Truseq-type oligos), with thermal cycling as follows: 12× (94°C, 80 sec; 65°C, 30 sec; 72°C, 30 sec).

For deep sequencing, libraries were sequenced with a single-end 100bp protocol on a HiSeq 2500 instrument (Illumina) using rapid run mode. For low-coverage sequencing of additional samples, libraries were sequenced on a MiSeq instrument (Illumina) until the desired depth (at least 15,000 mapped CG calls) was attained.

Detailed sequencing results are provided in Table S1.

Bioinformatic processing of WGBS-seq dataset

Mapped CG methylated calls for mouse liver[5] were downloaded from GEO (accession GSE42836, sample GSM1051157) and analysed directly. For zebrafish muscle[8] (SRA study SRP020008, run SRR800081) and sea squirt muscle[14] (GEO accession GSE19824, sample GSM497251), raw sequencing data was downloaded and processed along with elephant shark WGBS-seq data generated in this study as follows.

Trimming was performed to remove both poor-quality calls and adapters sequences using TrimGalore (v0.4.0, default parameters). For the elephant shark data, 10bp were also removed from the 5' end of reads to account for sequence biases associated with PBAT library construction.

Trimmed reads were aligned using Bismark[46] (v0.14.3, default parameters) with the --pbat option specified for elephant shark data. The following genome assemblies were used for alignment: zebrafish, GRCz10; elephant shark, 6.1.3; sea squirt, KH. For sea squirt and elephant shark, alignment was only performed against scaffolds larger than 277kb to avoid gene annotation issues and assembly artefacts. The deep-sequenced elephant shark data generated in this study was additionally mapped to the mitochondrial genome.

Bismark mapping reports were used to determine global methylation levels for low-coverage elephant shark data. All other datasets were deduplicated and CG methylation calls extracted using Bismark (--comprehensive and --merge_non_CG options specified).

The number of mapped cytosine calls for sequencing performed in this study are provided in Table S1. The frequency of non-CG methylation indicates the maximum rate of non-conversion during the bisulfite treatment step; by this measure, all libraries had a bisulfite conversion efficiency of at least 98.9%.

Bootstrap sampling to determine margin of error in low-coverage WGBS-seq

In order to determine the number of CG methylation calls required to accurately predict genome-wide methylation levels, bootstrap sampling of reads from the deep-sequenced male elephant shark dataset was performed to generate regular intervals of CG calls from approximately 100 to 30,000. These reads were trimmed, mapped and methylation quantified as described above, and following 1000 iterations, the proportion of data falling within the 0.5-99.5 percentiles was calculated to generate a 99% confidence interval. An asymptotic model described by the equation $y = 2.208/\sqrt{x}$ was used to fit a curve to the data. At our minimum sequencing depth of 15,000 CG calls, bootstrap sampling predicts a margin of error (99% confidence interval) of approximately ±1.8 methylation percentage points.

Analysis of deep-sequenced WGBS-seq datasets

CG methylation calls were imported into the SeqMonk program (v1.37.1) for analysis. For elephant shark and sea squirt, custom SeqMonk genomes were built using GFF annotation files downloaded from NCBI and Ensembl, respectively.

To analyse methylation at the level of individual CG dinucleotides, we generated an annotation track of each CG site using Bowtie v1.1.2[47]. A minimum of five methylation calls was required for inclusion of a CG site in analyses.

For mouse, zebrafish and elephant shark, precompiled annotation tracks of repetitive elements generated using the RepeatMasker program were downloaded from UCSC. For sea squirt, we generated these annotations by running the RepeatMasker program (v4.0.6) on the KH assembly with the -s option and specifying *Ciona intestinalis* as the species. The various classes of transposable elements were extracted from these annotation files and where indicated, merged for analysis. A minimum of five calls was applied as a threshold for inclusion when quantifying individual elements.

To examine methylation profiles across genes or TEs and neighbouring sequences, methylation was quantified at individual CGs and the mean plotted across a size-standardised gene or TE as well as 10kb upstream and downstream regions, using the quantitation trend plot function. Figures were produced using Prism (GraphPad, v7), with smoothing applied to flanking regions by averaging 100 neighbours.

Transcription start sites were defined as 200bp centred on the first nucleotide of an annotated mRNA, and a minimum of five methylation calls was applied as a threshold for inclusion in analyses. For analysis of gene bodies, 2kb running windows were quantified (with a minimum of 50 methylation calls applied for inclusion) within annotated mRNAs, excluding 1kb at the 5' end, and the mean was reported for each mRNA.

Violin plots and histograms were drawn using the ggplot2 package[48] in R.

Bioinformatic processing of RNA-seq datasets

We downloaded raw sequencing data from previous studies as follows; sea squirt muscle[14], GEO accession GSE19824, sample GSM497252; elephant shark liver[49], SRA study SRP013772, run SRR513760; zebrafish muscle[8], SRA study SRP020008, run SRR800045; mouse liver (ENCODE Consortium[50,51]), GEO accession GSE78583, sample GSM2072415.

Trimming was performed to remove both poor-quality calls and adapters sequences using TrimGalore (v0.4.0, default parameters). In addition, 12bp were removed from the 5' end of sea squirt reads and 10bp from the 5' end of both elephant shark and mouse reads to avoid sequence biases.

Trimmed reads were aligned to the reference genomes described above with HISAT2[52] (v2.0.5) using single-end or paired-end mode, as appropriate. Known splice sites were specified from a file built from GTF annotation files downloaded from Ensembl (release 87) using the HISAT2 python script. No GTF file was

available for elephant shark, so a GFF annotation file downloaded from NCBI was first converted to GTF format using the gffread program (https://github.com/gpertea/gffread).

Analysis of RNA-seq datasets

Alignments from HISAT2 were imported into the SeqMonk program, specifying a minimum mapping quality of 60 to select only uniquely aligned reads.

The RNA-seq quantitation pipeline was used to generate raw read counts across the exons of nuclear protein-coding genes with a correction for any DNA contamination. Counts were corrected by transcript length and genes were divided into quintiles according to expression level.

Results

Genome-wide methylation profiles of the elephant shark, *Callorhinchus milii*

To generate genome-wide methylation profiles, we extracted DNA from the liver tissue of one female and one male adult elephant shark and performed whole-genome bisulfite sequencing (WGBS-seq). Detailed sequencing results are provided in Table S1.

As described in the somatic tissues of other vertebrates, we found that methylation is much more prevalent in nuclear DNA at CG dinucleotides (69 – 71.6%) than in non-CG context (0.8 – 1%) or mitochondrial DNA (1.6 – 2.5%; Figure 2A). Low-coverage WGBS-seq demonstrated similar global methylation levels in three additional individuals for liver, and in spleen and pancreas samples (Figure 2B). While we observed a small trend for lower methylation in female samples (Figure 2B; female mean 66.4%, male mean 68.6%), this was not significant according to a *t*-test (p=0.2308) and within the margin of error expected at this sequencing depth (Figure S1).

We proceeded with further analysis of CG methylation in deep-sequenced liver datasets as an example of the elephant shark somatic methylome, and combined male and female samples to enhance sequencing coverage.

The elephant shark genome is pervasively methylated

Existing data indicate that methylation patterns differ markedly between vertebrates and invertebrates. In order to delineate the characteristics of these disparate systems and establish their relationship to the elephant shark methylome, we reanalysed published WGBS-seq data from two vertebrates, mouse (*Mus musculus*)[5] and zebrafish (*Danio rerio*)[8], as well as an invertebrate from the closest chordate outgroup, the sea squirt *Ciona intestinalis*[14] (Table 1A).

As expected from analysis of global levels, examination of methylation at individual CG dinucleotides in the elephant shark showed that the majority of sites are highly methylated (≥ 80%), and fewer than one tenth are unmethylated (Figure 3A). Both this pattern and the global methylation level are comparable to mouse and zebrafish (Figure 3A–B). In contrast, mean methylation in the invertebrate sea squirt is only 22.9%, and over two thirds of CG sites are unmethylated.

A further striking distinction is evident when the genome is profiled in 2kb running windows. High methylation levels are pervasive in the elephant shark genome (Figure 3C–D), resembling

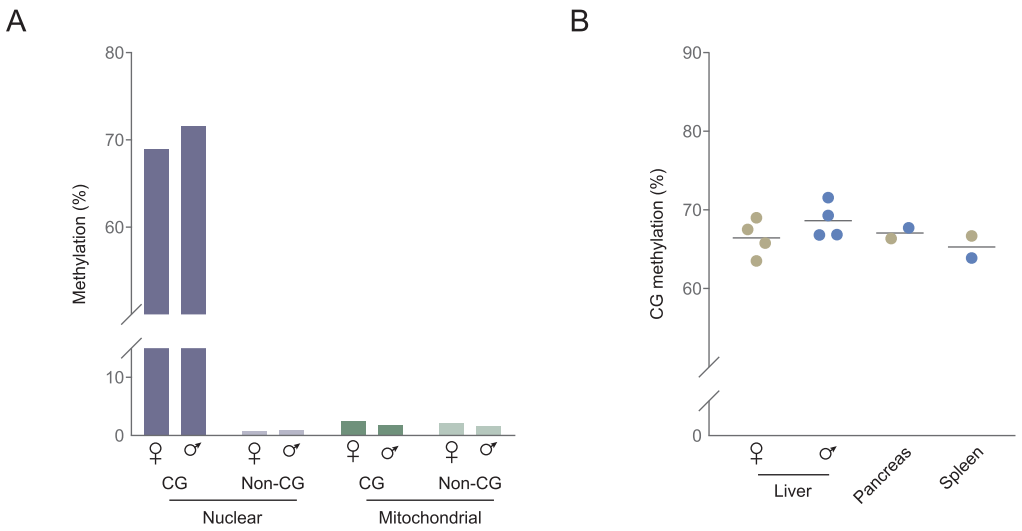

Figure 2. Global methylation levels in elephant shark somatic tissues. A: Global methylation levels of deep-sequenced liver samples in different contexts. 'CG' refers to symmetrical CG dinucleotides; 'Non-CG' indicates all other sequence contexts. **B:** Global CG methylation levels in elephant shark tissues examined by low-coverage sequencing. The horizontal bar indicates the mean; gold dots, female samples; blue dots, male samples. The difference between female and male liver samples is not significant according to a *t*-test, and within the technical margin of error expected at the threshold sequencing depth used (±1.8 methylation percentage points; Figure S1).

Table 1. Published WGBS-seq and RNA-seq datasets used for comparative analysis in this study. Accession numbers are provided in the methods.

	A. WGBS-seq Datasets		B. RNA-seq Datasets	
Species	**Reference**	**Tissue**	**Reference**	**Tissue**
Callorhinchus milii Elephant shark	This study	Liver	Venkatesh *et al.*, 2014[49]	Liver
Ciona intestinalis Sea squirt	Zemach *et al.*, 2010[14]	Muscle	Zemach *et al.*, 2010[14]	Muscle
Danio rerio Zebrafish	Potok *et al.*, 2013[8]	Muscle	Potok *et al.*, 2013[8]	Muscle
Mus musculus Mouse	Hon *et al.*, 2013[9]	Liver	ENCODE Consortium[50,51]	Liver

the structure of other vertebrate methylomes. In contrast, the sea squirt methylome is characterised by a bimodal but largely unmethylated distribution (Figure 3C), resulting from a mosaic pattern in which background hypomethylation is punctuated by shorter stretches of methylated sequences (Figure 3D). Interestingly, running windows show a broader distribution of methylation in elephant shark than in mouse or zebrafish (Figure 3C). Whether this is a feature of basal vertebrates generally or of elephant shark specifically will require analysis of methylation patterns in additional cartilaginous fish.

Transposable elements are hypermethylated in the elephant shark

Having established that the global structure of the elephant shark methylome is characteristic of vertebrates, we sought to determine the profile and impact of methylation at specific functional elements.

Transposable elements (TEs) are highly methylated in vertebrate genomes, a feature which is linked to the necessity of repressing their transcription to prevent destabilising transposase activity[8,9,16,22–24]. The generally low levels of methylation at TEs in invertebrates such as the sea squirt do not appear to regulate their activity[14,38,39].

Examination of methylation patterns at TEs and flanking sequences showed that the elephant shark exhibits hypermethylation at the large majority of TEs and a slight increase in mean methylation relative to adjacent regions (Figure 4A–B), conforming to the pattern of other vertebrates. While mean methylation levels of TEs in sea squirt are moderately elevated compared to flanking sequences, the large majority of TEs are hypomethylated. Little variation in methylation was observed between the two predominant TE classes in the elephant shark genome, long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs; Figure 4C–D), indicating that – as in other vertebrates[8,9,16,24] – hypermethylation of TEs is ubiquitous.

Methylation at elephant shark transcription start sites is associated with gene silencing

Silencing of gene expression through the deposition of methylation at transcription start sites (TSSs) constitutes an important regulatory mechanism in vertebrates, but appears to be absent from invertebrates[5–10,12–16,35,40,41]. To compare the relationship of methylation and transcription in elephant shark with higher vertebrates and the sea squirt, we made use of tissue-matched published RNA-seq datasets[8,14,49,50] (Table 1B) to classify protein-coding genes into expression quintiles.

Hypomethylation at the TSS of expressed genes constitutes a conspicuous exception to the otherwise pervasively methylated elephant shark genome, matching the higher vertebrates examined (Figure 5A–C). Significantly, we document an inverse relationship between TSS methylation and expression level in the elephant shark (Figure 5A, Figure 5E). A bimodal distribution in which a large proportion of sequences are methylated at low expression levels contrasts with negligible methylation at most TSSs of intermediate and highly expressed genes. The association of TSS methylation with transcriptional silencing is a distinguishing feature of higher vertebrate methylomes[5,8,12–16] that is recapitulated here for zebrafish and mouse (Figure 5B–C, Figure 5E), and its presence in the elephant shark indicates that methylation at the TSS induces repression in a similar manner. Consistent with reports showing that invertebrates lack this wide variation in TSS methylation as a function of expression level[14,35,41], the large majority of sea squirt TSSs are hypomethylated at all expression levels and methylation levels at the TSS are comparable to intergenic sequences (Figure 5D–E).

Interestingly, a larger number of TSSs at highly expressed genes remain methylated in elephant shark compared to mouse and zebrafish. This may suggest that the association of methylation with repression is less absolute than in higher vertebrates, but could also be attributed to poorer TSS annotation in the less intensively-studied and incompletely assembled elephant shark genome.

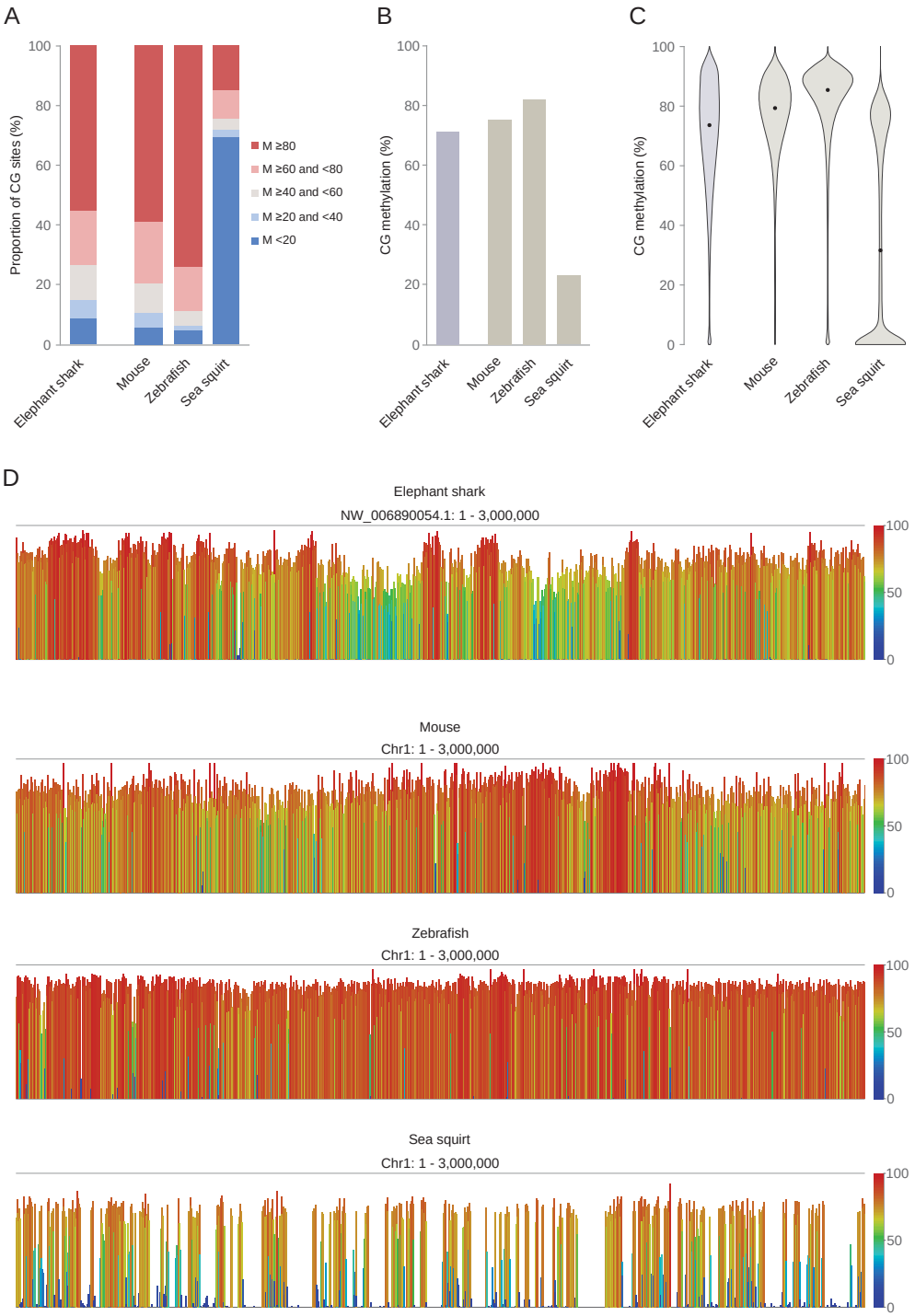

Figure 3. Global structure of the elephant shark methylome. A: Distribution of methylation at individual CG dinucleotides. 'M' denotes percentage CG methylation. **B:** Mean methylation of CG dinucleotides. **C:** Distribution of methylation within 2kb running windows covering the entire genome. Black dots denote the median. **D:** Genome screenshots of methylation quantified in 2kb running windows over the first 3Mb of chromosome 1 in sea squirt, zebrafish and mouse, and of the largest scaffold (NW_006890054.1) in elephant shark. These regions were arbitrarily chosen as an unbiased section of each genome.

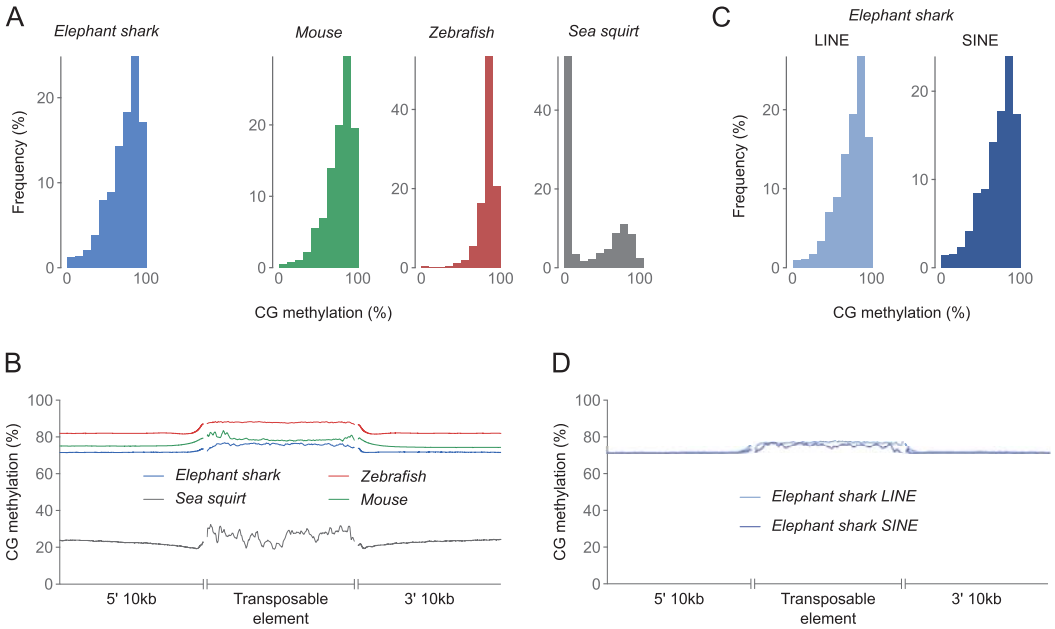

Figure 4. Methylation patterns at transposable elements. A: Distribution of methylation at transposable elements. Mean methylation values are divided into 10 bins. **B:** Mean CG methylation across transposable elements and 10kb flanking regions. Quantification was performed at the level of individual CG dinucleotides. Flanking regions were smoothed by averaging 100 neighbours. **C:** Distribution of methylation at long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) in the elephant shark genome. Mean methylation values are divided into 10 bins. **D:** Methylation at long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) in the elephant shark genome, plotted as in (**B**).

The methylomes of higher vertebrates and invertebrates also differ within gene bodies. While intragenic methylation in sea squirt forms the bimodal distribution reported in invertebrates[35,37], and most silenced genes lack methylation, vertebrate gene bodies are generally hypermethylated at all expression levels (Figure 5F). Intragenic methylation in the elephant shark is characteristic of this vertebrate pattern. In addition, higher expression levels are associated with moderately elevated gene body methylation in elephant shark liver, but not in zebrafish muscle or mouse liver. Given the limited understanding of the role played by intragenic methylation in the regulation of vertebrate gene expression, the functional relevance of this relationship is unclear.

Discussion

Methylation of CG dinucleotides forms a heritable but flexible epigenetic memory that constitutes a critical regulatory system in bony vertebrates, where it is employed in the modulation of gene expression and suppression of transposon element activity. The genomes of studied vertebrates are pervasively hypermethylated, with the exception of regulatory elements such as transcriptional start sites (TSSs), where the presence of methylation is linked to transcriptional silencing[1–10,12–16,22–25]. These features are not found in the sparsely methylated genomes of invertebrates, including chordates closely related to vertebrates[14,33–40], but establishing when this important regulatory system arose and the factors that drove its development has been has been impeded by a lack of methylation data from basal vertebrates (Figure 1).

In this study, we employ WGBS-seq to generate the first genome-wide methylation profiles of a cartilaginous fish, the elephant shark *Callorhinchus milii*. Through detailed comparison with published methylation and expression datasets, we demonstrate that the elephant shark methylome is characteristic of higher vertebrates and in clear contrast to the prevailing invertebrate configuration.

We first note that methylation in the elephant shark is primarily located in symmetric CG context, where comparable global methylation levels of approximately 65–70% were found by low-coverage WGBS-seq in the male and female liver, as well as in the spleen and pancreas (Figure 2). The similarity of male and female methylation indicates that, unlike certain bony fish species[33], the uncharacterised sex-determination mechanism in the elephant shark is not associated with large differences in global methylation. Examination of liver profiles at higher resolution demonstrated that – like higher vertebrates – the majority of elephant shark CG sites are methylated, and this is ubiquitous throughout the genome rather than concentrated in short stretches in the invertebrate mosaic pattern, typified by the sea squirt (Figure 3). The global hypermethylation of the elephant shark genome includes both major transposon classes, LINEs and SINEs (Figure 4), whose

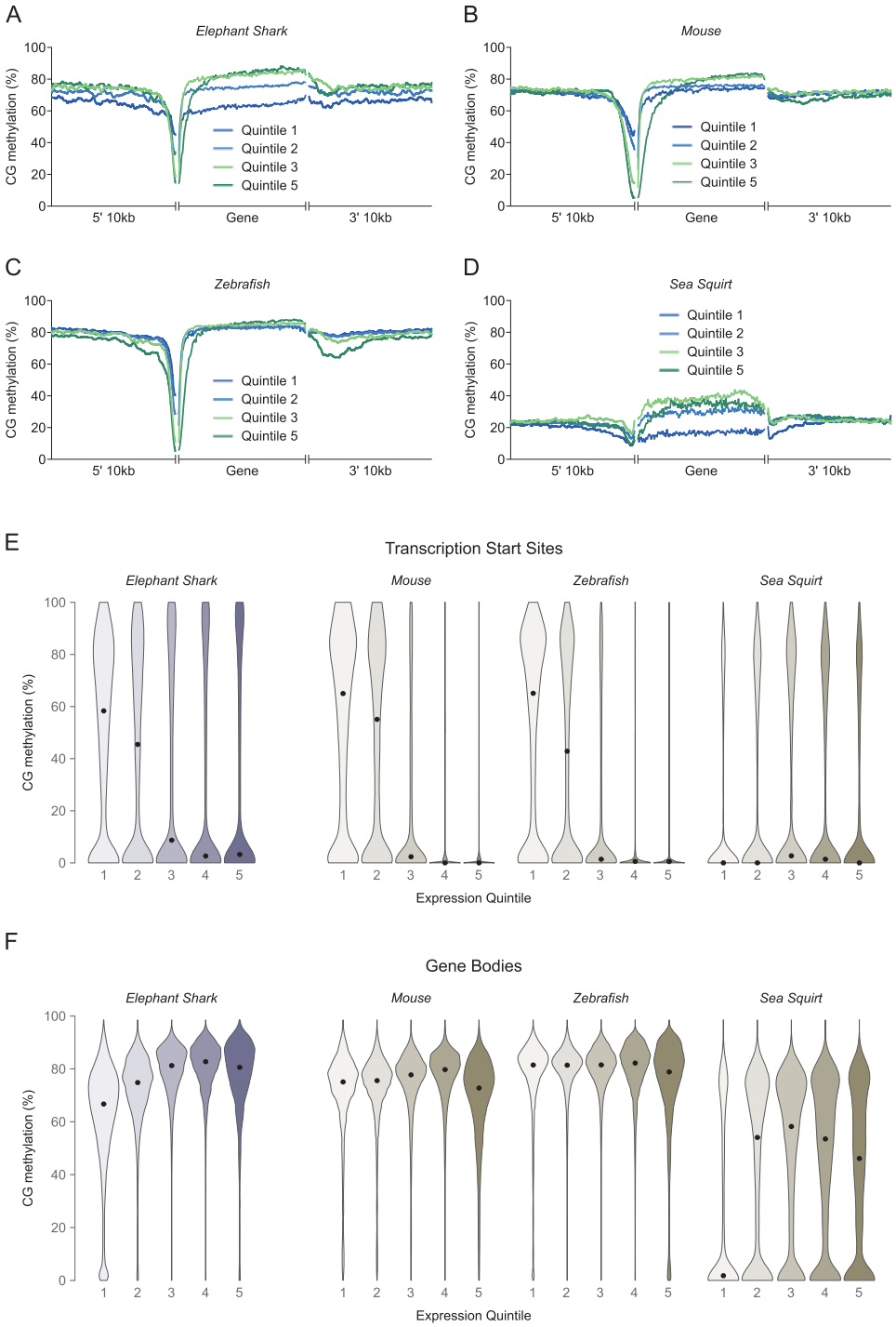

Figure 5. Relationship between methylation and gene expression. A – D: Mean CG methylation across genes and 10kb flanking regions, classified into quintiles according to expression level in RNA-seq datasets (5 = highest). Quintile 4 is omitted for clarity. Quantification was performed at the level of individual CG dinucleotides. Flanking regions were smoothed by averaging 100 neighbours. **E – F:** Distribution of methylation at the transcription start site (**E**) and within the body (**F**) of genes classified into quintiles according expression level (5 = highest). Each violin is scaled to the same maximum width (total area is not constant between violins) to demonstrate distributions for each quintile. Black dots denote the median.

transcriptional repression is thought to be an important function of vertebrate methylation systems as a safeguard against destabilising transposition activity.

Crucially, the elephant shark mirrors higher vertebrates in their inverse relationship of methylation with expression at the TSS (Figure 5); most expressed genes are unmethylated while a large proportion of inactive genes are hypermethylated at the TSS. This indicates that TSS methylation represses gene expression in a similar fashion in the elephant shark, and implies that this key regulatory mechanism – which is absent from invertebrates – is present in cartilaginous fish. While the association of TSS methylation with silencing is conserved across the vertebrates examined, we also observe that a greater number of expressed genes are methylated at the TSS in elephant shark than in mouse or zebrafish. It will be important to clarify whether this arises from the poorer annotation of the less intensively studied elephant shark genome, or a meaningful biological difference in the repressive potency of methylation in this system.

The hypermethylation of most gene bodies at all levels of transcription is a feature of higher vertebrate methylomes, that our data show is also shared by the elephant shark (Figure 5). We additionally document an interesting association between higher expression levels and elevated methylation in the elephant shark, a trend which is absent from the higher vertebrate tissues we examined. The relationship between intragenic methylation and expression is complex and appears to vary between vertebrate taxa and even within the tissues of a single species[5,8,12–16,26,28,29]. Indeed, although a variety of functions for intragenic methylation have been suggested, including suppression of spurious transcription and regulation of exon splicing[30–32], their generality is poorly understood, particularly outside mammalian systems. Significant further research will be required to uncover the impact of intragenic methylation in vertebrate genomes and determine the biological relevance of its positive relationship with expression in the elephant shark.

Evolutionary history of the vertebrate methylation system

The observation that methylation patterns in a cartilaginous fish are characteristic of higher vertebrates implies the conservation of a complex methylation system across jawed vertebrates separated by 465 million years of evolution (Figure 1). Of particular note, they support the common presence of a regulatory architecture that links methylation at the TSS to transcriptional repression.

Preprint methylome data from the sea lamprey *Petromyzon marinus*, a basal jawless vertebrate, indicate that this species lacks the genome-wide hypermethylation and functional relationships of higher vertebrates (https://doi.org/10.1101/033233). While the data from this study has not yet been released, the authors state that methylation patterns in sea lamprey more closely resemble those of the sea squirt and appear to represent a transitional intermediate. In the context of our findings, this implies that the evolution of the higher vertebrate methylation system was achieved after the emergence of jawed vertebrates (~600 Mya[43]), but before the divergence of bony and cartilaginous fish (~465 Mya[43]; Figure 1). These data further identify cartilaginous fish as the most divergent class to possess a DNA modification system similar to our own, and position the elephant shark as a valuable model to examine the function and evolution of the vertebrate methylation system. As the slowest evolving vertebrate documented[49], the elephant shark bears the closest resemblance to the most recent common ancestor of all jawed vertebrates, enhancing its appeal in this respect. Moreover, the extensive orthology of its small genome to those of tetrapods[49] facilitates comparative studies.

Transposon aggressiveness correlates with the degree of sexual outcrossing in the host, and repression of this destabilising activity has been proposed as a major reason for genome-wide hypermethylation in sexually-reproducing organisms such as plants and vertebrates[14,38,54]. This control mechanism appears to have been discarded as unnecessary in early asexual metazoans, and alternative suppression systems such as the piwi-piRNA pathway were developed in their sexually-reproducing invertebrate descendants[54,55]. The reason for the apparent reinvention of methylation-based silencing in vertebrates is unclear. Comparison of TE dynamics in the cells of elephant shark and basal chordates offers the opportunity to determine whether the need for additional control mechanisms was a primary driver for genome-wide hypermethylation in jawed vertebrates.

We note that in addition to substantial physiological changes, the emergence of jawed vertebrates was accompanied by major innovations in gene regulatory networks, notably non-coding RNA elements[49]. These advances may have facilitated, or conversely been enabled by, the development of a complex methylation system during the same time period. The role of the whole-genome duplications that occurred in vertebrate progenitors[56] in the acquisition of components that act downstream of the methylation signal, or as a stimulus for new mechanisms of regulating gene dosage, also merits further investigation.

Methylation of elements that modulate gene expression forms an epigenetic memory that plays an important role in defining and stabilising cell identity in higher vertebrates[5,17–21]. The reprogramming of this specification in the germline to regenerate full developmental competence after fertilisation, and the pathways employed to achieve this – such as active demethylation by ten-eleven-translocase (TET) enzymes, vary considerably across vertebrates[57]. Examination of these phenomena in the elephant shark will provide insight into the evolutionary history of epigenetic control in the life cycle and its consequences for vertebrate development.

Our findings provide fresh perspective on an important epigenetic modification. The elephant shark methylome delineates the evolutionary extent of the complex methylation system found in higher vertebrates, and sets the scene for comparative studies that will address longstanding questions about the primary purpose of this system and how these functions evolved from the mosaic pattern of invertebrates. It will be particularly pertinent

to understand the development of the mechanism that links TSS methylation to transcriptional repression. Epigenetic studies in the elephant shark also open promising avenues to explore the ways in which methylation is put to use during development and the specification of cell fate, and the conservation of these strategies amongst vertebrates.

Data availability

All raw WGBS-seq data (including low-coverage WGBS-seq data), as well as mapped CG call files for male and female liver deep-sequencing, are deposited in the GEO database under accession number GSE96683.

Source for published WGBS-seq datasets:

Mapped CG methylated calls for mouse liver[5] were downloaded from GEO, accession GSE42836, sample GSM1051157.

Raw sequencing data for zebrafish muscle[8] were downloaded from SRA, study SRP020008, run SRR800081.

Raw sequencing data for sea squirt muscle[14] were downloaded from GEO, accession GSE19824, sample GSM497251.

Source for published RNA-seq datasets:

Raw sequencing data for elephant shark liver[49] were downloaded from SRA, SRA study SRP013772, run SRR513760.

Raw sequencing data for mouse liver (ENCODE Consortium[50,51]) were downloaded from GEO, accession GSE78583, sample GSM2072415.

Raw sequencing data for zebrafish muscle[8] were downloaded from SRA, study SRP020008, run SRR800045.

Raw sequencing data for sea squirt muscle[14] were downloaded from GEO, accession GSE19824, sample GSM497252;

Author contributions

TAH conceived the project and JRP and TAH designed the study. JRP performed data analysis and wrote the manuscript. OK prepared WGBS-seq libraries. OO-R performed bootstrap sampling. TAH supervised the study, assisted with data analysis and contributed to the manuscript.

Competing interests

No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Supplementary material

Table S1: Whole-genome bisulfite sequencing of elephant shark somatic tissues.
The table lists the number of cytosine calls at either symmetric CG dinucleotides ('CG') or in other sequence contexts ('non-CG'), mapped against the elephant shark 6.1.3 genome assembly or mitochondrial DNA. Details of bioinformatic processing are provided in the methods section. For deep-sequenced samples, the number of calls following deduplication are given. The frequency of non-CG methylation indicates the maximum rate of non-conversion during the bisulfite treatment step; by this measure, all libraries had a bisulfite conversion efficiency of at least 98.9%.
Click here to access the data.

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Click here to access the data.

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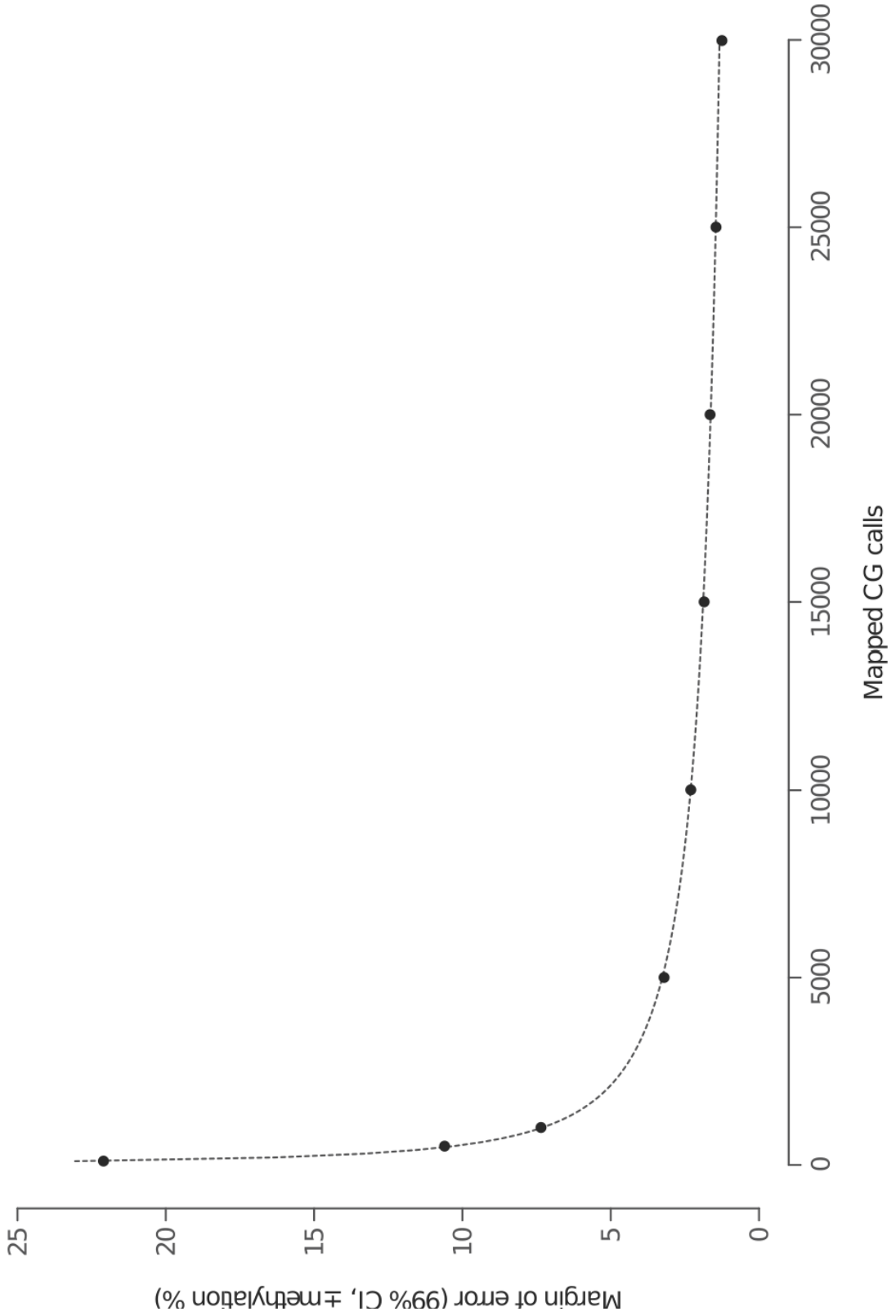
Supplementary material



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Supplementary material could be found at:

https://www.f1000research.com/articles/6-526/v1

Appendix 2

"Natural sex change in fish"

Contribution

This appendix consists of a full accepted review manuscript published in *Current Topics in Developmental Biology* (2019) and the cover image for the volume where this review was published.

Natural sex change in fish. Neil J. Gemmell[1], Erica V. Todd[1], Alexander Goikoetxea[1], **Oscar Ortega-Recalde**[1], Timothy A. Hore[1]. *Current Topics in Developmental Biology*. 2019, Vol. 134, Pag. 71-117, doi: 10.1016/bs.ctdb.2018.12.014

Waddington's epigenetic landscapes and sexual phenotype in fish. **Oscar Ortega-Recalde**[1], Timothy A. Hore[1]. *Current Topics in Developmental Biology*. 2019, Vol. 134, Cover image. ISBN: 978-0-12-811544-2

[1] Department of Anatomy, University of Otago, Dunedin, 9016, New Zealand

My contribution to this paper was the section of epigenetics in sex-change. Along with my primary supervisor Dr Timothy A. Hore, we proposed a novel theoretical model for sex change in fish and other vertebrates. This model uses Waddington's epigenetic landscape to explain the phenotypic plasticity underlying sex change in sequential hermaphroditic fish. The model and figure associated were selected as the cover image for the Sex Determination in Vertebrates Volume (**See pages 244 – 245**).

CHAPTER THREE

Natural sex change in fish

Neil J. Gemmell*, Erica V. Todd, Alexander Goikoetxea, Oscar Ortega-Recalde, Timothy A. Hore

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Contents

Abstract

Sexual fate can no longer be considered an irreversible deterministic process that once established during early embryonic development, plays out unchanged across an organism's life. Rather, it appears to be a dynamic process, with sexual phenotype determined through an ongoing battle for supremacy between antagonistic male and female developmental pathways. That sexual fate is not final and is actively regulated via the suppression or activation of opposing genetic networks creates the potential for flexibility in sexual phenotype in adulthood. Such flexibility is seen in many fish, where sex change is a usual and adaptive part of the life cycle. Many fish are sequential hermaphrodites, beginning life as one sex and changing sometime later to the other. Sequential hermaphrodites include species capable of female-to-male (protogynous), male-to-female (protandrous), or bidirectional (serial) sex change. These natural forms of sex change involve coordinated transformations across multiple biological systems,

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including behavioral, anatomical, neuroendocrine and molecular axes. Here we review the biological processes underlying this amazing transformation, focusing particularly on the molecular aspects, where new genomic technologies are beginning to help us understand how sex change is initiated and regulated at the molecular level.

1. Introduction

Sexual phenotype in most sexually reproducing organisms is dichotomous and static, a sexual mode termed gonochorism (Policansky, 1982). Most vertebrates are gonochoristic, for whom sexual fate is initiated during embryonic development through "sex determination," which triggers the initially bipotential gonad to develop as an ovary or a testis, and "sexual differentiation" the subsequent differentiation of the gonad and associated physiological traits (Capel, 2017; DeFalco & Capel, 2009). The primary sex of an individual may be determined chromosomally (as is seen in most mammals and birds), environmentally (e.g., temperature, pH, population density), or a combination of the two (as observed in many reptiles and fishes) (reviewed in Barske & Capel, 2008; Capel, 2017; Devlin & Nagahama, 2002; Gamble & Zarkower, 2012; Liu et al., 2017). This diversity in mechanism is startling when one considers the critical importance of sex determination for sexual reproduction and species persistence (Capel, 2017). However, despite the obvious diversity of sex-determining mechanisms, sexual fate was for many years considered to be fixed following downstream sexual differentiation cascades, which rely heavily on a few transcription factors and steroid hormones that are evolutionarily conserved (Barske & Capel, 2008; Guiguen, Fostier, Piferrer, & Chang, 2010; Herpin & Schartl, 2011a, 2011b; Kobayashi, Nagahama, & Nakamura, 2013; Matson & Zarkower, 2012; Piferrer & Guiguen, 2008). However, we increasingly recognize that gonadal sexual fate is not only established by competition for primacy between two sexes via antagonistic signaling pathways during embryonic development (Ungewitter & Yao, 2013; Windley & Wilhelm, 2015), but that it requires active maintenance to suppress the opposite sex during adulthood (Lindeman et al., 2015; Matson et al., 2011; Uhlenhaut et al., 2009). We are further aware that while the same genes may participate in sex determination and differentiation in multiple species, they are often expressed in different orders (Capel, 2017) or acquire completely different roles (Liu et al., 2017; Todd, Liu, Muncaster, & Gemmell, 2016).

These new studies establish a framework through which significant plasticity in phenotypic sex can readily emerge. Sex can no longer be viewed as

a static feature resulting from a deterministic switch, but rather must be viewed as a reaction norm, with sex attributes being phenotypically plastic (Ah-King & Nylin, 2010). To produce such phenotypic plasticity, the developmental process requires flexible regulatory systems, with switches that may be temporarily active that augment, inhibit or attenuate intrinsic and extrinsic signals (Capel, 2017; Liu et al., 2017; Todd, Liu, et al., 2016; West-Eberhard, 2003). In short, sexual development in vertebrates is actually a very plastic process, during which various genetic and/or environmental inputs can be integrated into the developmental scheme to influence the final sexual expression (Barske & Capel, 2008; Capel, 2017; DeFalco & Capel, 2009; Liu et al., 2017; Todd, Liu, et al., 2016).

This new perspective is strongly supported by an accumulation of evidence in non-mammalian vertebrates, particularly teleost fishes, that display huge plasticity in both gonadal development and sexual expression (Avise & Mank, 2009; Baroiller & D'Cotta, 2001; Devlin & Nagahama, 2002; Kobayashi et al., 2013; Liu et al., 2017; Thomas, Liu, Todd, & Gemmell, 2018; Todd, Liu, et al., 2016). Among teleosts sex is determined by genetics (e.g., XY, ZW, polygenic), environment (e.g., temperature, pH, population density) or their interaction (see Devlin & Nagahama, 2002; Kobayashi et al., 2013). However, in many gonochoristic fishes, the gonadal sexual fate established via primary sex determination can be interrupted, or even reversed, by endogenous and exogenous signals applied either during or after the embryonic period (Baroiller & D'Cotta, 2001; Bhandari, Nakamura, Kobayashi, & Nagahama, 2006; Kobayashi & Iwamatsu, 2005; Paul-Prasanth et al., 2013; Sato, Endo, Yamahira, Hamaguchi, & Sakaizumi, 2005). This plasticity, while incredible, looks tame in comparison to those fishes able to express both sexes at the same time (simultaneous hermaphroditism) or change their sexual expression (sequential hermaphroditism or sex change) according to environmental cues throughout their lifetime (reviewed in Avise & Mank, 2009; Devlin & Nagahama, 2002; Lamm, Liu, Gemmell, & Godwin, 2015; Liu et al., 2017; Sadovy de Mitcheson & Liu, 2008; Todd, Liu, et al., 2016).

Sequential hermaphroditism, where individuals begin life as one sex, changing sex sometime later to the other sex, is among the more stunning examples of phenotypic plasticity in response to environmental influences thus far described (Godwin, 2009; Lamm et al., 2015; Liu et al., 2017; Munday, Buston, & Warner, 2006; Nakamura, Kobayashi, Miura, Alam, & Bhandari, 2005; Thomas et al., 2018; Todd, Liu, et al., 2016; Vega-Frutis, Macías-Ordóñez, Guevara, & Fromhage, 2014; Warner, 1975, 1984). Among vertebrates, sequential hermaphroditism is restricted

to the Teleostei, but is phylogenetically widespread among that infraclass occurring in at least 27 families spread across nine teleost orders, which suggests multiple evolutionary origins (Avise & Mank, 2009; Devlin & Nagahama, 2002; Sadovy de Mitcheson & Liu, 2008). Sequential hermaphroditism occurs in three forms (Avise & Mank, 2009): protogynous (female-to-male), protandrous (male-to-female) and serial bidirectional sex change. In all cases, sex change is a usual event in the reproductive cycle of these fishes, is typically cued by changes in social structure or attainment of a critical age or size (Godwin, 2009; Kobayashi et al., 2013; Warner, 1984), and results in changes in reproductive behavior, gonadal anatomy, and external morphology, often within very short time frames (days to weeks) (Godwin, 2009; Lamm et al., 2015; Liu et al., 2017; Munday, Buston, et al., 2006; Nakamura et al., 2005; Thomas et al., 2018; Todd, Liu, et al., 2016; Vega-Frutis et al., 2014; Warner, 1975).

The broad phylogenetic occurrence, diversity of expression and speed of natural sex change make sequentially hermaphroditic fish very powerful models for studying the functioning and evolvability of sex determination and differentiation systems in vertebrates (Herpin et al., 2013; Herpin & Schartl, 2015; Matson & Zarkower, 2012; Wilhelm & Koopman, 2006). Knowledge of how sex change occurs would enhance our understanding of cellular differentiation, reprogramming and developmental commitment (Holmberg & Perlmann, 2012; Koopman, 2008; Orban, Sreenivasan, & Olsson, 2009), which are important for understanding both normal gonadal development in vertebrates and atypical sexual development in humans. Sequential hermaphroditism is also an extraordinary example of phenotypic plasticity in response to environment (see Aubin-Horth & Renn, 2009; Moczek, 2015; West-Eberhard, 2003). This linkage between gene-by-environment interactions and plastic developmental responses (Jaenisch & Bird, 2003; Zhang & Ho, 2011) is strengthening daily through our growing understanding of epigenetic regulation of gene expression (Feinberg, 2007), and its effect on sexual phenotype now emerging (Deveson et al., 2017; Ge et al., 2018; Holleley et al., 2015). Here we review our current understanding of sex change in sequentially hermaphroditic fish.

2. Why change sex?

The biological processes and adaptive benefits of natural sex change have fascinated scientists for decades (Ghiselin, 1969; Munday, Buston, et al., 2006; Robertson, 1972; Warner, 1975, 1984), with the ecological

and evolutionary contexts in which sequential hermaphroditism occurs in fishes well-studied and understood (reviewed by Avise & Mank, 2009; Godwin, 2009; Lamm et al., 2015; Nakamura et al., 2005; Warner, 1984).

2.1 Size advantage and the evolution of sequential hermaphroditism

The leading explanation for the adaptive benefit of sequential hermaphroditism, as well as the direction and timing of sex change in teleost fishes, is the Size-Advantage Model (SAM) (Ghiselin, 1969; Munday, Buston, et al., 2006; Warner, 1975). The SAM posits that sex change is adaptive when the reproductive value is greater as one sex when small and the other sex when larger (older), with sex change timed to maximize an individual's expected lifetime reproductive success. The benefits of sex change are tightly associated with a species' mating system and social structure, but in general if the reproductive value for females increases more with body size than for males, then male-first hermaphroditism is most adaptive, while female-first hermaphroditism predominates when the reproductive value for males increases more with body size than for females (Munday, Buston, et al., 2006). Evolutionary transitions between reproductive modes, for example, shifts from protogyny to gonochorism, and vice versa, are associated with changes in the mating system, as predicted by sex allocation theory (Erisman, Petersen, Hastings, & Warner, 2013). New work also demonstrates that the direction of sex change strongly affects effective population sizes, because the populations of protogynous systems are much smaller and more localized than those observed in protandrous systems (Benvenuto, Coscia, Chopelet, Sala-Bozano, & Mariani, 2017).

2.1.1 Protogyny

Protogyny is the most common form of sequential hermaphroditism in fish, occurring in 15 families, and is especially pervasive in social species with polygynous mating systems. In these species, large males aggressively defend territories to monopolize matings with females (Warner, 1984). Protogyny is ubiquitous in the wrasses (Labridae), of which the bluehead wrasse (*Thalassoma bifasciatum*) is among the best studied (Godwin, 2009; Lamm et al., 2015; Liu et al., 2017). Bluehead wrasse are small, polygamous reef fish with a lek-like mating system, in which dominant (terminal-phase, TP) males defend spawning sites to which females have high fidelity (Fig. 1A). Loss of a TP male stimulates sex change in (typically) the largest female of a social group, who then undergoes dramatic changes in behavior,

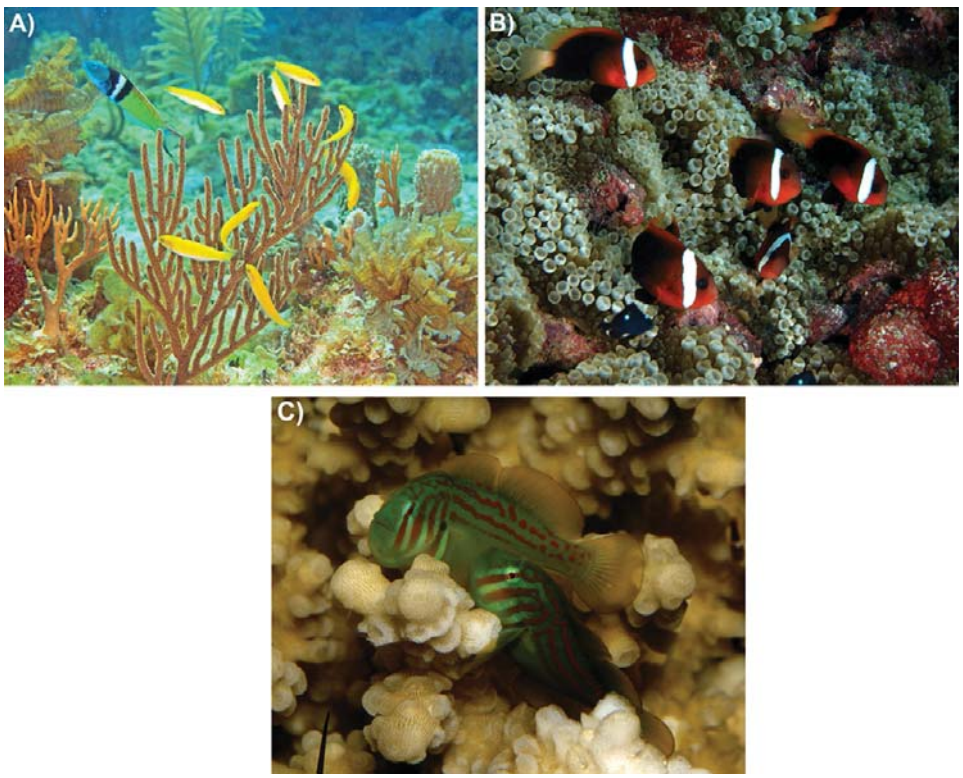

Fig. 1 Ecological context of (A) protogynous, (B) protandrous and (C) serial bidirectional sex-change in social fishes. (A) The bluehead wrasse (*Thalassoma bifasciatum*) is typical of many protogynous hermaphrodites. A brightly-colored terminal-phase (TP) male defends and courts a harem of smaller females on a tropical reef in the Caribbean sea, one of which will eventually change sex to replace him. (B) A colony of cinnamon clownfish (*Amphiprion melanopus*) inhabiting a bubble anemone. These protandrous hermaphrodites form monogamous breeding pairs consisting of a dominant female and a smaller male, who share shelter space with several immature subordinates. (C) A pair of Maori coral gobies (*Gobiodon histrio*), a species widespread on tropical reefs of the Indo-West Pacific. Monogamous coral gobies live a sessile lifestyle occupying spatially isolated coral colonies, where serial bidirectional sex change enables any two fish to form a heterosexual breeding pair. *Panel A: Image author: Kevin Bryant. Panel B: Image author: Nick Hopgood. Panel C: Image author: Mike Wesemann. Reused with permission from Todd, E. V., Liu, H., Muncaster, S., & Gemmell, N. J. (2016). Bending genders: The biology of natural sex change in fish.* Sexual Development, 10*, 223–241.*

anatomy and coloration (Warner & Swearer, 1991). Most juvenile bluehead wrasse develop as female, but a few develop directly as small, initial-phase (IP) males, that mimic the female phenotype and illicit "sneak" matings to achieve fertilization, albeit with a lower level of fertilization success than dominant TP males (Semsar & Godwin, 2004). IP male development is also under social control: more IP males develop on high-density reefs where

TP males can less-effectively monopolize mating opportunities (Munday, Wilson White, & Warner, 2006). In this and other diandric fishes, it remains unknown whether the same or different molecular pathways are involved in sex determination and differentiation of IP and TP males. Recent work shows that female mimicry by IP males has a transcriptional signature in both the brain and the gonad. Sneaker males shared striking similarity in neural gene expression with females, supporting the idea that males with alternative reproductive phenotypes have "female-like" brains (Todd et al., 2018), and because the trigger for sex change is undoubtedly neural it may well be that neural processes involved in the perception and transduction of social cues that initiate sex reversal in females and role change in IP males is similar.

The activators of sex change are less obvious in group-spawning species where social structure is not well-defined, such as the Groupers (Epinephelidae). These fish periodically form sizeable breeding groups where, typically, a single male and several females will break-off to spawn. In such species, whether an individual will undertake protogynous sex change likely depends on attaining a threshold age and/or size, and the sex-ratio and population density of the spawning group (Bhandari, Higa, Nakamura, & Nakamura, 2004; Shapiro, Sadovy, & McGehee, 1993).

2.1.2 Protandry

Protandry is rarer among the teleosts, occurring sporadically across six families. Protandry is usually associated with monogamous or random mating systems, where male territorial defense or intense sperm competition is absent (Munday, Buston, et al., 2006). Under such conditions protandry is adaptive because of the direct positive relationship between female fecundity and body size (Warner, 1975).

Protandrous sex change is socially controlled in monogamous anemonefish (*Amphiprion* and *Premnas* spp.) (Casas et al., 2016; Fricke & Fricke, 1977; Godwin, 1994; Hattori, 2012) (Fig. 1B). Anemonefish live in small social groups among sea anemones. Classically these consist 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 (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, Banh, Domingos, & Jerry, 2015). Australian barramundi (*Lates calcarifer*, Latidae) and gilthead seabream (*Sparus aurata*, Sparidae) both sexually mature as male before becoming female at an older age and larger size (Guiguen, Cauty, Fostier, Fuchs, & Jalabert, 1994; Liarte et al., 2007) and there are significant efforts ongoing to understand sex change in these species (Pauletto et al., 2018). However, most research on protandry has focussed on black porgy (*Acanthopagrus schlegeli*, Sparidae), which reproduce as male for the first 2 years of life before approximately 50% of fish change sex to female (Lee et al., 2001; Wu & Chang, 2013; Wu et al., 2010). Whether a threshold age and/or size also triggers sex inversion in protandrous group-spawning species remains unclear (Guiguen et al., 1994).

2.1.3 Bidirectional sex change

Bidirectional hermaphrodites have the capacity for sex change in either direction, potentially repeatedly during their lifetime. Bidirectional hermaphroditism is known to occur in 10 species spanning five families (Kuwamura, Kadota, & Suzuki, 2015; Manabe et al., 2013; Munday, Kuwamura, & Kroon, 2010). True serial sex change is observed in the monogamous coral-dwelling gobies (Fig. 1C) (e.g., *Gobiodon* and *Paragobiodon*). Sex change in gobies is poorly explained by the SAM—the popular explanation is that bidirectional sex change provides reproductive assurance for these fish in the face of niche specialization and a sessile lifestyle (Munday, Caley, & Jones, 1998; Nakashima, Kuwamura, & Yogo, 2010). Coral gobies experience limited mating opportunities and experience significant risks if moving between spatially isolated coral colonies. Thus, the ability to repeatedly change sex, in either direction, allows any two fish to form a heterosexual breeding pair, reducing both the effort to find a mate and the associated predation risk (Munday et al., 2010).

3. The anatomy of sex change

Detailed histological descriptions of gonadal sex change have been made for representative protandrous (Godwin, 1994; Guiguen et al., 1994; Lee et al., 2001; Wu et al., 2010), protogynous (Lo Nostro, Grier, Andreone, & Guerrero, 2003; Muncaster, Norberg, & Andersson, 2013; Nakamura, Hourigan, Yamauchi, Nagahama, & Grau, 1989) and bidirectional (Cole, 2011; Kuwamura et al., 2015; Sunobe, Nakamura, Kobayashi, Kobayashi, & Nagahama, 2005) sequential hermaphrodites.

In all cases, the process involves radical restructuring of the gonad, transforming a functional gonad of one sexual phenotype into that of the opposite sex. However, sex change follows diverse ontogenetic pathways in different sequentially hermaphroditic lineages (Kobayashi et al., 2013; Liu et al., 2017; Todd, Liu, et al., 2016; Warner & Robertson, 1978) reflecting its multiple independent evolutionary origins (Avise & Mank, 2009; Erisman et al., 2013; Sadovy de Mitcheson & Liu, 2008).

Gonadal restructuring is complete in protogynous wrasses; no testicular tissues are detectable in the ovary before sex change, and only a remnant ovarian lumen and lamellae structure is retained in the secondary testes following sex change (Bhandari et al., 2004; Muncaster et al., 2013; Warner & Robertson, 1978). Protogyny has a common evolutionary origin in wrasse and the key histological events during gonadal sex change are broadly comparable across species (Nakamura et al., 1989). The onset of sex change is heralded by ovarian follicle atresia and oocyte degeneration, followed by the proliferation of spermatogonia and Leydig cells in the peripheral ovarian lamellae before commencement of spermatogenesis characterizes the fully functional testis of a male fish capable of fertilizing eggs (Fig. 2A). This process can be completed rapidly. In tropical bluehead wrasse, which breed year-round and can be induced to change sex at almost any time point, the transformation can take just 8 days (Warner & Swearer, 1991). However, temperate species are slower taking several weeks or months to complete the process (Muncaster et al., 2013) and sex change is typically seasonal starting after spawning from a regressed ovary with only previtellogenic follicles (Bhandari et al., 2004; Jones, 1980; Muncaster et al., 2013).

Precisely where and from what gonadal cell type new testicular tissues derive in protogynous wrasse remains unresolved (Liu et al., 2017). However, in the protogynous seabass and grouper it is thought that crypts of resting spermatogenic tissues occur within the ovarian germinal epithelia prior to sex change (Bhandari et al., 2004; Sadovy de Mitcheson & Liu, 2008; Shapiro et al., 1993).

In other sequential hermaphrodites, sex change proceeds from a bisexual gonad, or "ovotestis" (Liu et al., 2017; Sadovy de Mitcheson & Liu, 2008; Todd, Liu, et al., 2016). In porgies (Sparidae), which include both protandrous and protogynous species, connective tissues strongly demarcate male and female regions in the ovotestis (Sadovy de Mitcheson & Liu, 2008). In the protandrous black porgy, the ovotestis is dominated by active testicular tissue during the first and second spawning seasons when fish are functionally male (Wu et al., 2010). At this time, the ovarian portion of the

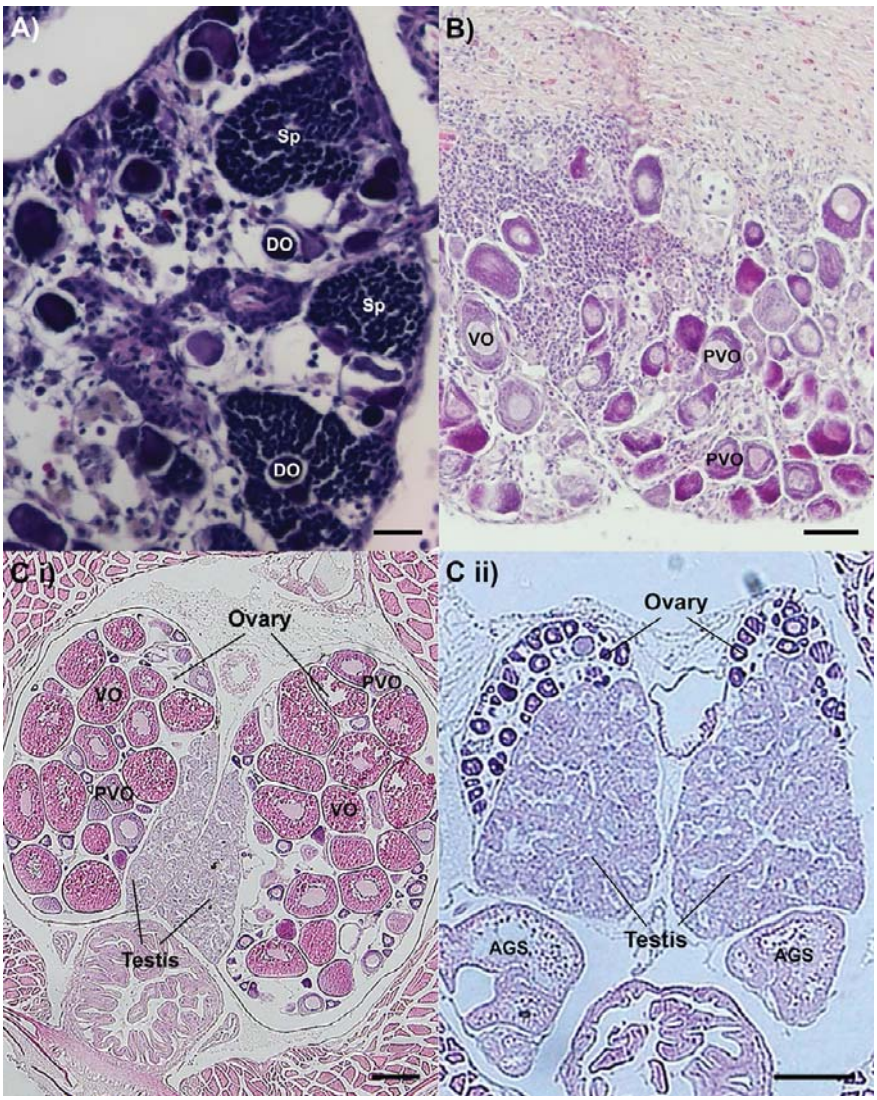

Fig. 2 Histological details of gonads from (A) protogynous, (B) protandrous and (C) bidirectional sex-changing fish. (A) A transitional bluehead wrasse (*Thalassoma bifasciatum*) gonad in the later stages of protogynous sex change (stage 5 of Nakamura et al., 1989), showing degenerating oocytes (DO) and crypts of proliferating spermatocytes (Sp). Scale bar: 20 µm. (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. (C) The paired ovotestis of a (i) female-phase and a (ii) male-phase individual of the bidirectional sex-changing goby, *Trimma kudoi*. In the female phase, the ovotestis consists of an enlarged ovarian portion containing many vitellogenic (VO) and previtellogenic (PVO) oocytes, and a small testicular portion. In the male phase, 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. *Panel A: Image author: Hui Liu. Panel B: Image author: Quyen Bahn. Panel C: Image authors: Tomoki Sunobe and Hisaya Manabe. Reused with permission from Todd, E. V., Liu, H., Muncaster, S., & Gemmell, N. J. (2016). Bending genders: The biology of natural sex change in fish.* Sexual Development, 10*, 223–241.*

ovotestis is small, containing oogonia and a few primary oocytes, but subsequently matures and expands during non-spawning (intersex) periods (Lee, Huang, & Chang, 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 those fish that change sex to female (Lee et al., 2008). Male anemonefish also possess a bisexual gonad that contains both active spermatogenic tissue and previtellogenic oocytes, but in this protandrous species the tissues, while topographically distinct, are not separated by boundary tissues (Godwin, 1994; Shapiro, 1992).

Bidirectional sex-changing gobies also possess an ovotestis that contains ovarian and testicular portions simultaneously (Fig. 2C), but either portion can be reproductively fully functional in a given season (Cole, 2011; Kuwamura et al., 2015; Sunobe et al., 2005). Maintaining a bisexual gonad affords these species enormous flexibility to rapidly adjust their sexual phenotype and gain maximum benefit from reproductive opportunity (Sunobe et al., 2005). Sequential hermaphroditism has multiple evolutionary origins in gobiid fishes and gonadal configurations show substantial taxon-specific variation; from the undelimited ovotestis of *Eviota* gobies, where male and female cells are intermixed, to an increasingly complex and compartmentalized organ (as in *Lythrypnus*, *Trimma*, *Gobiodon* and *Paragobiodon* spp.) (Cole, 2010).

While sex change is an extraordinary phenotype, a bisexual gonadal (Orban et al., 2009), or an immature ovary (Devlin & Nagahama, 2002) is a common feature of the juvenile stage for many non-sex changing fish, before they develop fully as either males or females. In zebrafish the gonad initially develops as a bipotential organ, with a few primordial germ cells in all juveniles initially developing as oocytes (Orban et al., 2009). In some individuals oocytes survive leading to ovary formation, while in others the oocytes are eliminated leading to the formation of a testis (Rodríguez-Marí et al., 2010). Fancl, a member of the Fanconi anemia/BRCA DNA repair pathway appears to be critical to oocyte retention; *fancl* mutation leads to female-to-male sex reversal through an increase in Tp53-mediated germ cell apoptosis (Rodríguez-Marí et al., 2010).

That germ cells of both sexes coexist at least transiently in the gonad of many gonochoristic fishes, and that sexual fate can be altered through the knock out of a single gene (Rodríguez-Marí et al., 2010) implies considerable evolutionary scope for plasticity in reproductive mode (Todd, Liu, et al., 2016). Given this potential for plasticity a reasonable question is why more species do not change sex naturally?

4. The possible cellular origins of gonadal restructuring in protogynous species

Precisely from where and from what gonadal cell type new gonadal tissues derive remains unresolved for species where no germinal tissues of the secondary sex are discernible prior to sex change (Nakamura et al., 1989). For most protogynous species, no testicular tissues can be found in the ovaries before sex change and very few ovarian tissues remain in the secondary testes after sex change (Muncaster et al., 2013; Nakamura et al., 1989; Nozu, Kojima, & Nakamura, 2009). Tracing cellular origins and fates during gonadal sex change is complicated because correctly identifying and staging germ cells in these sexually plastic species is difficult when based solely on histology (Lo Nostro et al., 2003). In protogynous hermaphrodites, early male tissues are often first observed at the periphery of the ovarian lamellae in the vicinity of the germinal epithelium (Lo Nostro et al., 2003). However, establishing whether proliferating spermatogonia arise from a dormant, but sexually differentiated, germ cell population (i.e., co-existence of oogonia and spermatogonia) or from a population of latent sexually bipotent primordial germ cells is hard to establish (Lo Nostro et al., 2003).

In protogynous species, it is currently assumed that testicular construction begins with bipotential germ (gonia) and somatic (epithelial) cells residing within the ovarian germinal epithelium (Lo Nostro et al., 2003; Nakamura et al., 1989; Nozu et al., 2009). Gonial cells clearly proliferate and differentiate into spermatocytes during later stages of sex change, yet it remains to be confirmed whether these are oogonia, spermatogonia or undifferentiated stem cells (Liu et al., 2017). However, the bipotentiality of transplanted spermatogonia and oogonia in rainbow trout (Okutsu, Suzuki, Takeuchi, Takeuchi, & Yoshizaki, 2006; Yoshizaki et al., 2010), a strictly gonochoristic species with an XY sex-determining system, indicates that gonial cells routinely retain sexual plasticity, and are reprogrammable depending on the somatic microenvironment (Todd, Liu, et al., 2016; Yoshizaki et al., 2010).

The sources of the somatic cells that cause testicular restructuring are equally obscure. The somatic cells surrounding the gonial cells near the periphery of ovarian lamellae appear a likely source of presumed Sertoli cells because *gsdf* (gonadal somatic derived factor), a genetic marker involved in testicular differentiation, has been detected in these supporting cells at low levels prior to sex change (Shibata et al., 2010). Furthermore, during ovarian atresia, all vitellogenic and previtellogenic oocytes degenerate, but relatively few follicle cells appear to go through apoptosis (Nozu, Horiguchi, Murata, Kobayashi, &

Nakamura, 2013). Instead the near stages of sex change are characterized by the proliferation of the granulosa cells that surround and support the degenerating oocyte (Nozu et al., 2013). These remaining follicle layers also show positive signals of *cyp11b2* (11β-hydroxylase) a key enzyme responsible for synthesis of 11-ketotestosterone (11-KT) the key male sex steroid in fish (Alam et al., 2006; Li et al., 2013). Whether these cells can be reprogrammed into Sertoli or Leydig cells (steroidogenic cells in testes) to form testicular tubules warrants further investigation (Liu et al., 2017).

Following ovarian atresia, the space left by degenerated oocytes is invaded by the remaining follicle cells and vascularized connective tissues, which appear to give rise to the yellow-brown bodies and cell mass in the central areas of ovarian lamellae (Muncaster et al., 2013; Nakamura et al., 1989). Various types of cells are observed in this cell mass, including macrophages that clean up the degenerating oocytes and connective tissues that form testicular lobules (Muncaster et al., 2013; Nakamura et al., 1989). At this stage, Leydig cells cluster and proliferate around loose connective tissues (Nakamura et al., 1989). The origin of these Leydig cells is not clear, but a likely source is the inner layer of the gonadal wall, which becomes thicker with active cell proliferation as gonadal sex change proceeds (Liu et al., 2017). In the testis of bluehead wrasse this layer contains all somatic cell types, is continuous with the interstitial tissue layer, and may contribute to gonadal restructuring by cell invasion or exchange (Koulish, Kramer, & Grier, 2002). Studies in honeycomb grouper also show that androgen-producing cells form clusters in the inner layer of the gonadal wall near blood vessels of both ovaries and testes as well as transitioning gonads (Alam et al., 2006; Nakamura et al., 2005). Whether and how these cells are reprogrammed or relocated to form Leydig cells during the gonadal transition remains unknown (Liu et al., 2017).

The lack of detailed knowledge about cellular origins and fates in transitioning or sex changing gonads remains a major gap in our understanding of sexual differentiation and sex change in fishes. While tracking cell fates remains challenging, it should become increasingly more feasible with the continued development of cellular markers for fishes, likely strongly empowered by comparative transcriptomic studies in sex-changing species (Liu et al., 2015; Todd, Black, & Gemmell, 2016; Todd et al., 2018) and new single-cell approaches (Stévant et al., 2018). Understanding how the developmental bipotentiality of gonial and somatic cell populations is retained into adulthood is important not only for understanding sex change in sequential hermaphrodites, but also the evolvability of sexual plasticity in teleosts generally (Todd, Liu, et al., 2016).

5. The endocrine regulation of sex change

Sexual differentiation and gonadal development are directed by the balance between gonadal oestrogen and androgen production. In teleosts, 11-ketotestosterone (11-KT) and 17β-estradiol (E2) are the principal androgen and oestrogen that respectively promote testicular and ovarian function (Fig. 3). The relationship between them is especially close, as production of either 11-KT or E2 depends on the bioconversion of testosterone (T) via the 11β-hydroxylase (*cyp11b*)/11β-hydroxysteroid dehydrogenase (11β-HSD, coded by *hsd11b*) and aromatase (*cyp19a1a*) pathways (see Guiguen et al., 2010). The relative expression of these opposing pathways in the gonad determines the sex steroid balance and ultimately decides gonadal fate (Guiguen et al., 2010; Liu et al., 2017; Todd, Liu, et al., 2016).

In sex-changing fishes, dramatic shifts in plasma sex steroids accompany gonadal transition (Devlin & Nagahama, 2002; Godwin, 2010) (Fig. 3),

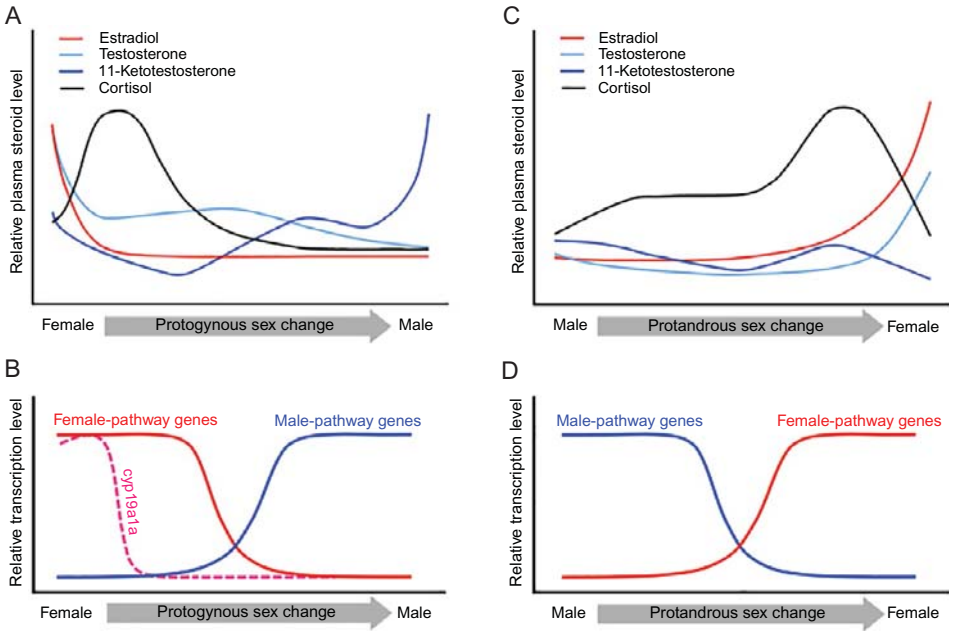

Fig. 3 Shifts in steroid profiles and sex-specific gene expression accompany sex change in each direction. During protogynous sex change, (A) steroid profiles shift from an oestrogenic to an androgenic environment. (B) Following shutdown of the aromatase gene (*Cyp19a1a*), female-specific gene expression is down-regulated before expression profiles become increasingly male-biased. The opposite pattern is observed during protandrous sex change: (C) there is transition from an androgenic to an oestrogenic environment and (D) from male-specific to female-specific expression. *Adapted with Permission from Todd, E. V., Liu, H., Muncaster, S., & Gemmell, N. J. (2016). Bending genders: The biology of natural sex change in fish.* Sexual Development, 10, *223–241.*

yet what tips the balance in favor of the opposing hormonal environment remains unclear. A sharp drop in plasma E2 heralds ovarian degeneration and protogynous sex change, which leads onto a gradual increase in 11-KT production at the onset of spermatogenesis (Bhandari et al., 2006; Bhandari, Komuro, Nakamura, Higa, & Nakamura, 2003; Muncaster et al., 2013; Nakamura et al., 1989; Ohta, Mine, Yamaguchi, & Matsuyama, 2008). The opposite pattern characterizes protandrous sex change; 11-KT levels fall and subsequently plasma E2 increases (Godwin & Thomas, 1993; Lee et al., 2001). In bidirectionally sex changing gobies, E2 levels follow this sexually dimorphic pattern, but there is no apparent association between 11-KT concentrations and gonadal development (Kroon, Munday, & Pankhurst, 2003; Kroon, Munday, & Westcott, 2009; Lorenzi, Earley, & Grober, 2012). The role of 11-KT in gobies is unclear, but routinely low 11-KT levels may reflect the lack of secondary male characteristics in gobies and enable rapid switching between sexual phenotypes (Godwin, 2010; Kroon et al., 2009).

Exogenous manipulation of sex steroids can be used to induce masculinization or feminization in fish (e.g., Chang, Lau, & Lin, 1995; Higa, Ogasawara, Sakaguchi, Nagahama, & Nakamura, 2003; Yeh, Kuo, Ting, & Chang, 2003); reviewed by (Baroiller & D'Cotta, 2016; Budd et al., 2015; Devlin & Nagahama, 2002; Lee et al., 2017), but in some systems this is only successful when steroids are administered during the "sex determination" period, when the gonadal fate remains labile (Brion et al., 2004). Application of non-aromatizable androgens down-regulates the aromatase pathway in female fish leading to sex change (Bhandari et al., 2006; Govoroun et al., 2001; Li, Liu, Zhang, & Lin, 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, Miura, Nozu, & Kobayashi, 2015) and bidirectional hermaphrodites (Kroon, Munday, Westcott, Hobbs, & Liley, 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, Li, Luo, Chen, & Chang, 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.

6. Neuroendocrine regulation of sex change

The neuroendocrine regulation of sex change has been most intensively studied in protogynous wrasses that display socially-controlled sex change (Lamm et al., 2015). In these species, behavioral changes frequently

precede gonadal sex change and appear to be independent of the gonad (Godwin, Crews, & Warner, 1996; Nakamura et al., 1989; Warner & Swearer, 1991). The established view is that changes in the brain drive both behavioral and gonadal sex change, although through different neuroendocrine pathways (Godwin et al., 1996; Lamm et al., 2015) (Fig. 4). Cross-talk between these pathways and interactions among the neurochemical signals that mediate them likely coordinate the behavioral and physiological responses during sex change.

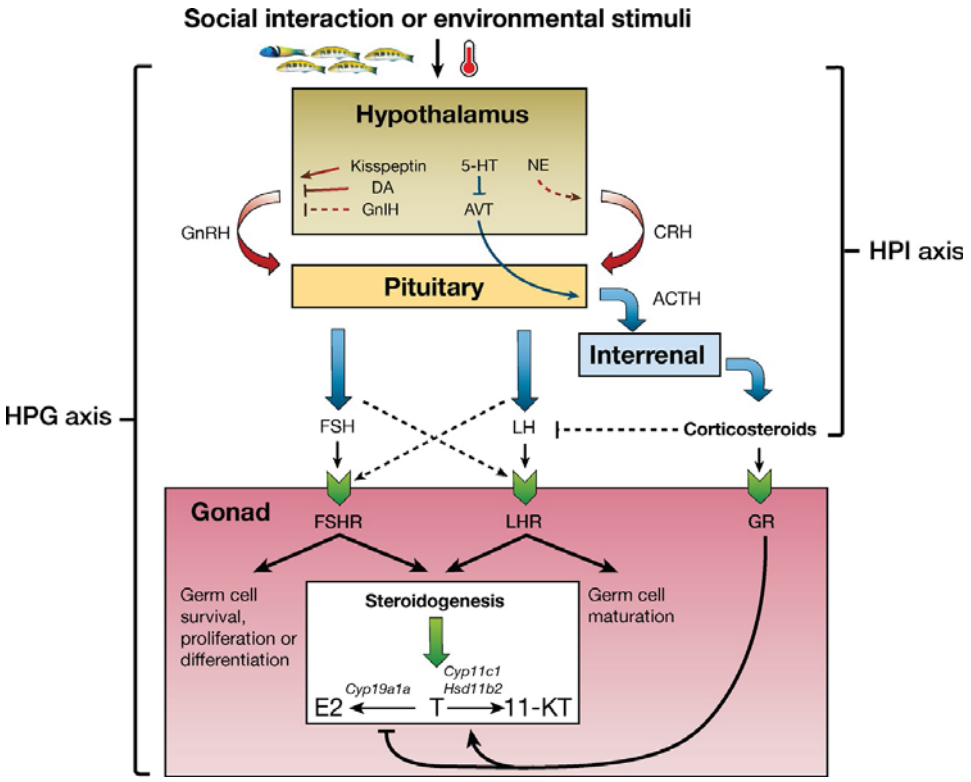

Fig. 4 Schematic illustration of neuroendocrine cross-talk between the HPG and HPI axes regulating steroidogenesis and behavior in teleosts. Solid lines indicate interactions with support from fish models; dashed lines indicate interactions with support from non-teleost systems. Abbreviations: ACTH, adrenocorticotropic hormone; AVT, arginine vasotocin; CRH, corticotropin-releasing hormone; DA, dopamine; E2, 17beta-estradiol; GnIH, gonadotropin inhibitory hormone; GnRH, gonadotropin releasing hormone; FSH, follicle-stimulating hormone; FSHR, FSH receptor; LH, luteinizing hormone; LHR, LH receptor; NE, norepinephrine; T, testosterone; 5-HT, serotonin; 11-KT, 11-ketotestosterone. *Adapted with permission from Todd, E. V., Liu, H., Muncaster, S., & Gemmell, N. J. (2016). Bending genders: The biology of natural sex change in fish.* Sexual Development, 10, *223–241.*

The oestrogen-androgen balance, and thus gonadal fate, is ultimately regulated through the Hypothalamic-Pituitary-Gonadal (HPG) axis and its interaction with the neighboring stress axis (Fig. 4). Gonadotropin releasing hormone (GnRH) is released in pulses from the hypothalamus, stimulating the pituitary to produce and release gonadotropins (GtHs), follicle stimulating hormone (FSH) and luteinizing hormone (LH), into the circulation. GtHs directly regulate gonadal steroidogenesis via receptor-mediated stimulation of ovarian follicle cells or somatic Leydig cells in the testis (reviewed by Devlin & Nagahama, 2002; Weltzien, Andersson, Andersen, Shalchian-Tabrizi, & Norberg, 2004).

The expression of GtH subunits and their receptors (LHR, FSHR) alter during sex change in protogynous (Kobayashi, Alam, Horiguchi, Shimizu, & Nakamura, 2010), protandrous (An, Lee, & Choi, 2010; An, Lee, Yun, & Choi, 2009) and bidirectional (Kobayashi et al., 2009) species. Direct manipulation of GnRH or GtH signaling induces partial or complete sex change in protogynous (Kobayashi, Alam, et al., 2010; Reinboth & Brusle-Sicard, 1997) and protandrous hermaphrodites (see Lee et al., 2001). However, the patterns of GnRH and GtH signaling across sex change are inconsistent even among closely-related species (e.g., contradictory patterns of expression for GtH receptors are observed in protogynous groupers (Alam, Kobayashi, Hirai, & Nakamura, 2010; Hu et al., 2011) making it hard to understand the precise roles of GnRH and GtH signaling in controlling sex change. One possibility is that this variability reflects species-specific gonadotropin functioning in teleosts (see Levavi-Sivan, Bogerd, Mananos, Gomez, & Lareyre, 2010).

The perception and processing of external cues into the coordinated physiological response that characterizes sex change remain poorly understood (reviewed by Lamm et al., 2015; Liu et al., 2017). In bluehead wrasse, rapid neurochemical changes in the brain drive behavioral sex change (Godwin, 2010; Lamm et al., 2015), which precedes by several days the gonadal restructuring coordinated via the HPG axis (Larson, Norris, Gordon Grau, & Summers, 2003; Larson, Norris, & Summers, 2003; Semsar & Godwin, 2003); reviewed by (Godwin & Thompson, 2012; Lamm et al., 2015). It seems probable that cross-talk among distinct, but likely overlapping, neuroendocrine pathways mediates the effects of the social environment on gonadal state to coordinate sex change at the whole-body level (Lamm et al., 2015).

Removing a TP male from its social group triggers dramatic neuroendocrine changes in the brain of large females that stimulates behavioral sex

change (Godwin, 2010; Lamm et al., 2015). These neuroendocrine changes include fluctuations in neurochemicals known to modulate social rank and sexually-dimorphic reproductive behaviors (Godwin, Sawby, Warner, Crews, & Grober, 2000; Godwin & Thompson, 2012; Lamm et al., 2015; Semsar, Perreault, & Godwin, 2004) which are known to elicit rapid behavioral and reproductive changes in response to environmental and/or physiological signals (Godwin & Thompson, 2012; Lamm et al., 2015; Liu et al., 2017; Maruska & Fernald, 2013; Zohar, Muñoz-Cueto, Elizur, & Kah, 2010).

Norepinephrine (NE) and arginine vasotocin (AVT) stimulate behavioral sex change, while dopamine (DA) and serotonin (5-HT) have inhibitory effects (Kramer, Caddell, & Bubenheimer-Livolsi, 1993; Larson, Norris, Gordon Grau, et al., 2003; Larson, Norris, & Summers, 2003; Perreault, Semsar, & Godwin, 2003; Semsar & Godwin, 2003, 2004; Semsar, Kandel, & Godwin, 2001). AVT appears especially important in promoting male-typical behaviors, such as courtship and aggression, in protogynous wrasses (see Godwin & Thompson, 2012; Semsar et al., 2001, 2004). However, methodological differences across studies and species-specific variation highlight the need for further research in a variety of species exhibiting different sex change strategies.

Kisspeptins and isotocin (IT) are also likely regulators of early sex change. Kisspeptins regulate vertebrate reproduction by stimulating GnRH release (Elizur, 2009; Espigares, Zanuy, & Gómez, 2015; Mechaly, Vinas, & Piferrer, 2013), but may also mediate transitions in social status (see Maruska & Fernald, 2011) via their receptors on AVT and IT neurons (Kanda, Akazome, Mitani, Okubo, & Oka, 2013). Isotocin, the teleost homolog of mammalian oxytocin, is strongly associated with social status and sex-specific reproductive behaviors across a range of species (Goodson & Bass, 2000; Lema, Sanders, & Walti, 2015). These hormones are of interest, as social rank may serve as a primer for sex change in haremic breeders (Lamm et al., 2015; Liu et al., 2017; Todd, Liu, et al., 2016). In a recent transcriptomic 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 et al., 2017). Further work exploring the transcriptomic differences between TP males, IP males and females found that IT expression was characteristic of the TP male phenotype and expressed specifically in the preoptic area of the hypothalamus (POA), a primary site for socio-sexual behavioral integration in vertebrates (Todd et al., 2018). However, bidirectionally sex changing gobies show the opposite pattern; males and late-stage sex-changers

show lower IT activity in the POA compared with females (Black, Reavis, & Grober, 2004). To date, fluctuations in the expression of kisspeptin and its receptors (kiss2, kiss1r) across sex change are known only for orange spotted grouper, *Epinephelus coioides* (Shi et al., 2010). Recent transcriptome analyses have, somewhat unexpectedly, not uncovered significant expression changes for these and other candidate neuroregulatory genes during sex change (Casas et al., 2016; Liu et al., 2017; Pauletto et al., 2018). Such studies have sampled at either a whole brain or forebrain level, and so may fail to pick up subtle and/or localized expression changes. The social-rank hypothesis, and a potential regulatory role for kisspeptins and IT in socially controlled sex change, warrants further research. Future work should employ more fine-scale sampling of specific brain regions, of which the POA should be a primary target.

Gonadotropin-inhibitory-hormone (GnIH) a key regulator of the HPG axis, may also have a role in sex change, but as yet is poorly studied. GnIH is known to inhibit GnRH and LH release in birds (Ubuka et al., 2014) and has been reported to reduce serum LH levels in goldfish, although the effects on GtH synthesis and release are stage specific and complicated (Moussavi, Wlasichuk, Chang, & Habibi, 2013; Qi et al., 2013). In protogynous groupers, opposite expression patterns of *gnih* (Wang et al., 2015) and *kiss2* (Shi et al., 2010) were observed in the hypothalamus following MT-induced sex change.

Neurochemical regulation of sex change in protandrous and bidirectional species, and species where sex change is not under social control, remains largely uninvestigated. Targeted manipulations of these neurohormones in the brain are required to clarify their roles in sex change.

7. The stress response may mediate sex change

The stress response modulates many processes central to major life-history transitions, including changes in behavior, metabolism and growth (Goikoetxea, Todd, & Gemmell, 2017; Solomon-Lane, Crespi, & Grober, 2013; Todd, Liu, et al., 2016). The hypothalamic-pituitary-interrenal (HPI) axis (Fig. 4), a major part of the neuroendocrine system, controls reactions to stress through the actions of corticotropic releasing hormone (CRH) and glucocorticoid steroids (GCs) (Goikoetxea et al., 2017; Liu et al., 2017; Todd, Liu, et al., 2016). Cortisol, the main glucocorticoid in fish and the hormone most directly associated with stress, may act as a key factor linking social environmental stimuli and the onset of sex change by initiating a shift

in steroidogenesis from estrogens to androgens (Goikoetxea et al., 2017). That cortisol apparently also mediates agonistic behavior and reflects social status, strongly implicates the HPI axis in socially-induced sex change (Solomon-Lane et al., 2013).

In a variety of gonochoristic fishes, such as pejerrey or Japanese flounder, elevated temperatures and other environmental stressors result in increased cortisol levels and the down-regulation of aromatase, which leads to the activation of androgen pathways and gonadal masculinization (Fernandino, Hattori, Moreno Acosta, Strussmann, & Somoza, 2013; Goikoetxea et al., 2017; Hattori et al., 2009; Hayashi et al., 2010; Yamaguchi, Yoshinaga, Yazawa, Gen, & Kitano, 2010). Cortisol likely stimulates 11β-HSD expression, resulting in the production of both 11-KT and cortisone, the deactivated metabolite of cortisol (Fernandino et al., 2013; Fernandino, Hattori, Kishii, Strussmann, & Somoza, 2012). Thus, through its influence on steroidogenic gene expression and by communicating environmental and social status information along the HPI axis, cortisol may regulate natural sex change in sequential hermaphrodites (Goikoetxea et al., 2017; Solomon-Lane et al., 2013). Studies in several sequentially hermaphroditic species suggest cortisol production fluctuates across sex change. A transient spike in serum cortisol was observed in protandrous cinnamon clownfish undergoing sex change (Godwin & Thomas, 1993). Bluebanded gobies, bidirectional hermaphrodites, experiencing a "permissive" social environment showed a spike in serum cortisol as they transitioned from female to male (Solomon-Lane et al., 2013). Sustained cortisol administration promoted protogynous sex change in three-spot wrasse (Nozu & Nakamura, 2015).

The exact ways in which cortisol may act as a mediator in the sex change process remains unclear. Goikoetxea et al. (2017) considered three non-exclusive mechanisms through which cortisol might trigger female to male sex change: (i) cross-talk between the corticosteroid and androgen pathways, (ii) the inhibition of aromatase expression through the binding of cortisol to the glucocorticoid response elements (GRE) in the *cyp19a1a* promoter, and (iii) the depletion of primordial germ cells (PGCs) via upregulation of *amh* (the gene encoding anti-Müllerian hormone) (Fernandino et al., 2013; Goikoetxea et al., 2017; Pfennig, Standke, & Gutzeit, 2015; Todd, Liu, et al., 2016).

Transcriptome sequencing in bluehead wrasse strongly supports a role for cortisol in sex change. Liu (2016) observed early upregulation of *cyp11c1*, responsible for cortisol production, concurrent with inhibition of *cyp19a1a* and activation of *amh*, inhibition of *cyp19a1a* and activation

of *amh*, followed by rapid upregulation during mid-sex change of *hsd11b2*, which encodes the enzyme responsible for metabolizing cortisol to inactive cortisone and downregulation of *hsd11b1a* which converts cortisone to cortisol.

Recent evidence in other vertebrates suggests stress may play a broader role in sexual fate transitions. In the bearded dragon, sex-reversed females could be distinguished transcriptionally from normal chromosomal females and males by upregulation of the proopiomelanocortin (POMC)-mediated environmental stress response and the preferential retention of introns from each of two *Jumonji* family genes, JARID2 and JMJD3, that are important epigenetic regulators. The working hypothesis is that high-temperature induced stress, presumably mediated by cortisol, results in the retention of intron sequences containing premature stop codons that alter or abolish epigenetic regulation, thereby over-riding chromosomally determined sexual fates (Deveson et al., 2017). This work, and other studies linking thermal stress with sex reversal (Navarro-Martín et al., 2011; Ribas et al., 2017; Shao et al., 2014), suggest that cortisol may be a long-sought-after mediator translating environmental signals into downstream genetic and physiological responses resulting in sex change (Goikoetxea et al., 2017). The possible role of cortisol as a proximate regulator of natural sex change warrants further attention (Goikoetxea et al., 2017).

8. The molecular regulation of sex change

Sexual fate appears increasing less an irreversible developmental commitment made during early embryo development, and more an ongoing tug-of-war for primacy between competing male and female developmental trajectories (Fig. 5). Nowhere among the vertebrates is this battle for primacy and its effect on sexual phenotype more obvious that in fish (Capel, 2017; Liu et al., 2017; Todd, Liu, et al., 2016).

Multiple genes have been shown to act as master sex-determining genes in fish and other vertebrates (Capel, 2017). While these sex-determining switches vary, a common set of downstream effectors act antagonistically in feminizing (e.g., *cyp19a1a*, *foxl2*, *wnt4*) and masculinizing (e.g., *dmrt1*, *amh*, *sox9*) networks to, respectively, promote ovarian or testicular development (Munger & Capel, 2012). Consequently, sexual fate is not simply dependent on the activation of one or other sex-specific network, but also contingent on the suppression of the opposing network to maintain that fate throughout life (Herpin & Schartl, 2011a). This dynamic antagonism

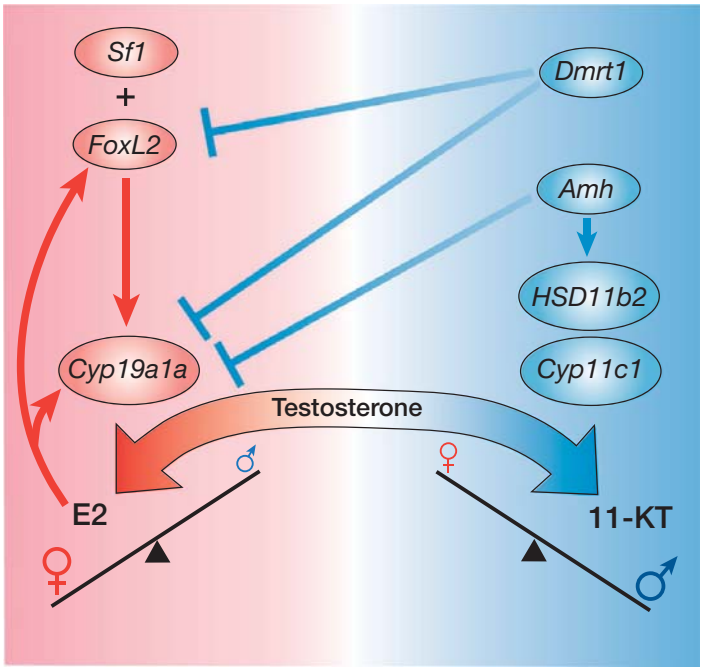

Fig. 5 Antagonistic sex-specific gene networks maintain sexual fate in fishes by promoting either an oestrogenic (left) or androgenic (right) environment. In females, *cyp19a1a* expression produces aromatase, which converts testosterone to estradiol (E2) and maintains the auto-regulatory feed-forward loop sustaining the high oestrogen levels that support ovarian function (Guiguen et al., 2010). Within this loop, transcription factors Foxl2 and Sf1 interact to up-regulate *cyp19a1a* expression (Wang et al., 2007), which also is 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, *cyp19a1a* expression and aromatase production is suppressed and *hsd11b2* and *cyp11c1* promote production of 11KT; such that androgenesis prevails supporting testicular function and male-specific gene expression. Inhibitory and activating effects of androgens on *Amh* are reported (+/−) (Pfennig et al., 2015). Dmrt1 suppresses *cyp19a1a* promoter activity directly (Wang et al., 2010) and indirectly via its antagonistic relationship with *foxl2* (see text). *Adapted with permission from Todd, E. V., Liu, H., Muncaster, S., & Gemmell, N. J. (2016). Bending genders: The biology of natural sex change in fish.* Sexual Development, 10, *223–241.*

makes sexual fate highly susceptible to manipulation by factors that interrupt the supremacy of the prevailing sexual network, enabling that of the opposing sex to gain dominance. The factors that might interrupt sex-specific networks are predominantly undetermined, but the actions of cortisol on epigenetic regulators, may be central to their regulation by environmental and physiological cues in sex-changing fish (Lamm et al., 2015; Liu et al., 2017; Todd, Liu, et al., 2016).

Sex change has evolved repeatedly in teleosts (Avise & Mank, 2009), but the molecular mechanism that triggers sex change remains unknown for any fish species (Todd, Liu, et al., 2016). In fishes, two widely conserved components of the molecular machinery essential for vertebrate sexual development, *cyp19a1a/b* (coding for gonadal/brain aromatase that catalyses conversion of androgens to oestrogens) and *dmrt1* (doublesex and mab-3 related transcription factor 1), are each vital to female- and male-promoting networks, respectively (Fig. 5). In females, *cyp19a1a* expression sustains an auto-regulatory loop that maintains the high oestrogen environment critical for ovarian function (Guiguen et al., 2010). In males, *dmrt1*, a critical transcriptional regulator, activates male-promoting genes (e.g., *sox9*, *sox8*) and suppresses ovarian pathways (e.g., *foxl2* and *rspo1/wnt/β-catenin*) (Herpin & Schartl, 2011a, 2011b).

In mice, under- or over-expression of either *dmrt1* or *foxl2* induces reprogramming of sexual cell fate and gonadal sex reversal (Lindeman et al., 2015; Matson et al., 2011; Uhlenhaut et al., 2009). Functional analyses, employing natural mutations, knockdown or genome editing of *dmrt1* have demonstrated its critical role in male development of various fish including medaka (Nanda et al., 2002), black porgy (Wu et al., 2012) sole (Cui et al., 2017). Impairing *dmrt1* expression alters *foxl2* or *cyp19a1* expression and leads the male gonad to develop as an ovotestis (Cui et al., 2017; Wu et al., 2012). Mutation of either *foxl2* or *cyp19a1* in Nile Tilapia (*Oreochromis niloticus*) resulted in female to male sex reversal, demonstrating that *foxl2* promotes ovarian development by upregulating *cyp19a1a* expression and repressing male pathway gene expression (Zhang et al., 2017). These, and other data, suggest that d*mrt1* interacts antagonistically with the female-specific transcription factor *foxl2* to influence *cyp19a1a* expression to control oestrogen production and the sexual fate of the gonad (Cui et al., 2017; Guiguen et al., 2010; Herpin & Schartl, 2011a; Kobayashi et al., 2013; Li et al., 2013; Zhang et al., 2017).

Thus, regulation of the androgen/oestrogen balance that determines sexual fate in fish seems to be governed predominantly by *dmrt1, foxl2* and *cyp19a1a* (Fig. 5). *E*xpression of these genes is consistently sex-specific depending on which gonadal phenotype is developing, in both gonochoristic and sex-changing fishes (Alam, Kobayashi, Horiguchi, Hirai, & Nakamura, 2008; Liu et al., 2015; Wu, Tomy, Nakamura, & Chang, 2008; Xia, Zhou, Yao, Li, & Gui, 2007). The expression pattern of these genes also changes predictably in response to hormonal manipulations: *cyp19a1a/foxl2* is downregulated and *dmrt1* upregulated in fish treated with androgens,

oestrogen antagonists or aromatase blockers, while treatment with oestrogen shows the opposite effect (Guiguen et al., 2010; Herpin & Schartl, 2011b).

Evidence that the expression patterns of a variety of genes linked to sexual fate (*cyp19a1a*, *dmrt1* and *amh*) alter in response to external environmental fluctuations, such as temperature and density (Fernandino et al., 2008; Guiguen et al., 2010; Pfennig et al., 2015; Ribas et al., 2017), raises the possibility that these and other key sex genes may be similarly sensitive to environmental cues that initiate sex change in sequential hermaphrodites (Todd, Liu, et al., 2016).

Investigations into the molecular control of sex change in sequential hermaphrodites, have predominantly focused on candidate gene approaches (Liu et al., 2017; Todd, Liu, et al., 2016). Extensive investigations into the patterns of expression for a core suite of genes in the vertebrate sex determination and differentiation cascade (e.g., *amh*, *foxl2*, *dmrt1*, *cyp19a1a*) and their likely proximate regulators have been undertaken in protogynous wrasse and grouper (Alam et al., 2008; Horiguchi et al., 2013; Kobayashi, Horiguchi, Nozu, & Nakamura, 2010; Li et al., 2006; Liu, Guiguen, & Liu, 2008; Miyake, Sakai, & Kuniyoshi, 2012; Nozu, Horiguchi, Kobayashi, & Nakamura, 2015; Xia et al., 2007), and protandrous porgy and clownfish (Nakamura et al., 2015; Wu et al., 2012, 2015; Wu & Chang, 2009).

From prior work in other vertebrates, there are a variety of promising candidates through which sex change could conceivably be initiated and controlled (Capel, 2017). However, to date the expression patterns observed for genes proposed as key initiators (e.g., *foxl2* and *dmrt1*) do not readily fit the pattern of natural sex change observed in those species studied. In protogynous groupers, gonadal *foxl2* expression decreases while *dmrt1* expression increases during natural sex change (Alam et al., 2008). However, the shifts in *foxl2* and *dmrt1* expression emerge only during the late transition stage (Alam et al., 2008), well downstream of the decline in E2 levels (Bhandari et al., 2003). More surprisingly, there was no sex-bias in gonadal expression of *foxl2* in protogynous three-spot wrasses; *foxl2* expression actually increased during sex change induced using aromatase inhibitor (Kobayashi, Horiguchi, et al., 2010). In bluehead wrasses, *foxl2* expression was much higher in ovaries than testes (Liu et al., 2015), but its expression peaked during mid-late sex change leading us to suggest it is not an initiator of sex change (Liu et al., 2017). Similarly, *dmrt1* expression during protogynous sex change occurs too late for this to be a key initiator of the process; rather its expression pattern seems to be driven by decreasing

E2 levels (Kobayashi, Nozu, Horiguchi, & Nakamura, 2014; Liu et al., 2007). Taken together, *foxl2* and *dmrt1* may be more important for progressing, as opposed to initiating gonadal sex change in protogynous fishes (Liu et al., 2017).

More recently, whole-transcriptome expression analysis has been used to understand sex differences in protogynous (Liu et al., 2015; Todd et al., 2018; Tsakogiannis et al., 2018) and protandrous systems (Casas et al., 2016). Two studies have also begun to explore how the transcriptome-wide expression landscape alters across natural sex change, in the brains and gonads of protogynous bluehead wrasse (Liu et al., 2017) and in clownfish (Casas et al., 2016). Collectively, these studies have identified several genes and regulatory factors that may be important orchestrators, and possible initiators, of natural sex change in sequential hermaphrodites. These studies are also shedding new light on how the balance between masculinizing and feminizing gene networks is altered to enable sex change in fishes (Todd, Liu, et al., 2016).

Whole-transcriptome expression profiling in bluehead wrasse (Liu et al., 2017) shows female-related gene expression gradually declines in the gonad (e.g., *dax1*, *figla*, *sox3*, *gdf9*, *hsd7b1*, *hsd11b3*) before the expression profiles become increasingly masculinized (e.g., *dmrt1*, *gsdf*, *cyp11c1*, *sox9*) (Fig. 3A). Candidate gene studies in protandrous black porgy (Wu & Chang, 2013) show the opposite pattern: male-related gene expression (e.g., *dmrt1*, *amh, amh2r*) declines coincidentally with testis volume, before expression profiles are increasingly feminized as the bisexual gonad becomes purely ovarian (e.g., *cyp19a1a*, *foxl2*, *wnt4*). Transcriptomic analyses in clownfish across sex change (Casas et al., 2016) also identified a steady decline in male-related genes (*dmrt1*, *amh, sox8*) and upregulation of female-pathway genes (*cyp19a1a*, *foxl2, wnt4*) as the gonad transitioned from an ovotestis to an ovary. In bluehead wrasse, the greatest shift in sex-specific gene expression occurs during mid-to-late sex change (Fig. 3C). Thus, it appears that the prevailing sexual network must first be shut down, and that sex change progresses as the suppression of the opposing network is lifted (Todd, Liu, et al., 2016). Genes that show changes in expression immediately prior to this shift are particularly interesting as potential elements of the switch that initiates sex change. As of now, it is not known whether expression changes in wrasses are due to reprogramming of existing cellular populations, or reflect broad scale replacement of the cells of one sex, with latent progenitor cells of the other. Future single cell genomic studies may resolve this issue.

9. Aromatase appears to trigger gonadal sex change in protogynous species

The gonadal aromatase gene, *cyp19a1a*, stands out in transcriptome studies as the only gene among those commonly known to influence sexual differentiation that is rapidly and completely shut-down in early protogynous sex change (Liu et al., 2017; Todd, Liu, et al., 2016). In bluehead wrasse, *cyp19a1a* expression plummets from the first sign of ovarian atresia, prior to the collapse of the feminizing expression landscape (Liu et al., 2017) (Fig. 3A). *Wnt4a* and *sf1* expression drop off concurrently, but are not completely arrested. This sharp decline in *cyp19a1a* expression has been reported in prior candidate gene studies of multiple protogynous species (Li et al., 2006; Zhang, Zhang, Liu, Zhang, & Zhang, 2013) and is completely consistent with the halt in E2 production observed at this stage (Fig. 3A). It therefore seems likely that *cyp19a1a* downregulation is the switch needed to initiate the gonadal transformation observed during protogynous sex change. However, there must also be upstream factors that act to negatively regulate its expression and so are potential triggers of sex change.

The promoter regions of teleost *cyp19a1a/b* genes contain putative DNA-binding motifs for multiple transcription and endocrine factors that have known roles in vertebrate sex differentiation (Todd, Liu, et al., 2016) including: FOXL2, steroidogenic factor 1 (SF-1), 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) (Gardner, Anderson, Place, Dixon, & Elizur, 2005; Guiguen et al., 2010). Thus, *cyp19a1a* expression appears to be regulated, or influenced, by numerous factors. Evidence is accumulating for (and against) a role of several of these during sex change, with epigenetic modification to *cyp19a1a* promoters one mechanism through which its responsiveness to these factors can be altered (see below).

As discussed earlier, candidate gene studies in protogynous fish show that *dmrt1* and *foxl2* expression alter only after serum E2 decreases, presumably in response to changes in *cyp19a1a* expression (Alam et al., 2008; Bhandari et al., 2003; Nozu et al., 2015). Further support that alterations in the expression of *dmrt1*, *dax1* and *foxl2* all occur well-downstream of *cyp19a1a* shut down come from transcriptomic studies in bluehead wrasse (Liu et al., 2017).

One possible regulator of *cyp19a1a* is steroidogenic factor 1 (*sf1* or *nr5a1*) (Liu et al., 2017), a transcription factor that has multiple roles in sex determination and differentiation networks in mammals (Bashamboo & McElreavey, 2015). Gonadal expression of *sf1* declined sharply during early female-to-male sex change in the bidirectional sex changing goby *Trimma okinawae* (Kobayashi et al., 2005). In bluehead wrasse gonads transitioning from ovary to testis, *sf1* expression also declined abruptly and concurrently with *cyp19a1a* expression, but recovered thereafter (Liu et al., 2017). The association of *sf1* with *cyp19a1a* in the initiating stages of female-to-male sex change is exciting in the wake of new evidence linking it to ovotesticular disorders of sexual development (Baetens et al., 2017). The role of *sf1* in sex changing fish needs further investigation.

10. Protandrous sex change and the role of dmrt1, amh and sox8

Studies of protandrous sex change have thus far focused strongly on single-gene studies, most of which have been undertaken in black porgy. A sharp decrease in *dmrt1* expression occurs at the onset of testicular degeneration in black porgy (Wu et al., 2012) and gilthead seabream (Liarte et al., 2007; Pauletto et al., 2018). Casas et al. (2016) also report a decrease in *dmrt1* across sex change in clownfish. It is possible that *dmrt1* may be as important in initiating protandrous sex change as *cyp19a1a* appears to be for protogynous species. Knocking down *dmrt1* 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., 2010). Clearly, downregulation of both *dmrt1* and *amh* is important for protandrous sex change, but which acts earlier in the process is unclear. Transcriptome analysis of clownfish suggests that the *dmrt1* expression changes may precede those of *amh* (Casas et al., 2016), although the factors that affect *dmrt1* expression in early protandrous sex change remain to be determined. *Dmrt1* expression in the male testis appears to be maintained by androgens and gonadotropin signaling (e.g., LH) via the HPG axis (Herpin & Schartl, 2011b; Wu & Chang, 2013; Wu et al., 2012).

Further transcriptomic studies of the brain and gonad during sex change, particularly the early stages, are needed to identify the likely upstream regulators of *dmrt1,* followed by tests of regulatory interactions to determine just how the negative regulation of *dmrt1* is achieved in early protandrous sex change.

Anti-Müllerian hormone (Amh) is a multifunctional member of the Tgfβ superfamily of growth factors (Massagué, Seoane, & Wotton, 2005) that plays a critical role in vertebrate gonadal development for both males and females (reviewed by Pfennig et al., 2015). Amh is best known in mammals for its role in the regression of the Müllerian ducts in male embryos, from which the uterus and other female organs would otherwise develop (Behringer, 1994). Teleost fish have no Müllerian ducts, but possess orthologs of *amh* that are thought to have roles in early testicular differentiation, inhibition of germ cell proliferation and steroidogenesis to promote maleness (Pfennig et al., 2015).

Many of the physiological roles of *amh* in the ovary, testis and transitioning gonad during sex change remain speculative and need further investigation (Pfennig et al., 2015). Elevated levels of *amh* in male gonads are common during sexual differentiation in fish; and, as is observed in mammals, *amh* appears to directly inhibit *cyp19a1a* expression leading to an inverse relationship between *cyp19a1a* and *amh* expression (Pfennig et al., 2015). This inhibition is typical, but not universal for teleosts, as is inhibition of *amh* expression by oestrogens and FSH (Pfennig et al., 2015).

Transcriptomic analysis of protandrous clownfish (Casas et al., 2016) showed heightened expression of two different transcripts of *amh* in males compared to females, and expression of *amh* declined during the transition from male to female. Amh is also linked to sex change in protandrous black porgy, with expression of *amh* in cells bordering ectopic oocytes thought to suppress ovarian development in ovotestes (Wu et al., 2015).

In the protogynous hermaphroditic ricefield eel, a*mh* was also found to be upregulated early in the transitioning gonad (Hu, Guo, Gao, Tang, & Li, 2015), although the timing in relation to other male pathway genes or *cyp19a1a* was not investigated. However, whole-transcriptome data from bluehead wrasse showed that *amh* and its receptor *amhr2* are the first male-pathway genes upregulated in the gonad, and their initial surge is concurrent with the interruption of *cyp19a1a* expression (Liu et al., 2017). Whether *amh* contributes to the suppression of *cyp19a1a* at this time, or is simply responding to falling oestrogen levels and is a proximate effector of male-specific pathways, is not yet clear. More experimental evidence is

needed to elucidate the exact functions of *amh* signaling in early protandrous and protogynous sex change. 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., 2013), suggesting a potential role in regulating brain-gonad communication via the HPG axis.

Sox8 is an important factor in the maintenance of cellular identify in mouse testis (Matson et al., 2011), and also an important regulator of adult Sertoli cell function and thus male fertility (O'Bryan et al., 2008). The role of *sox8* in sex change has not been widely considered, but it is known to be both a target of *dmrt1* and a regulator of *amh* expression (Salmon, Handyside, & Joyce, 2005). In the protandrous clownfish, *sox8* was strongly upregulated in males versus females alongside *dmrt1* and *amh* (Casas et al., 2016).

11. The role of epigenetics in sex change

Epigenetics provides a mechanism for cells to stably alter their gene expression patterns without requiring any underlying change in DNA sequence. Most modern epigenetic studies focus on modifications of DNA (e.g., methylation of cytosine bases) and histones (e.g., acetylation) which can regulate gene expression by reversibly altering the availability of genes, or specific exons, to transcription, and typically inhibit and promote transcription, respectively (Duncan, Gluckman, & Dearden, 2014; West-Eberhard, 2003). However, when the term epigenetics was first coined by Waddington (Goldberg, Allis, & Bernstein, 2007; Waddington, 1957) in explaining the process of cellular differentiation, whereby a multitude of cell- and tissue-types are created from essentially the same genetic code (Fig. 6). Essentially, Waddington imagined "epigenetics" representing a landscape within which cells progressively commit to a dedicated function, much like a marble rolling down a valley.

Natural sex change in fish almost certainly occurs without any underlying change in genetic content or DNA sequence. Thus, it seems likely that epigenetic mechanisms will play a central role in orchestrating sex change (Todd, Liu, et al., 2016). In this way, Waddington's epigenetic landscape may be helpful in conceptualizing sexual canalization and how it is reprogrammed during sex change. For example, in standard gonochoristic species, the bipotential gonad during early development could be imagined as a marble approaching the junction between two steep ravines, where entry into one makes transition to the other highly unlikely. When using this

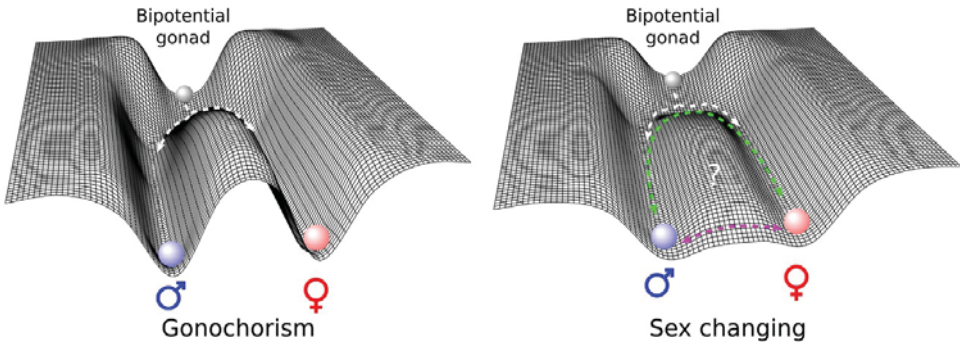

Fig. 6 Waddington's epigenetic landscapes and sexual phenotype. In gonochoristic and sex changing fish, the bipotential gonad undergoes sex determination and it is canalized through either a female or male pathway. For gonochoristic species, a strong genetic and epigenetic barrier precludes sex reversal during adult life. In sex changing species this barrier is reduced, sexual phenotype is more plastic and can be reprogrammed by environmental and social cues, resulting in sex change. The mechanistic process by which these barriers are overcome may involve transdifferentiation (Pink line), where cells of definitively one sex transition directly into cells of the other. Alternatively, a more complex reprogramming path, perhaps involving some form of dedifferentiation or developmental regression (Green line) may instead be involved.

analogy to consider sequential hermaphroditism, the critical question that emerges is what molecular processes form the barriers that normally separate males from females, and how are they routinely overcome?

11.1 Epigenetic barriers separate the sexes

DNA methylation at CG dinucleotides is unique compared to other epigenetic modifications because it has a well described mechanism by which it is propagated (Goll & Bestor, 2005). Essentially, the maintenance methyltransferase DNMT1 recognises methylated cytosines on the template strand, and copies this mark to the cognate cytosine on the newly synthesized strand. This elegant form of epigenetic memory allows stable transmission of (potentially lifelong) biological information. Sexually dimorphic DNA methylation patterns could represent a primary mechanism used to establish and maintain sexually divergent gene expression in species that are sexually isogenic. Alternatively, DNA methylation remodeling could be used to alter the expression of the prevailing sex pathway in favor of the other, possibly in an environmentally-sensitive manner, thus driving sex reversal.

For example, although the half-smooth tongue sole (*Cynoglossus semilaevis*) primarily has a ZW chromosomal sex determination system, analysis of gonads from ZZ and ZW fish revealed that sex-pathway genes differentially expressed between ovary and testis are major targets of methylation

(Shao et al., 2014). Thus, in addition to the genetic barriers separating male and female tongue sole, additional epigenetic barriers also exist. Interestingly, when ZW females become sex-reversed to "pseudomales" by temperature exposure, their DNA methylation signatures are indistinguishable from ZZ males and expression of female-specific W chromosomal genes is suppressed. ZW offspring in the F1 generation (produced by a cross between a ZW pseudomale and a normal ZW female) retain male epigenetic marks in their gonadal cells, and ~90% spontaneously sex-reverse in the absence of thermal influence (Shao et al., 2014). These results indicate that environmentally-induced sex reversal can reset heritable epigenetic marks, and that this reprogramming of the genome can override the opposing sexual pathway (Capel, 2017; Shao et al., 2014; Todd, Liu, et al., 2016).

It is likely that this is not an isolated example. The Nile Tilapia has a chromosomal sex determination system and with similar temperature-dependent sex reversal. It has recently been shown that *cyp19a1a* methylation levels are significantly higher in phenotypic males compared to females, irrespective of genotypic sex-type (Wang et al., 2017). Similarly, genetically female European sea bass (*Dicentrarchus labrax*) show down-regulation of *cyp19a1a* expression that is associated with the accumulation of DNA methylation and masculinization (Navarro-Martín et al., 2011).

In addition to these associations, bulk alteration of DNA methylation patterns has been shown to influence natural sex change. For example, in the 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 sf1, and blocked gonadotropin-induced cAMP activation of *cyp19a1a* expression in vitro. Importantly, *cyp19a1a* promoter methylation increased as *cyp19a1a* expression decreased during sex change in ricefield eel, and implantation with DNA methylation inhibitors (5-aza-2′-deoxycytidine) could prevent or reverse gonadal sex change toward male. In a similar fashion, gonadal explants from Nile Tilapia treated with 5-aza-2′-deoxycytidine show increased expression of *cyp19a1a*, and subsequent feminization. Therefore, it seems likely that methylation of *cyp19a1a* DNA is an essential component of the epigenetic barrier separating males from females in these fish, and its remodeling is also likely essential for maintaining secondary male gonadal fate in protogynous hermaphrodites.

While histone modification does not have the same well-described memory system as DNA methylation, it has been linked to environmental sex determination in bearded dragons (Holleley et al., 2015), American

alligator (Yatsu et al., 2016) and the red-eared slider turtle (Czerwinski, Natarajan, Barske, Looger, & Capel, 2016). KDM6B, a lysine-specific demethylase with a central role in epigenetic regulation of gene expression, is an early responder to male incubation temperature in both turtles and alligators (Capel, 2017) and a key regulator of sex in red-eared slider turtles (Ge et al., 2018). Suppressing *kdm6b* expression reduces demethylation of its target, trimethylated lysine 27 on histone 3 (H3K27), repressing *dmrt1* promoter activity. Thus, high amounts of KDM6B activate *dmrt1* expression and determine male sex, whereas reduced amounts of KDM6B repress *dmrt1* expression (Ge et al., 2018).

It is as yet unknown if *kdm6b*, a Jumonji gene family member, responds directly to temperature or is regulated by upstream temperature-sensitive elements yet to be discovered, such as CIRBP (cold-inducible RNA binding protein) (Schroeder, Metzger, Miller, & Rhen, 2016). However, in the red-eared slider turtle, American alligator, and the bearded dragon an intron is alternatively retained or excised during transcription of *kdm6b* and another Jumonji family member, *Jarid2* (Jumonji and AT-rich interaction domain containing 2) in response to temperature (Deveson et al., 2017). Alternative splicing of Jumonji genes is hypothesized to alter the targets of gene silencing, gene activation, and the recruitment of chromatin remodeling complexes [for example, Polycomb repressive complex 2 (PRC2)] in ways that are not yet fully understood to determine sex (Georges & Holleley, 2018).

Epigenetic modifications, such as those driven by *kdm6b*, which alter the expression of key sex-pathway genes is clearly 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 attention. In whole-transcriptome data from bluehead wrasse, several genes encoding DNA methyltransferases (e.g., *dnmt1*, *dnmt3ab*, *dnmt3bb*) and histone acetyltransferases (e.g., *kat8*, *hat1*) or deacetylases (e.g., *hdac2*, *hdac10*) showed significant expression changes at the extremities of gonadal sex change (Liu et al., 2017). Specific functions for these genes during sex change cannot be inferred until more is known regarding epigenetic regulation of vertebrate sexual differentiation pathways. Moreover, much more attention should be focussed upon the nature of the intermediate states in these fish. Do the gonads of sequential hermaphrodites regress to some earlier developmental time point and re-differentiate into the other sex? Or perhaps a latent (germline?) stem cell population constantly present within the gonad reactivates at sex change to replace sexually divergent cells

that are undergoing apoptosis? Or is there a more straightforward reprogramming of ovarian cells to a testicular fate (Fig. 6)? It is likely that novel genome-wide techniques such as single-cell RNA-seq (Stévant & Nef, 2018) will assist with answering these questions.

12. A new model of sex change

We have previously proposed a mechanistic model, featuring cross-talk between the HPG and HPI axes, through which a normal female reproductive cycle may be interrupted to initiate sex change in socially protogynous fishes (Goikoetxea et al., 2017; Liu et al., 2017). Briefly, following loss of a dominant male, rapid neurochemical changes in the hypothalamus of large females promote behavioral sex change (see previous section). Elevated AVT and NE levels specifically may then perturb GnRH and LH dynamics via the HPG axis to induce follicle apoptosis in the ovary, while driving up serum cortisol via the HPA/I axis (Liu et al., 2017). Elevated cortisol, together with epigenetic regulators, could block *cyp19a1a* transcription directly, and via activation of specific transcription factors (e.g., Amh), instigate the chain-reaction of falling E2 levels that accelerate ovarian degeneration and interrupt female-specific gene expression. Thereafter, elevated cortisol may stimulate 11-KT production to activate male-pathway genes and support testicular differentiation (Goikoetxea et al., 2017; Liu et al., 2017). There is much yet to test, but this current hypothesis is the first to link neurochemical changes occurring in the brain that are associated with behavioral sex change and stress, with the changes in gene expression and endocrine production during gonadal sex change in protogynous sequential hermaphrodites.

13. Conclusions

Studies from divergent systems are beginning to clarify how natural sex change may initiate and progress. Sex change clearly begins in the brain, but where this trigger lies, what this trigger is and how this then initiates gonadal sex change remain unknown (Lamm et al., 2015). Across many systems, re-direction of gonadal fate is initiated when expression of critical sex-maintenance genes (e.g., *cyp19a1a* in protogynous, and *dmrt1* in protandrous species) is interrupted causing a cascading collapse of the prevailing expression landscape, endocrine environment, and gonadal anatomy. Once antagonistic suppression of the opposing sexual network lifts, establishment of a

new sex-specific expression and endocrine environment drives gonadal development toward the secondary sex (Todd, Liu, et al., 2016). A tightly regulated, multi-component, trigger mechanism appears to sit at the top of this cascade to ensure sex change proceeds only under specific circumstances (Todd, Liu, et al., 2016). C*yp19a1a* appears to initiate gonadal sex change in protogynous systems and we know many factors that appear to reduce the expression of this critical sexual pivot (e.g., cortisol, DNA methylation, *amh*). However, most of the data we have obtained to date is simple association, linking changes in gene expression levels and timing to key phenotypic events. We need more sophisticated experimental validation, as has been seen in mouse and other model systems, if we are to disentangle cause from effect. A fuller understanding of how complex neurological systems in the brain cross-talk with the stress (HPI) and reproductive (HPG) axes to translate external environmental signals into internal physiological responses is also needed.

Such work takes time—even in mouse our understanding of sex determination continues to evolve (Capel, 2017; Harris et al., 2018). In fish we know this process is also controlled by antagonistic interactions. In mice, the key players are the SRY-SOX9-FGF9 network that promotes testis determination partly by opposing pro-ovarian pathways, while RSPO1/WNT-β-catenin/FOXL2 signals control ovary development by inhibiting SRY-SOX9-FGF9 (Harris et al., 2018). The molecular basis of this mutual antagonism is unclear, but recent work suggests that ZNRF3, a WNT signaling antagonist and direct target of RSPO1-mediated inhibition, is involved (Harris et al., 2018). Male mice lacking ZNRF3 exhibit complete or partial gonadal sex reversal (Harris et al., 2018). Perhaps ZNRF3 plays a role in the dynamic antagonism we observe in sex changing fish?

Current insights into sex change derive from investigations into a handful of protogynous systems and studies on the protandrous black porgy and clownfish (Liu et al., 2017; Todd, Liu, et al., 2016). Contrasting across diverse representatives of each sex change strategy will be vital to determining how the switch between sex-specific developmental networks arises during sex change, and whether common mechanisms trigger and regulate the processes of female to male and male to female sex change.

The establishment of model systems for sex change remains a challenge, particularly where we seek to understand molecular genetic mechanisms. However, ongoing refinements in genomic sequencing and manipulative approaches are making the investigation and establishment of novel systems easier. RNA-sequencing (RNA-seq) (Todd, Black, & Gemmell, 2016;

Wang, Gerstein, & Snyder, 2009), especially, enables us to rapidly describe the co-ordinated pattern of expression, including that of uncharacterized genes and isoforms, across a time-series without the need for prior genetic resources. The value of this approach for identifying candidate triggers of sex change and genes with novel and unexpected roles in vertebrate sexual development has already been established for sex changing fish (Casas et al., 2016; Liu et al., 2015; Todd et al., 2018). Further refinement through the latest single-cell RNA-seq technologies are especially exciting with regards to characterizing genetic cascades involved in cellular reprogramming and cell fate determination (Farrell et al., 2018; Stévant & Nef, 2018; Stévant et al., 2018). This precision approach to understanding cell specific expression patterns is likely to herald significant advances in our understanding of sex change in both the brain and gonad. Single cell approaches will be especially valuable in determining the cellular origins of the secondary sex in protogynous species undergoing complete sex reversal, and in answering questions regarding the potential reprogramming or retained bipotency of gonia and somatic cells in teleost gonads. Further refinements through simultaneous genome-wide analysis of transcriptomes (RNA-seq) and methylomes (whole-genome bisulfite sequencing, BS-seq) from single cells (Angermueller et al., 2016) and chromatin accessibility (single-cell nucleosome, methylation and transcription sequencing, scNMT-seq) (Clark et al., 2018) will generate new testable hypotheses as we continue to search for components of the trigger mechanism used by sequential hermaphrodites to alter sexual fate in adulthood. However, manipulative studies will always be the acid test and an integrative approach will be necessary to answer 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 manipulations have historically been confined to a handful of model systems, but once husbandry challenges are circumvented, gene editing approaches (Cong et al., 2013) will 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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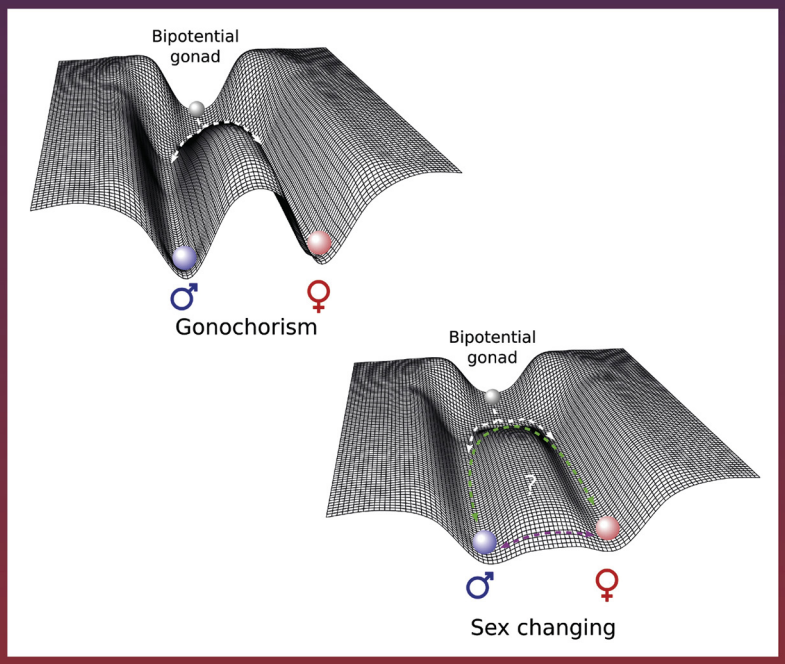
SEX DETERMINATION IN VERTEBRATES



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SEX DETERMINATION IN VERTEBRATES

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Cover image:

Waddington's epigenetic landscapes and sexual phenotype in fish (see Gemmell et al. Chapter 3). Males and females are separated by formidable genetic and epigenetic barriers in gonochoristic species (top). However, in sex changing fish these barriers are much smaller, allowing reprogramming of sexual phenotype to occur (bottom) courtesy of Oscar Ortega Recalde and Tim Hore.

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