Epigenetic Regulation and Environmental Sex Determination in Cichlid Fishes

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Abstract

Studying environmental sex determination (ESD) in cichlids provides a phylogenetic and comparative approach to understand the evolution of the underlying mechanisms, their impact on the evolution of the overlying systems, and the neuroethology of life history strategies. Natural selection normally favors parents who invest equally in the development of male and female offspring, but evolution may favor deviations from this 50:50 ratio when environmental conditions produce an advantage for doing so. Many species of cichlids demonstrate ESD in response to water chemistry (temperature, pH, and oxygen concentration). The relative strengths of and the exact interactions between these factors vary between congeners, demonstrating genetic variation in sensitivity. The presence of sizable proportions of the less common sex towards the environmental extremes in most species strongly suggests the presence of some genetic sex-determining loci acting in parallel with the ESD factors.

Sex determination and differentiation in these species does not seem to result in the organization of a final and irreversible sexual fate, so much as a life-long ongoing battle between competing male- and female-determining genetic and hormonal networks governed by epigenetic factors. We discuss what is and is not known about the epigenetic mechanism behind the differentiation of both gonads and sex differences in the brain. Beyond the well-studied tilapia species, the 2 best-studied dwarf cichlid systems showing ESD are the South American genus *Apistogramma* and the West African genus *Pelvicachromis*. Both species demonstrate male morphs with alternative reproductive tactics. We discuss the further neuroethology opportunities such systems provide to the study of epigenetics of alternative life history strategies and other behavioral variation.

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Natural selection normally favors parents who invest equally in the development of male and female offspring, but conditions may favor deviations from this 50:50 ratio [Fisher, 1958; Hamilton, 1967]. If reproductive skew is greater in males, higher quality males have a competitive advantage, and females in above average condition are

more able to invest in making males with such an advantage, then females should evolve to bias the sex ratio of their offspring as a function of their condition [Trivers and Willard, 1973; Charnov and Bull, 1977; Leimar, 1996]. If the environmental conditions in which an individual develops later influences their competitiveness in adulthood, then some environments should favor development as the sex with the greatest reproductive skew, and other environments should favor development as the sex with the smaller reproductive skew [Trivers and Willard, 1973; Leimar, 1996]. These processes should result in the evolution of diverse sex-determining mechanisms, including those sensitive to environmental variables which foreshadow the prospects of different sexes and alternative life history strategies [Charnov and Bull, 1977].

The relatively consistent and simple outcome of differentiating into either a male (sperm-producing) or female (egg-producing) form is determined by a wildly diverse set of mechanisms across different animal species [Bachtrog et al., 2014]. Sex determination systems range from varieties of genetic sex determination (GSD) with sex chromosomes and master sex-determining genes, or polygenic sex determination systems (PSD) with multiple sex-determining loci, to haplodiploidy and paternal genome elimination, to non-genetic environmental sex determination (ESD) systems. In some taxa, such as mammals and birds, the sex determination mechanism is relatively conserved. Teleost fish, however, provide full-spectrum service to the student of sexual differentiation, the full gamut from XY and/or ZW GSD systems, PSD systems, and ESD factors such as pH and temperature. In addition, there are teleost species in which various GSD and ESD systems have simultaneous action [Devlin and Nagahama, 2002; Cnaani et al., 2008; Avise and Mank, 2009; Godwin and Roberts, 2018; Piferrer, 2018].

Here, we review what is known about the environmental influences on sex determination within the teleost family Cichlidae. We pay particular attention to environmental variables which have demonstrable effect within ranges normally encountered in the natural history of the animals to focus on traits reflecting the influence of natural selection on the mechanisms of sex determination. Since ESD does not rely on base-pair sequence polymorphisms to determine sexual fate, the mechanisms determining sex must be plastic and therefore epigenetic. We briefly examine what is known about these epigenetic mechanisms and outline the major questions remaining to be addressed with this powerful comparative system for the study of evolutionary forces acting on sex determination. We discuss how the mechanism underlying

ESD may be linked to the development of alternative reproductive tactics and how they differ from sequential hermaphroditism.

Genetic versus Environmental Sex Determination

GSD is typically associated with a master sex-determining gene, which creates 2 broad categories of sex chromosomes, XX/XY if the males are heterogametic for the master sex-determining gene and ZW/ZZ if females are the heterogametic sex. The specific gene serving as the master sex determiner varies across taxonomies but is drawn from a pool of usual suspects of genes involved in sexual differentiation [Valenzuela et al., 2013; Shen and Wang, 2014]. Polygenic sex-determining systems lack a master sex-determining gene. Sexual fate in PSD systems is determined epistatically by independently segregating alleles, a parliamentary outcome from the cast of usual suspects interacting among themselves [Roberts et al., 2016; Godwin and Roberts, 2018; Vandeputte and Piferrer, 2018]. In species with ESD, external environmental conditions, acting during an early sensitive period, influence gene expression within this parliament of sex genes to determine sexual fate [Piferrer et al., 2019]. While sex determination is evolutionarily relatively static in some taxa, such as mammals and birds, in other lineages, such as teleost fish, even closely related species exhibit a diversity of sex determination systems and a diversity of master sex determiner genes [Godwin and Roberts, 2018]. As an example of this rapid evolution, in the family Cichlidae alone, environmental influences of temperature and pH exist alongside striking diversity of genetic factors including B chromosome, XY and ZW heterogametic sex chromosome systems, as well as complex polyfactorial systems where several of these factors have simultaneous effects [Baroiller et al., 2009; Ser et al., 2010; Yoshida et al., 2011].

The most studied environmental factor is temperature, and species can be grouped according to the pattern of sex ratio response. By far the most common in fishes is more males at higher temperatures, with few species showing the opposite pattern of more females at higher temperatures – the common pattern found in reptiles [Ospina-Álvarez and Piferrer, 2008]. More complex patterns with a sex ratio skew at both high and low extremes are seen less often [Devlin and Nagahama, 2002; Ospina-Álvarez and Piferrer, 2008]. Though discrete processes, GSD and ESD systems are not mutually exclusive, and both genetic differences and environmental conditions

may have interacting effects on sexual fate [Capel, 2017]. For many species with demonstrated ESD (usually temperature effects), the data derive from lab-based experiments, and in some cases the presence of GSD has not been eliminated [Ospina-Álvarez and Piferrer, 2008].

Among the very earliest reports of ESD is that by Walter Heiligenberg [1965] on the cichlid *Pelvicachromis pulcher*, which we discuss below. This predates virtually all of the highly influential work on ESD in reptiles [reviewed in Bull, 1980], as well as the first definitive field-based evidence of temperature-dependent sex determination in fishes [Atlantic silverside: Conover and Kynard, 1981]. While recent work in cichlids has focused on the evolution of polygenic sex-determining systems, including multiple ZW and XY loci segregating within a single species and their evolution among cichlids from Lake Malawi [Ser et al., 2010], even those data leave room to hypothesize a role for environmental factors. There is also ample evidence for both ESD [Baroiller et al., 2009] and GSD [Devlin and Nagahama, 2002; Cnaani et al., 2008] in tilapiine cichlids, and in fact the majority of fish species with demonstrated ESD are from the cichlid clade [Römer and Beisenherz, 1996; Ospina-Álvarez and Piferrer, 2008].

Sex Determination versus Sex Reversal

Sex reversal is a form of ESD in which development of a genetically determined sex is reversed through environmental, often artificial, manipulation to produce the other sex. This practice is common in aquaculture for economic reasons [Budd et al., 2015; Baroiller and D'Cotta, 2016]. A great deal has been learned about the mechanisms of sex determination through the study of sex reversal. In species where sex is normally determined through a straight-forward XX/XY system, the XX males and XY females resulting from hormonal treatments are clearly sex reversed. Species with polygenic sex determination, or mixed GSD-ESD systems, may also show XX males and XY females [Bezault et al., 2007] and are said to be naturally sex reversed. Among ZW genetic females of the half-smooth tongue sole about 14% are reversed to phenotypic males under normal natural temperature conditions, with a correspondingly masculinized DNA methylation pattern [Shao et al., 2014]. The appropriateness of the term reversal in this instance lies on a spectrum. If an environmental variable, such as temperature, reverses genetic sex and this environmental variable needs to be outside the natural history range for that spe-

cies, then the term seems entirely appropriate. This is not necessarily the case for all species with both genetic (either XX/XY or ZW/ZZ) sex determination and other factors, where PSD, or GSD-ESD may be more accurate terms.

The study of sex reversal, whether artificially manipulated or natural, provides a valuable experimental system to address the role of sex-determining genes as well as molecular cascades in sex differentiation. While the temporal patterns of gene expression may differ dramatically between genetically and environmentally driven reversal, some of the specific genes and gene functions including epigenetic regulation are shared [e.g., bearded dragon: Whiteley et al., 2021]. A growing number of studies reveal epigenetic mechanisms by which environmental sex reversal can override the sexual fate determined by genetic factors [Ortega-Recalde et al., 2020]. In any case, our primary interests in the present article are cases where normal-range ecological variation in environmental factors influences sex determination and leave the topic of reversal to other manuscripts in this special edition (see other reviews in this issue for detailed treatment of sex reversal).

Abiotic Environmental Effects on Sex Determination in Cichlids

Research on environmental influences on sex ratios in cichlid fish begins with Walter Heiligenberg's [Heiligenberg, 1965] study of alternative male morphs in the East African cichlid *P. pulcher* (then *Pelmatochromis*). Heiligenberg anecdotally reported that sex ratios in this genus ranged from 90% males to 10% males across the range of breeding pHs from 4 to 5 to neutral. This was confirmed for 3 species of *Pelvicachromis* (including *P. pulcher*) as well as 2 species of the South American dwarf cichlid *Apistogramma* by Rubin [1985] across pH conditions ranging from 5 to 6 to 7, under which all species showed male-biased sex ratios at low pH and female-biased ratios at neutral pH. The exact length of the critical period for pH exposure on sex ratio in *P. pulcher* is not known, but treatment for the first 30 days after hatching are effective in influencing sex ratio [Reddon and Hurd, 2013]. Subsequent work has also shown an effect of temperature treatments over the first 60 days post-hatching, with broods incubated in ranges of 20–22°C producing 13% males, while broods incubated in the range of 26–28°C produced 39% males at neutral pH [Hurd unpubl. data].

In a large comparative study of *Apistogramma* cichlids, Römer and Beisenherz [1996] manipulated both pH and water temperature for over 30 species of *Apistogramma*. Nearly uniformly, these species showed more male-biased sex ratios at lower pH and high temperatures and female-biased sex ratios at neutral pH and lower temperatures. For one of these species, *A. trifasciata*, they determined that the critical period for temperature effects was a linear function of time spent, up to 800 h of development. Seven species in that study were subjected to a more rigorous test in which at least one clutch was bred at each of 9 combinations of 3 temperatures and 3 pHs. Temperature effects were stronger in some species and pH effects stronger in others. The only species to show a significant “reversed” temperature effect showed it only at the lowest pH and showed far stronger pH effects in the typical direction than other species. Temperature effects generally overshadowed pH effects in the 7 closely studied species. Temperature and pH values for microhabitats in which *Apistogramma* are found in the field range from 11.6 to 36°C, and 3.9–7.6 pH, with typical values of $26.6 \pm 4.4^\circ\text{C}$ and 5.5 ± 1.1 pH [Römer, 2001]. The experimental ranges selected for both their pH and temperature treatments are centered close to the mean field values, and the span of the treatments amounts to approximately 1.5 standard deviations of between site variation. The relative importance of temperature over pH effects seen in these experimental results probably reflects the relative importance of these variables in the wild. The interspecific differences in relative magnitude of these 2 effects, and in the nature of their interaction, clearly indicates genetic differences among these close relatives in environmental sensitivity to these 2 variables.

Another abiotic factor which may influence sex in cichlids is oxygen concentration. A parenthetical mention in a study examining the response to hypoxic rearing environment in the cichlid *Pseudocrenilabrus multicolor victoriae* notes that hypoxic environments produced female-biased sex ratios [Chapman et al., 2008]. This effect is in the reverse direction to that seen in zebrafish where hypoxia results in male-biased sex ratios [Shang et al., 2006].

Temperature, pH, and oxygen concentration are all expected to be interrelated. Raising the temperature of water may lower its pH by a trivial amount, but in a biological system, many linked processes will be affected, and the effects may be large. At the level of the ecosystem these variables can be expected to correlate. For example, across the 6 sites in the study of Nwadiaro [1985] of *P. pulcher* habitat on the Rio Sombrierio, mean temperature and pH are highly positively correlated ($r = 0.99$), and dissolved O_2 and temperature are highly negatively

correlated ($r = -0.97$). While these samples track geographical variation, and we might expect ESD effects to have evolved to track seasonal variation, if pH and temperature are consistently correlated that strongly, then the ESD mechanism has clearly evolved to track one distal variable (maybe predicting some seasonal aspect), even though 2 proximate variables may be used by the physiological mechanism. On the other hand, across the many *Apistogramma* habitat sites surveyed by Römer [2001], temperature and pH are, if anything, negatively correlated ($r = -0.30$, 36 sites with specific temperature and pH values). The interspecific differences in the interaction of temperature and pH seen across the species in Römer and Beisenherz’s [1996] study demonstrate that these 2 factors are having independent effects at the level of the organism. The variation in the strength of pH and temperature effects may reflect selection for tracking more than one distal variable.

It is also worth noting that population sex ratios in the field may be quite different from the sex ratios predicted from lab studies manipulating water chemistry. Nwadiaro [1985] reported approximately 1:1 sex ratios in the field across a range of sites with mean pH ranging from about 5.5 to 6.2, and temperatures of 24.8–27°C. One site with mean pH of about 5.5 and temperature of about 24.0°C showed approximately a 1:2 M:F ratio, a female-biased deviation from the 1:1 sex ratio in cold, acidic water. From water chemistry, it seems a male-biased, not female-biased, sex ratio would be expected. The next site downstream, with a 1:0.97 M:F ratio was 0.8°C warmer on average, with the same dissolved oxygen and trivially (0.03 pH difference) more acidic water.

Temperature and pH influence sex ratio in both the large South American genus *Apistogramma*, and the West African genus *Pelvicachromis*. Ospina-Álvarez and Piferer [2008] previously reviewed the magnitude of TSD in fish, describing the effects when they exist as being from 1:1 to 3:1 over 1 or 2 degrees. While there are certainly some species that show this extreme, the majority of them show more modest effects with considerable proportions of the rarer sex in excess of 20% at the most extreme environmental conditions. We suggest that such patterns indicate coincident action of genetic variation.

Social Effects on Sex Determination in Cichlids

In addition to abiotic environmental effects, social factors in the environment may determine sex, a phenomenon termed behavioral sex determination (BSD). Work

by Francis and Barlow [1993] suggested that Midas cichlid (*Amphilophus citrinellus*) sex was determined by their position within their cohort's size hierarchy during juvenile development. That study relied on the assumption that the size hierarchy within a clutch is stable to suggest that the larger fish in a cohort become male. Francis and Barlow [1993] divided a cohort of 74 six-month-old juveniles into 2 groups based on size, then measured and sexed the fish at 1 year of age when they were expected to be sexually mature. Those fish that had been below median size at the time of separation were larger on average at adulthood than those that had been in the top half of the size hierarchy, and both groups had non-significantly female-biased sex ratios, with males being larger on average than the females in both groups. The experiment tested whether larger fish become male either because sex is determined early and males grow faster than females or variation in growth rate is the initial variable and the faster growing fish decide to become male at some later point. The results support the hypothesis that relative size within the cohort determines sex. Attempts to replicate this work have failed. Oldfield [2007] split a cohort of 4-month-old Midas cichlids along the size distribution into 13 groups of 7 fish, then randomly removed 1 fish from each group to be raised in isolation. The results would support Francis and Barlow [1993] if the largest 3 fish in each group matured as males and the smallest as females. However, there was no significant association between eventual sex and position within the size hierarchy in these groups at adulthood nor was there a pattern of variation in sex ratio across the 13 groups that might be expected if growth rate and sex were associated at the time of separation. In another attempt to replicate the original Francis and Barlow [1993] experiment, 4 cohorts separated according to the original protocol showed no evidence of social influence on sex and showed differential growth rates between the sexes only after sexual differentiation [Oldfield, 2009]. Attempts to replicate the Francis and Barlow study using convict cichlids (*Amatitlania nigrofasciata*) in the Hurd lab have also failed. Creation of cohorts by splitting and joining broods of near same age leucistic and wildtype colored fry at young ages have revealed size hierarchies to be very unstable at young age and unlinked to the eventual sex. While cichlids may not provide a system for investigating socially driven sex determination, the richness of cichlid social behavior and the existing plasticity of cichlid sex determination might create opportunities to comparatively examine how mechanisms associated with social sex determination in other species act on traits other than sex in cichlids.

Molecular Mechanisms to Encode Environmental Factors

When environmental factors determine, override, or interact with genetic factors to impact the phenotype, these external signals must be recorded or translated into differential gene expression to create the phenotypic plasticity [Aubin-Horth and Renn, 2009; Renn and Aubin-Horth, 2019]. How this information is encoded is not fully understood, but stable modifications of DNA and its associated histone proteins play an important role. Because these changes do not alter the genetic sequence, they are collectively referred to as epigenetic modifications. More broadly, epigenetic phenomena include intra- and interindividual variation in response to environmental and endogenous cues across an individual's ontogenesis, life cycle, and even descendants [Burggren and Crews, 2014]. As ESD is clearly a dramatic example of environmentally induced phenotypic plasticity, it is not surprising that epigenetic mechanisms are involved [Piferrer, 2018]. However, at this time a comprehensive picture of which mechanisms, which pathways, and which environmental factors is not yet available.

Epigenetic marks at the DNA level are mitotically, and in some cases meiotically, stable and thus can play a relatively long-term role, being set at one time point to impact the phenotype later in ontogeny [Duncan et al., 2014]. The most studied form of DNA modification is the attachment of a methyl (CH_3) group at a 5'-carbon of the pyrimidine ring of a cytosine nucleotide when it is 5' to a guanine nucleotide (CpG), but additional modifications such as oxidation of methylated cytosine and methylation of adenine are also important epigenetic marks that can impact gene expression [Klungland and Robertson, 2017]. The genomic architecture of epigenetic marks at the DNA level is most studied in mammalian species identifying regions of high density in the upstream regulatory region and first intron of genes [Antequera, 2003]. These modifications are generally (but not exclusively) associated with a decrease in gene expression in vertebrates [Deaton and Bird, 2011; Anastasiadi et al., 2018]. Specific enzymes are responsible for different aspects of DNA methylation initiation and maintenance through cell division [Law and Jacobsen, 2010]. However, both passive and enzyme-mediated active "erasure" of DNA methylation creates dynamic regulation that, while the core machinery is highly conserved, shows different rates and modes of regulation across species [de Mendoza et al., 2020]. Furthermore, the means by which epigenetic marks are made to be tissue (cell type)-specific is variable

and less well understood [Zhong, 2016]. While genome-wide techniques [Chatterjee et al., 2013] are often applied to developmental studies and tissue type differentiation, targeted gene-promoter specific techniques [e.g., Bernstein et al., 2015] have been fruitful in understanding how environmental signals are transduced to shape physiology. With regard to ESD, such studies have largely focused on the aromatase gene promoter [Navarro-Martín et al., 2011; Parrott et al., 2014; Driscoll et al., 2020]. While methylated DNA is thought to directly interfere with transcription factor binding in some cases [Watt and Molloy, 1988], methyl-CpG-binding proteins have also been characterized as “readers” of the methylation mark to recruit protein complexes that impact general accessibility of the DNA region [Bogdanović and Veenstra, 2009].

DNA is wound around nucleosomes, a histone octamer composed of 2 copies each of the histone proteins H2A, H2B, H3, and H4, each of which has a protein tail that can be modified via phosphorylation, ubiquitylation, methylation, or acetylation at different amino acid sites. These modifications create a complex and not fully understood code that determines the packing and accessibility to allow or inhibit transcription [Jenuwein and Allis, 2001; Rando, 2012]. While there is general consensus that DNA methylation directs the formation of nuclease-resistant chromatin leading to repression of gene activity, the DNA methylation machinery is also sensitive to (i.e., reads) the histone marks in this bi-directional regulatory process [Cheng, 2014]. With regard to the field of comparative physiology, although enzymes responsible for histone modifications are identified and their expression tracked through development in some species [Kratochwil and Meyer, 2015; Fellous et al., 2019; Seebacher and Simmonds, 2019; Kirfel et al., 2020], much less is known about the dynamics and combinatorial code for histone modifications outside of mammals. The most common techniques to assess chromatin structure and accessibility of the DNA for transcription rely on immunoprecipitation directed to specific histone modifications followed by high-throughput sequencing (ChIP-seq) of the associated DNA [Park, 2009; Furey, 2012]. Further research is needed to definitely link these modifications with the epigenetic state in most organisms as has been done for humans [Guenther et al., 2007; Zhou et al., 2011].

In addition to the DNA level processes described above, several post-transcriptional RNA level regulatory mechanisms involving untranslated RNA molecules targeting mRNA for degradation or silencing are often considered under the heading of epigenetic regulation, be-

cause these molecules have been shown to be transmitted trans-generationally in either the egg [Martinez et al., 2019] or sperm [Rodgers et al., 2013]. Thus, in addition to epistatic gene interactions within an individual, they can impact physiology and phenotype of subsequent generations. Also, longer untranslated RNAs play a role in regulating histone modifying proteins to impact physiology, some of which are known to be involved in sex differentiation in mammals [Rastetter et al., 2015] and turtles [Zhang et al., 2018]. As such, these post-transcriptional RNA level regulatory mechanisms may play a role in the epigenetic mechanisms that encode early life experiences and environmental factors to impact later phenotypic expression [Gowthaman et al., 2020].

Mechanisms of ESD in (Non-cichlid) Fish

In vertebrates, aromatase, encoded by the *cyp19a1* gene, is a key steroidogenic enzyme that converts androgens into estrogens. It is essential both in the brain and in female gonads where it is necessary for ovarian differentiation [Callard et al., 2001; McCarthy et al., 2009]. In non-teleost vertebrates, male-biasing temperatures correspond to decreased gonadal aromatase expression and higher *cyp19a1* promoter methylation, with female-producing temperatures showing the opposite pattern [American alligator: Parrott et al., 2014; Reeves' turtle: Ru et al., 2017; red-eared slider turtle: Ramsey et al., 2007]. Fish have 2 copies of the gene; the more studied A-copy is expressed in female gonads, while the less well studied B-copy is expressed predominantly in the brain, with contradictory reports with regard to sex bias [Kallivretaki et al., 2007; Okubo et al., 2011]. Again, male-biasing temperatures lead to increased methylation of the A-copy promoter within sexes for several fish species including European sea bass [Navarro-Martín et al., 2011] and mangrove killifish, *Kryptolebias marmoratus* [Ellison et al., 2015]. European sea bass (like Nile tilapia discussed below) are farmed species in which the sexes differ in size. For the bass it is the females that are preferred [Pavlidis et al., 2000], making sex ratio manipulation a matter of economic interest [Stelkens and Wedekind, 2010] and driving research on mechanisms of ESD in teleosts despite both being a combined system of ESD and GSD. The mangrove killifish *K. marmoratus* is androdiecious; most individuals are hermaphrodites and the rest are males. Males become all the more rare when eggs are incubated at higher temperatures [Harrington, 1967]. This decreasing male sex ratio at higher temperatures is the reverse to

most other fish with temperature effects on sex determination where higher temperatures produce more males. This species has been subject to a half century history of research and has recently been used for the identification of candidate genes whose differential methylation or histone modifications may underlie ESD [Ellison et al., 2015; Fellous et al., 2019].

In vitro studies show that temperature-sensitive methylation at the A-copy impacts a flounder Nr5a2 binding site [Fan et al., 2017], the sea bass SF-1 and Foxl2 sites [Navarro-Martín et al., 2011], and cAMP binding in rice eel *Monopterus albus* [Zhang et al., 2013]. The degree to which species-specific results reflect underlying differences in biology or differences in experimental approach requires further comparative and functional analyses. Data from turtles, alligators, and fish show environmental effects on the expression of several transcription factors, many of which are known to impact expression of aromatase, or them be regulated by estrogens justifying future genome-wide analyses. Specifically, functional studies in the red-eared slider turtle [Ramsey et al., 2007] demonstrated that a temperature-based increase in female-biased *cyp19a1* expression is attributable to demethylation impacting binding for FOX and SF-1 transcription factors as well as at the TATA box [Matsumoto et al., 2013].

What little developmental work has addressed the timing of brain aromatase expression or epigenetic regulation thereof has failed to show a clear and simple pattern [Blázquez and Piferrer, 2004; Chang et al., 2005; Kallivretaki et al., 2007; Patil and Gunasekera, 2008; Vizziano-Cantonnet et al., 2011], suggesting the need for in-depth studies examining single species in greater detail rather than a comparative approach seeking a universal pattern. In red-eared slider turtles, the brain initiates sexual differentiation and is sensitive to temperature effects prior to gonadal differentiation [Crews et al., 1996; Czerwinski et al., 2016]. This system is analogous to sex change in teleosts (see below). More work is necessary to determine the relationship between brain aromatase and environmental factors that impact sex differentiation.

Mechanisms of ESD in Cichlids

The majority of research related to environmental influence on sex determination in cichlids has taken advantage of the ability to experimentally induce sex reversal (see above) in tilapia through hormone [Gennotte et al., 2014; Zaki et al., 2021] or temperature [Baroiller et al., 2009] manipulations to create sex-reversed “neomales.”

Variants of sex-influencing loci are found on several chromosomes [Palaikostas et al., 2015; Baroiller and D’Cotta, 2018] in both inbred strains and wild populations [Sissao et al., 2019; Triay et al., 2020]. These loci have also been linked to family level variation in temperature sensitivity, suggesting that the molecular cue for TSD may coincide with mechanisms for GSD [Lühmann et al., 2012; Wessels et al., 2017].

One of these loci, an XY system determiner on linkage group 23, contains a duplication of the *amh* gene and subsequent smaller deletion that is male-specific in some lineages [Wessels et al., 2017] and variable in wild populations [Sissao et al., 2019; Triay et al., 2020]. The typically male-biased *amh* gene has previously shown to also be upregulated in sex-reversed neomales along with the *dmrt1* gene, followed in time by reduced expression of the typically female-biased *foxl1* and *cyp19a1a* (gonad form) as well as downregulation of *cyp19a1b* (brain form) [Poonlaphdecha et al., 2013].

Recent transcriptome level studies of either gonad [Wang et al., 2019] or brain [Zhao et al., 2019; Yao et al., 2021] reveal global patterns of gene expression in sex-reversed neomales that generally correspond to male-typical expression. While the majority of these studies target late time points (100+ days), reflecting post-differentiation gene expression, one study [Yao et al., 2021] produced a time course dataset to investigate “fast-response temperature genes” as early as 6 h post temperature treatment to identify genes directly involved in the masculinization process. Interestingly, several of the genes identified are involved in epigenetic regulation. These include *kdm6b*, which catalyzes the demethylation of histones [Lan et al., 2007] to promote transcription of target genes, one of which is the male sex determiner *dmrt1* [Ge et al., 2018], also implicated in alligator TSD [Deveson et al., 2017]. Similarly, *Cbx7* and *Jarid2*, which are components of the histone modifying complexes PRC1 and PRC2, respectively, were also upregulated at these early timepoints and have been linked to sex determination in other systems [Kuroki et al., 2013; Deveson et al., 2017]. This demonstrates that epigenetic mechanisms are involved in temperature-based sex reversal in tilapia. The degree to which sex differentiation of the brain follows or drives differentiation of the gonads in fish remains an open question [Senthilkumaran et al., 2015].

In addition to histone modification mechanisms for epigenetic regulation, DNA methylation has also been addressed in tilapia sex determination and sex reversal. Roughly 1–2% of the genome shows differential methylation in response to high temperature sex reversal in tilapia

gonads [Sun et al., 2016] in a pattern that generally shows the expected negative correlation with gene expression [Wang et al., 2019]. Pharmacological manipulation with fadrozole at a later timepoint demonstrated the upregulation of the DNA-methyltransferase genes themselves correlated with upregulation of *dmrt1* and downregulation of *cyp19a1a* [Wang et al., 2018].

Hormonal and physiological studies of sex-reversed neomales suggest that, despite similar gonadal development and sex steroid production, the size and number of specific neurons involved in sociosexual pathways are different [Dussenne et al., 2020], which may explain the differences in behavior, such as aggression [Gennotte et al., 2017]. The degree to which these physiological and behavioral phenotypes seen in response to early hormone treatment also manifest under natural sex reversal or ESD remains to be determined. Juvenile tilapia have been shown to choose an extreme, but not ecologically unrealistic, masculinizing temperature for a period which is short but sufficient to skew sex ratio, which suggests that behavior can influence sex determination for this system [Nivelle et al., 2019].

The existence of pH, temperature, oxygen, and possible socially mediated systems in non-tilapia cichlids provides an untapped opportunity to address the evolution of these mechanisms. Furthermore, the rich behavioral repertoire and ability to reproduce naturalistic behaviors in the lab offers the opportunity to address both neural and gonadal mechanisms in a controlled setting.

By sequencing both copies of the *cyp19a1* aromatase gene and performing gene expression analysis and epigenetic analysis of both brain and gonad tissues in both adult and fry, Driscoll et al. [2020] have established *P. pulcher* as a system for the comparative study of mechanisms underlying ESD and alternative male morph development. The canonical teleost pattern of elevated expression of the A-copy of aromatase in the female ovary and elevated expression of the B-copy in the brain was explained by methylation patterns most strongly for the A-copy in gonads, according to an amplicon sequencing strategy. This is consistent with the relative density of CpGs upstream of these 2 genes. While specific individual functional methylation sites were not identified, the results suggested that different CpG-rich regions of the A-copy gene are acting independently, one for tissue specificity and one for sex specificity. While epigenetic analyses of ESD have largely focused on CpG sites within 500 bp of the gonadal aromatase promoter [red-eared slider turtle: Matsumoto et al., 2013; European sea bass: Navarro-Martín et al., 2011; black porgy: Wu et al., 2012; Nile tilapia:

Wang et al., 2017; flounder: Fan et al., 2017], results from cichlids suggest that sites farther upstream may also be involved [Driscoll et al., 2020].

While that study did not address the ability to encode the pH environmental variable known to impact sex determination, it did importantly demonstrate that the discrete methylation patterns at the A-copy that differentiate adult male and female gonad sex are present among fry. In fact, the male and female methylation patterns were among the most explanatory patterns for A-copy methylation pattern variation among individual fry. This supports the hypothesis of epigenetic marking of sex prior to sexual differentiation and gene expression of the A-copy. A more detailed developmental time course and discrete dissection of gonads in fry will be helpful in identifying the epigenetic marks introduced by environmental pH and leading to male versus female development or male morph expression.

What ESD Research Can Learn from Sex Change

Individuals of gonochoristic species make an irrevocable life history decision to develop into either one sex or the other. Individuals of sequentially hermaphroditic species, on the other hand, initially develop into one sex, then may later reverse in a subsequent change. The timing of the reversal may be under some environmental control, but the initial choice of sex can be seen as less of a choice than in gonochoristic species, where a decision about sexual fate is made early in development. Sequential hermaphroditism represents a most fascinating example of phenotypic plasticity. Such malleability is known in 27 taxonomic families spanning 9 orders of fish with 3 strategies occurring; protogynous (female-to-male change), protandrous (male-to-female change), and sequential bidirectional change [Awise and Mank, 2009]. In clades that exhibit true sex change, behavioral and physiological analysis far exceeds the more recent work at the transcriptomic and epigenetic level. It is known that sex determination and maintenance require opposing regulation of sex-specific gene networks which are largely conserved pathways downstream of master sex-determining genes [Munger and Capel, 2012]. In part because behavioral changes precede changes in gonadal morphology, studies suggest that sex change begins with the brain, where the reproductive axis and stress axis are integrated [Liu et al., 2017]. Changes in neurohormones and cortisol may mediate the regulation of opposing sex-specific gene networks [Ortega-Recalde et al., 2020]. The remarkable

plasticity seen in sequentially hermaphroditic fish demonstrates that the process of differentiation is not irreversible but rather reflects a life-long ongoing battle between competing male- and female-determining genetic and hormonal networks [Paul-Prasanth et al., 2013; Todd et al., 2016; Ortega-Recalde et al., 2020]. Under ESD in non-sex changing fish, the same molecular battle is waged earlier in ontogeny and with a more decisive outcome, thus study of these 2 phenomena will inform each other.

Just as aromatase (*cyp19a1*) plays an important role in ESD, its expression is downregulated at the initiation of sex change in several protogynous species [ricefield eel: Zhang et al., 2013; red spotted grouper: Li et al., 2006] due to an increase in promoter region methylation [Zhang et al., 2013; Todd et al., 2019] which may directly interfere with transcription factor binding. While changes in expression of several key genes have been noted in both protogynous and protandrous systems, further study is needed to elucidate the exact sequence and timing. Here, the study of mechanisms related to primary ESD may also shed light on sex change. Because the brain is thought to initiate sex change, brain aromatase has been a target of study, but results are inconsistent or contradictory, highlighting the importance of species-specific functions [blue banded gobies: Black et al., 2005; black sea bass: Breton et al., 2015; bluehead wrasse: Todd et al., 2019; ricefield eel: Zhang et al., 2008].

These interesting exemplars span the teleost clade and represent different patterns of sex change [Godwin, 2010]. What they lack is a rigorous phylogenetic comparative approach at the mechanistic level with which to explore the evolution of mechanisms mediating sex change and transdetermination. Though more is known at behavioral and physiological levels [Kazancıoğlu and Alonzo, 2010; Erisman et al., 2013], such comparative approaches at the genetic and even epigenetic level have begun in wrasses [Kuwamura et al., 2020], gobies [Sunobe et al., 2017], and clownfish [Southey et al., 2020]. Thus far, findings suggest that key components of the molecular machinery controlling gonadal sex change are phylogenetically conserved at the family level, while neural pathways governing behavioral sex change may be more variable [Thomas et al., 2019].

Sex Change in Cichlids

Among cichlids, Peters [1975] suggested the possibility of adult protandrous sex change in *Pseudotropheus lombardoi* based on field sampling. However, controlled

experiments attempting to reproduce this failed at the histological level, revealing only immature oocytes in all males' testes, which are thought to be a remnant of a intersexual juvenile period [Naish and Ribbink, 1990]. Stauffer and Ruffing [2008] reported a study of female mouthbrooders, *Pseudotropheus livingstonii*, in which females in an iso-female population developed male-like coloration and head shape. In 2 instances, females produced viable broods said to be the result of egg fertilization by these transformed individuals. This claim should be interpreted with caution, because no histological analysis, which is an important criterion for claims of sex change [Sadovy and Shapiro, 1987], was conducted. Protogynous sequential hermaphroditism was suggested in the South American cichlid *Crenicara punctulata* based on behavioral observations and further supported by an experimental manipulation of social isolation and verified through histological analysis of gonads to reveal testicular tissue [Carruth, 2000]. Despite initial support [Oldfield, 2005], even this last example is generally not considered to be an example of true sex change by experts in the field.

Despite the rarity of sequential hermaphroditism in cichlids, their plasticity with regard to sex-typical behavioral and secondary sexual characteristics provides valuable insight to the underlying physiological and neural mechanisms. The use of iso-female populations to induce male-typical secondary sexual characteristics and behavior in otherwise functional females has proven useful in the study of neural gene expression associated with these behaviors [O'Connell et al., 2013; Renn et al., 2016]. Similarly, the ability to induce behavioral reversal of sex-biased behaviors that parallel evolutionary reversal of sex-biased behaviors has been used to address behavior, hormone regulation, and neural gene expression [Schumer et al., 2011; Wood et al., 2014].

Intrasexual Variation and ESD

An underexamined aspect of ESD in fishes is the effect that the same environmental factor may have on traits other than gonadal sex, including behavioral traits. Both the *Apistogramma* and *P. pulcher* systems feature alternative reproductive tactics (ARTs) [Taborsky and Brockmann, 2010] in which males are of one or another alternative morphs, each with different life history strategies. Römer [2001] reports that *Apistogramma* cichlid species always have crypto-female sneaker males, who resemble females in external morphology and achieve fertilizations

by sneaking in to inseminate the spawn of a female and her territorial male. Among *P. pulcher*, the 2 male color morphs, red and yellow, identified by Heiligenberg [1965], have been shown to follow different life history strategies, with the red morph biased towards harem breeding and the yellow towards monogamous breeding [Martin and Taborsky, 1997]. Those same low pH environmental conditions that produce more males also result in a greater proportion of males being of the red morph, suggesting that the mechanism of sexual differentiation is linked to intra-sexual variation [Reddon and Hurd, 2013]. Red and yellow males show a range of behavioral differences in traits such as aggression and activity levels, some but not all, consistent with the pattern that red males are “hypermasculinized” compared to yellow males [Seaver and Hurd, 2017].

This pattern is similar to effects seen in some lizards. Variation in environmental sex-determining factors, e.g., temperature during the critical period of sex determination, in leopard geckos (*Eublepharis macularius*) has a life-long organizational effect on sexual and aggressive behavior variation within sexes [Rhen and Crews, 1999; Huang and Crews, 2012]. The alternative male morphs seen in many lizards, with alternative male reproductive tactics associated with different colored throat badges [reviewed in Stuart-Fox et al., 2020], is strongly reminiscent of the *P. pulcher* system. Research into the relationship between the mechanisms of sexual differentiation and the organization of male morph differences in these species in the framework of the organizational and activation effects of hormones has been extremely fruitful [Moore et al., 1998]. Theoretical work [Lande et al., 2001] based on laboratory and field observations of ecology, male and female mating behavior, and inheritance of sex determination and color polymorphisms has suggested multiple pathways for rapid sympatric speciation. In the model, when the evolution of novel color morphs was combined with strong assortative mating, it would promote speciation when both sex reversal and suppressor genes are incorporated. Empirical data support the connection between color morphs, assortative mating, and rapid speciation for species in the genus *Apistogramma* [Estivals et al., 2020]. It could be argued that ESD could speed up or inhibit such speciation via sex reversal. Given that ESD and environmentally determined alternative color morphs are commonly coincident in cichlids, this system could be used to test the theoretical model.

Because brain aromatase expression has a demonstrably complex relationship with behavioral variation, epigenetic regulation of this gene provides a logical link be-

tween ESD and environmentally induced behavioral effects. There is higher expression of the B-copy of *cyp19a1* in the brains of territorial male peacock blenny *Salarias pavo* [Goncalves et al., 2008], black-faced blennies *Tripterygion delaisi* [Schunter et al., 2014], and bluegill sunfish *Lepomis macrochirus* [Partridge et al., 2016]. However, the opposite effect is seen in plainfin midshipman *Porichthys notatus* [Fergus and Bass, 2013], and no significant difference is seen in wrasse alternative male morphs [Todd et al., 2018]. In teleost fish, sex differences in the brain may be more plastic and dependent on immediate social and environmental factors, following a similar process of “on-going battle between 2 competing gene networks” as does primary sex determination. The combination of both ESD and alternative reproductive tactics in cichlids could provide an opportunity to disentangle the processes of gonadal sex determination and differentiation from the differentiation of behavioral life histories in the brain.

ESD as Phenotypic Plasticity

As is evident from the topics covered above, ESD is a fascinating case of developmental plasticity, a field with a rich treatment in evolutionary biology. Developmental plasticity is considered to either buffer the effects of environmental variation, slowing an evolutionary response to selection, or to promote evolutionary diversification by allowing populations to persist. Just as we see in ESD, the mechanisms that orchestrate the response to the environment are themselves under selection [Beldade et al., 2011]. Though there is strong selection for an equal sex ratio, ESD can allow sex ratio skew under favorable conditions. In some instances, selection may favor genetic changes that alter the nature or sensitivity of plasticity, a process referred to as genetic accommodation [West-Eberhard, 1989; Pigliucci and Murren, 2003]. At the genetic level, it has been suggested that sex chromosomes could evolve from ancestral autosomes when one chromosome hosts more epigenetic methyl groups near sex controlling regions [Gorelick, 2003]. Because methylation also can suppress recombination, the rate of chromosome evolution could itself be impacted by the mechanism we see in ESD [Jablonka, 2004]. Understanding the epigenetic regulation of ESD systems may therefore shed light on the evolution of sex determination systems more generally. Cichlids, where the number of described sex chromosome systems far exceeds the diversity in other vertebrate systems, evolved over a similar timescale [Gammerdinger

and Kocher, 2018] and are thought to contribute to species diversity through post-zygotic barriers [da Costa et al., 2019], providing particularly rich comparative fodder. A complete understanding of these processes will benefit from emerging technologies that allow for the combination of transcriptomics and epigenetic analyses as the relevant epigenetic marks will differ by tissue and even down to the single cell level [Angermueller et al., 2016; Linker et al., 2019].

Conclusion

By studying ESD in cichlids we have the opportunity to use a phylogenetic and comparative approach to understand the evolution of sex determination mechanisms and their impact on the evolution of sex-determining systems. There is abundant variation in the mechanisms of differentiation evidenced by the varying effects of environmental variables and their interaction on congener species. The behavioral richness of the cichlid system further allows for the investigation of the processes behind the epigenetic differentiation of the brain into different life history strategies. A more detailed developmental time course and discrete dissection of tissue in fry is needed to identify the epigenetic marks introduced by environmental factors, leading to male versus female development or male morph expression. These marks are more than likely to be part of the ongoing balance between competing networks of masculinizing and feminizing genes. The ever-growing genomic

resources for cichlids [Brawand et al., 2014; Salzburger, 2018; Faber-Hammond et al., 2019] and the integrative and active research community surrounding the behavior [York et al., 2015; Maruska and Fernald, 2018], ecology [Golcher-Benavides and Wagner, 2019], and evolution [Wagner et al., 2012; Kornfield and Smith, 2000] as well as commercial aspects of this group provide exciting opportunities.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

The authors contributed equally to the conception and writing of this review.

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