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## Epigenetic mechanisms in sex determination and in the evolutionary transitions between sexual systems

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## 2 3 **Epigenetic mechanisms in sex determination and in the** 4 **evolutionary transitions between sexual systems**

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### 10 11 12 **Abstract**

13  
14 The hypothesis that epigenetic mechanisms of gene expression regulation have two  
15 main roles at least in vertebrate sex is presented. First, and within a given generation,  
16 by contributing to the acquisition of: a) the male *or* female function once during the  
17 lifetime in gonochoristic species, b) the male *and* female function in the same  
18 individual, either at the same time in simultaneous hermaphrodites, or first as one sex  
19 and then as the other in sequential hermaphrodites. Second, if environmental conditions  
20 change, epigenetic mechanisms may have also a role across generations, by providing  
21 the necessary phenotypic plasticity to facilitate the transition: a) from one sexual system  
22 to another, or b) from one sex determining mechanism to another. Furthermore, if  
23 environmental change lasts enough time, epimutations could facilitate assimilation into  
24 genetic changes that stabilize the new sexual system or sex determining mechanism.  
25 Examples supporting these assertions are presented, caveats or difficulties and  
26 knowledge gaps identified, and possible ways to test this hypothesis suggested.

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29 **Keywords:** Epigenetics, DNA methylation, Histone modifications, genetic  
30 assimilation, sexual system, hermaphroditism, sex determining mechanisms, sex  
31 chromosomes, evolutionary transitions, Williams' paradox

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34 **Running head:** Epigenetics, sex fate and plasticity  
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## 1. Introduction

To explain how epigenetics can contribute to sex determination and to the evolutionary transitions between different forms of sex, first it is necessary to briefly define some terms used throughout the text, since hermaphroditism, one of several types of sexual systems, has been regarded as epigenetic sex determination (e.g., Beukeboom and Perrin, 2014). Next, examples concerning the increasing evidence for a role of epigenetics in sex determination, maintenance and plasticity in different taxa will be presented. Finally, the idea that epigenetics participates in the evolutionary transitions between different sexual systems and between different sex determining mechanisms will be discussed.

### 1.1. Sexual systems

To describe sex, two scales of classification have traditionally been used: 1) the sexual system and 2) the sex determining mechanism (see section 1.2. below). A sexual system, according to Leonard (2018), is defined as the pattern of sex allocation that exists among individuals of a given species. In general, the two most stable and abundant sexual systems, both in plants and animals, are dioecy (gonochorism) and hermaphroditism. In the former, male (sperm production) and female (egg production) functions are separated in different individuals, while in the latter male and female functions take place in the same individual, either simultaneously or sequentially. Sequential hermaphrodites reach adulthood and sexually mature first as one sex. Then the gonads experience extensive tissue reorganization and the animal changes sex to the opposite sex. This process is termed sex change. However, there are other mixed systems such as androdioecy (species consisting of males and hermaphrodites), that are less stable and thus less widely distributed (Leonard, 2018; Weeks, 2006).

Sexual systems in organisms can be viewed as exhibiting different degrees of phenotypic plasticity, with gonochorism and genetic sex determination at one end (e.g., mammals, birds and many insects), having a minimum of sexual plasticity; and, at the opposite end, simultaneous hermaphroditism (e.g., some fishes, many different types of invertebrates), with a maximum of sexual plasticity (Leonard, 2013, 2018). Transitions between different sexual systems are common and have been documented in different groups of vertebrates (Leonard, 2018; Pennell et al., 2018). Leonard (2018) argued that evolutionary transitions between different sexual systems, specifically between gonochorists and simultaneous hermaphrodites, would occur through intermediate stages consisting of species with environmental sex determination or sequential hermaphroditism.

Based on what has been discussed so far, it seems clear that phenotypic plasticity is an inherent property in the diversity of sexual systems and is necessary for the evolutionary transitions among them. As we will see below, epigenetics underlies phenotypic plasticity.

### 1.2. Sex determining mechanisms

Sex determining mechanisms can be classified into two major types according to the nature of the main sex determining factor: 1) genotypic sex determination (GSD), where the factor is genetic, with different mechanisms (chromosomal, polygenic), and

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3 87 2) environmental sex determination (ESD), where the main factor is an environmental  
4 88 cue. In ESD, at least in fish (Ospina-Álvarez and Piferrer, 2008) and reptiles  
5 89 (Valenzuela et al., 2003), temperature is the most common environmental factor  
6 90 (temperature-dependent sex determination, TSD). However, in many species sex  
7 91 determination actually depends on the contribution of both genetic and environmental  
8 92 factors (Devlin and Nagahama, 2002; Uller and Helanterä, 2011; Piferrer et al., 2012;  
9 93 Holleley et al., 2015). Nowadays, sex determining mechanism are viewed across a  
10 94 gradient of possibilities in which “pure” GSD and ESD species just represent opposite  
11 95 ends of this continuous gradient, with many possible intermediate combinations (Sarre  
12 96 et al., 2004).  
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15 97

16 98 It may be argued that hermaphrodites can be regarded as a form of ESD. However,  
17 99 simultaneous hermaphrodites develop gonads with male and female function taking  
18 100 place at the same time or within a short period of time, so ESD controlling primary sex  
19 101 determination here is problematic. Or at least should not be considered as done for  
20 102 gonochoristic species because all individuals develop gonads having both male and  
21 103 female tissues. It may be argued that in the same way that sex determination in a GSD  
22 104 gonochoristic species takes place at the individual level among individuals of a  
23 105 population, in simultaneous hermaphrodites sex determination takes place at the  
24 106 gonadal level between the male and female parts of the gonads of an individual. On the  
25 107 other hand, in sequential hermaphrodites, individuals of a given species typically first  
26 108 reproduce as females (protogynous species) or as males (protandrous species) and  
27 109 hence the first sex seems also genetically established. However, the genetic factors  
28 110 responsible for primary sexual development and the underlying molecular mechanisms  
29 111 are poorly understood, although based on current evidence is safe to assume that they  
30 112 use the same toolkit and gene networks involved in sexual development in  
31 113 gonochoristic species (Liu et al., 2017). The influence of the environment in primary  
32 114 sexual development in hermaphrodites is, in contrast, plausible in protogynous diandric  
33 115 (two types of males: males can differentiate directly or from females through sex  
34 116 change) and protandrous digynic (two types of females: females and differentiate  
35 117 directly or from males through sex change) species (Leonard, 2018). For example, in  
36 118 the diandric wrasse, *Halichoeres poecilopterus*, terminal phase (TP) males are large  
37 119 territorial males with bright body coloration and are derived either from initial phase  
38 120 (IP) females that change sex to male or from IP primary males that change colour and  
39 121 behaviour, but do not change sex. By performing cohabitation experiments involving  
40 122 different types of fish, it was found that TP transition in primary males was related to a  
41 123 dominance relationship (or size order) within social groups (Sakai et al., 2007). Aside  
42 124 from these cases, in sequential hermaphrodites, then, what is usually environmentally  
43 125 controlled is not the first sex that differentiates but, rather, the process of sex change,  
44 126 which can be regarded as a trans-differentiation of the adult gonad from the production  
45 127 of one type of gametes to the opposite type. Sex change in sequential hermaphrodites  
46 128 is thus environmentally controlled, but the environmental factor is usually biotic or  
47 129 social (presence of dominant conspecifics, population density, population sex ratio)  
48 130 (Liu et al., 2017), and thus different from the abiotic factors such as temperature  
49 131 controlling sex determination in gonochoristic species with ESD. In sequential  
50 132 hermaphrodites, be it either protogyny (female-to-male sex change) of protandry (male-  
51 133 to-female sex change), there is a complete reorganization of the gonadal tissues (Liu et  
52 134 al., 2017). With these considerations made, **figure 1** provides a picture that combines  
53 135 sexual systems and sex determining mechanisms under the same framework.  
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3 137 There is abundant literature on the evolution of sex chromosomes and new sex  
4 138 determining mechanisms. Models include genetic drift, pleiotropic selection of sex  
5 139 determining genes, sex ratio selection, and sexually antagonistic selection (Werren and  
6 140 Beukeboom, 1998; Sarre et al., 2011; Kamiya et al., 2012; van Doorn, 2014; Sander  
7 141 van Doorn, 2014; Pennell et al., 2018) (See also the paper by Perrin in this issue). Thus,  
8 142 transitions between different mechanisms of sex determination are not only common  
9 143 but, importantly, can take place in a relatively short time. For example, turnover in the  
10 144 sex chromosomes and the sex-determining gene in medaka, *Oryzias latipes*, and its  
11 145 closely related species can occur in short evolutionary times (Kikuchi and Hamaguchi,  
12 146 2013). The divergence of *O. curvinotus*, which has *dmY* as its sex determining gene,  
13 147 from *O. luzonensis*, which has *gsdf*, was calculated to occur ~10 million years ago  
14 148 (Kondo et al., 2004). If sex reversal (i.e., animals with a sexual phenotype opposite to  
15 149 their sex chromosome constitution) is brought in, then changes from GSD to TSD is  
16 150 possible in a single generation, as shown in the central bearded dragon, *Pogona*  
17 151 *vitticeps*, in which elevated temperatures sex reverse ZZ males into functional females  
18 152 eliminating the W chromosome and becoming TSD (Holleley et al., 2015).  
19 153

20 154 It has been argued that current models do not take into account the underlying  
21 155 developmental mechanisms, and that the increasing availability of molecular data will  
22 156 help to clarify how selection and developmental architecture interact to direct the  
23 157 evolution of sex determination genes (van Doorn, 2014). Epigenetics, in addition to  
24 158 phenotypic plasticity, also underlies cell fate commitment (as in sex determination and  
25 159 differentiation) and tissue reorganization (as in sex change and possibly also in sex  
26 160 reversal). Thus, the following section presents examples of the accumulating evidence  
27 161 for a role of epigenetic regulatory mechanisms in sex acquisition, maintenance and  
28 162 plasticity.  
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## 30 164

### 31 165 **2. Epigenetics and sex determination and differentiation**

#### 32 166

#### 33 167 2.1. Brief introduction to epigenetics

34 168  
35 169 Epigenetics involves a set of chemical modifications either directly to the cytosine  
36 170 bases of DNA or to the histone proteins that constitute the chromatin and package the  
37 171 genome. The main epigenetic mechanisms are DNA methylation and histone  
38 172 modifications (methylation, acetylation, etc.) and variants. These modifications  
39 173 influence how genes are expressed by regulating chromatin structure and gene  
40 174 accessibility (Deans and Maggert, 2015). Epigenetic regulatory mechanisms have a key  
41 175 role in controlling gene expression across a diverse array of developmental stages,  
42 176 tissue types, physiological states and environmental signals (Carja and Feldman, 2012).  
43 177 Epigenetic changes can be inherited not only during mitosis from mother to daughter  
44 178 cells but also through meiosis from parents to offspring, thus contributing to the  
45 179 transmission of acquired states of gene expression during cell and tissue differentiation  
46 180 within and across generations (Dupont et al., 2009). Furthermore, epigenetic  
47 181 mechanisms are susceptible to environmental change and thus have a major role in  
48 182 integrating genomic and environmental information to bring about the phenotype  
49 183 (Turner, 2009; Vogt, 2017). Species with sexual lability, and hermaphrodites in  
50 184 particular, constitute clear examples of phenotypic plasticity since both sexes can be  
51 185 produced from the same genotype. Thus, hermaphroditism has been considered as the  
52 186 product of an epigenetic sexual determination system (Beukeboom and Perrin, 2014).



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188 2.2. Epigenetics and sex chromosome evolution

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190 The implication of epigenetics on sex chromosome evolution was first proposed by  
191 Gorelick (2003) who argued that initial differences between sexes (without focusing on  
192 any particular group) are determined by differential methylation in nuclear DNA  
193 between females and males, driving Muller's ratchet. The same principle has also been  
194 proposed for the situation in mammals, with male heterogamety (Jablonka, 2004), and  
195 in birds, with female heterogamety (Uller and Badyaev, 2009). According to this view,  
196 differences in methylation of sex chromosomes lead to recombination suppression  
197 increasing mutation rate and further accelerating the speed of Muller's ratchet  
198 (Gorelick, 2003). Evolution of sex chromosomes via methylation is challenging to test,  
199 as recently discussed by Furman et al. (2020), for three main reasons: 1) because the  
200 relationship between DNA methylation and gene expression can be dependent on the  
201 genomic region being considered (e.g., Anastasiadi et al., 2018), 2) the fact that  
202 methylation regulation can occur in *trans*, i.e., it may affect distant loci with enhancer  
203 or repressor activity, and 3) that methylation of specific loci may be erased during  
204 gamete formation (Furman et al., 2020). Nevertheless, using whole genomic bisulfite  
205 sequencing (WGBS), different DNA methylation profiles between the sex  
206 chromosomes have been found, for example, in the half-smooth tongue sole,  
207 *Cynoglossus semilaevis* (ZW/ZZ system) (Chen et al., 2014; Shao et al., 2014), and in  
208 the threespine stickleback, *Gasterosteus aculeatus* (XX/XY system) (Metzger and  
209 Schulte, 2018). Taken together, these observations clearly indicate sex-related  
210 differences in DNA methylation of sex chromosomes but whether these differences are  
211 a cause or a consequence of sex chromosome differentiation still remains to be  
212 elucidated.

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215 2.3. Epigenetics and sex fate commitment

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217 In the last years, evidence has been accumulating on the implication of epigenetics in  
218 regulating the expression of key genes involved in sexual development, from plants to  
219 animals (Piferrer, 2013). The rest of this section will mostly deal with examples on the  
220 epigenetic differences between those genes, focusing on vertebrates.

221  
222 The involvement of epigenetic regulatory mechanisms in sexual development is now  
223 supported by studies conducted in both GSD and ESD species in different taxa. The  
224 first evidence linking temperature to gene expression via epigenetics in vertebrates was  
225 obtained in the European sea bass, *Dicentrarchus labrax* (polygenic sex determination,  
226 PSD), where elevated temperature results in an increase in the number of males. This  
227 was related to hypermethylation of the promoter of gonadal aromatase (*cyp19a1a*), the  
228 enzyme responsible for oestrogen synthesis, and concomitant transcriptional  
229 downregulation of *cyp19a1a* (Navarro-Martín et al., 2011). Subsequent work on the  
230 same species showed that lasting effects of elevated temperature involved alterations  
231 in the expression of genes involved in different type of epigenetic regulation including  
232 euchromatic histone-lysine N-methyltransferase 2 (*ehmt2*), the histone demethylase  
233 Jumonji (*jarid2a*) and polycomb group ring finger 2 (*pcgf2*) (Díaz and Piferrer, 2015).  
234 In the half-smooth tongue sole (ZZ/ZW), elevated temperature resulted in  
235 hypomethylation of the *dmrt1* promoter, leading to masculinisation of ZW females, a  
236 change that was inherited in the unexposed ZW offspring, i.e., spontaneous sex reversal

237 without environmental stimuli (Shao et al., 2014). In this species, *dmrt1* is involved in  
238 sex determination (Chen et al., 2014). These results provide evidence of a common  
239 mechanism regulating GSD and ESD and their coexistence in the same species.

240  
241 In the red-eared slider turtle, *Trachemys scripta*, similar findings were made regarding  
242 the *cyp19a1* promoter, which was also hypomethylated in female-promoting  
243 temperature (FPT) (Matsumoto et al., 2013). Interestingly, FPT in turtles are elevated  
244 when compared to male-producing temperatures (MPT), whereas the situation is  
245 reversed in fish (Ospina-Álvarez and Piferrer, 2008). Nevertheless, in both fish and  
246 reptiles, MPT consistently involve hypomethylation of *cyp19a1a*. This suggests that  
247 signal transduction of the initial cue (elevated temperature) can have different outcomes  
248 between turtles and fish. Subsequent work in *T. scripta* confirmed previous findings  
249 and showed an association of promoter region hypomethylation with canonical  
250 transcriptional activation markers, H3K4me3 and RNA polymerase II (Matsumoto et  
251 al., 2016), indicating multi-layer epigenetic modifications in the regulation of sexual  
252 development. It was also demonstrated that *dmrt1* has a temperature-dependent,  
253 sexually dimorphic expression pattern, that is both necessary and sufficient to initiate  
254 male development in *T. scripta*, and that DNA methylation dynamics of its promoter  
255 were also correlated with temperature, suggesting that *dmrt1* is a candidate master male  
256 sex-determining gene in this TSD species (Ge et al., 2017). Furthermore, temperature  
257 is able to increase the transcription of lysine-specific demethylase 6B (*kdm6b*), a  
258 chromatin modifier gene that eliminates the trimethylation of H3K27 in the promoter  
259 of *dmrt1*, leading to upregulation of its expression and male development (Ge et al.,  
260 2018; Georges and Holleley, 2018) (see also the paper by Weber and colleagues in this  
261 issue). In mice, *Jmjd1a*, a H3K9 demethylase, controls expression of the mammalian  
262 Y chromosome sex-determining gene *Sry* by regulating H3K9me2 marks (Kuroki et  
263 al., 2013). Sex reversal is common in some species of rodents. In the Akodon grass  
264 mice, *Akodon azare*, some XY males with an intact Y chromosome with a non-mutated  
265 *Sry* gene develop as fertile females and the underlying cause is the result of epigenetic  
266 modifications in sex chromosomes (Sánchez et al., 2010). Thus, epigenetic regulatory  
267 mechanisms are also involved in mammalian sex determination (Garcia-Moreno et al.,  
268 2018).

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#### 271 2.4. Epigenetics, sex and mechanistic models

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273 Based on the current knowledge on the role of epigenetics in sexual development some  
274 mechanistic models have recently been proposed. Analysing data gathered in more than  
275 a dozen different species of fish, including both gonochoristic and hermaphroditic  
276 species, Piferrer (2019b) and Piferrer et al. (2019a) proposed the model of the  
277 Conserved Epigenetic Regulation of Sex (CERS). This model, based on the regulation  
278 of gene expression by DNA methylation (Anastasiadi et al., 2018a), contemplates sex-  
279 specific differences in DNA methylation and expression levels in genes involved in  
280 sexual development and, importantly, that these sex-specific differences are conserved  
281 regardless of taxa or the sexual system, i.e., whether one considers gonochoristic or  
282 hermaphrodite species. Thus, patterns of DNA methylation and gene expression are  
283 similar for primary male sex differentiation in gonochoristic species and for  
284 protogynous (female-to-male) sex change in hermaphrodite species because in both  
285 cases the end result is the same: a testis. Likewise, patterns are similar for female sex  
286 differentiation and protandrous sex change (male-to-female) with an ovary as the end



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3 287 result. Of note, the pattern is particularly consistent for key genes in the sexual  
4 288 development network such as *dmrt1* and *cyp19a1a*, and thus CERS likely can also be  
5 289 applied to reptiles based on the findings discussed earlier, while for other genes such as  
6 290 *amh*, *foxl2*, *sox9* and *gsdf* data in additional species is needed (Piferrer et al., 2019a).  
7 291

8 292 As stated above, CERS is based on data collected mostly from gonochoristic fish  
9 293 species but also from a few hermaphrodites, and is also compatible with findings so far  
10 294 in reptiles. Recently, further evidence for distinct epigenetic reprogramming and the  
11 295 involvement of the stress axis has been gathered concerning environmentally-induced  
12 296 sex plasticity in vertebrates in general (Geffroy and Douhard, 2019) and sex change in  
13 297 fish in particular (Hattori et al., 2020; Todd et al., 2019). The emerging picture is that  
14 298 epigenetic modifications constitute a critical link between environmental stimuli, the  
15 299 onset of sex change in hermaphrodites, and subsequent maintenance of sexual  
16 300 phenotype (Ortega-Recalde et al., 2020).  
17 301

18 302 Regarding the proximal sensor of environmental stimuli, it has been proposed that  
19 303 environmental cues are sensed through conserved elements of calcium and redox status  
20 304 that are transduced to cellular signal pathways, and/or influence epigenetic processes,  
21 305 to ultimately drive the differential expression of sex genes (the CaRe model) (Castelli  
22 306 et al., 2020). If proven correct, this would provide information on how cues such as  
23 307 temperature are transduced. However, it would need to be tested also in other  
24 308 circumstances, e.g., cues such as population density or population sex ratio driving sex  
25 309 change in sequential hermaphrodites. In any case, the CERS and CaRe models are  
26 310 compatible and complementary since in fact concern different parts and aspects of the  
27 311 pathway, from the initial environmental cue to the ultimate sexual fate. Histone  
28 312 modifications may be induced before methylation changes that then serve as more  
29 313 stable epigenetic marks (Duncan et al., 2014). The proposed mechanistic connection  
30 314 between environmental signals and sex determination pathways can be useful to direct  
31 315 further research.  
32 316

33 317 Most of the examples above concern DNA methylation and histone modifications but  
34 318 there is also evidence on long-term changes in miRNA expression—considered also a  
35 319 type of epigenetic regulation of gene expression—in response to environmental cues  
36 320 (Bizuayehu et al., 2012). Taken together, the findings discussed above provide  
37 321 mounting evidence for the involvement of epigenetic regulatory mechanisms in sex  
38 322 chromosome evolution and in sex determination and differentiation across different  
39 323 taxa. Next, the implication of epigenetics on evolutionary transitions will be discussed.  
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### 42 326 **3. Epigenetics as a hub for evolutionary transitions**

#### 43 327 44 328 3.1. Epigenetics and phenotypic responses to environmental variation 45 329

46 330 Epigenetic mechanisms can respond to environmental variation and facilitate  
47 331 phenotypic plasticity (Turner, 2009; Vogt, 2017). Novel phenotypic variants generated  
48 332 by epigenetic modifications in response to environmental change increase the  
49 333 evolutionary potential of a population because they promote genetic adaptation by  
50 334 different means (O’Dea et al., 2016). In addition, epigenetic modifications allow for  
51 335 more rapid phenotypic responses to novel environments than are possible via the  
52 336 accumulation of genetic variation (Kilvitis et al., 2017). Thus, it is well established that

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3 337 epigenetic variation is one of the most important contributors to phenotypic variation  
4 338 in a population (Biwer et al., 2020). The causal relationships between genetic,  
5 339 environmental, epigenetic and phenotypic variation are shown in **figure 2a**.  
6 340

### 8 341 3.2. Epigenetics and evolution

9 342  
10 343 The possibility that an epigenetic modification might give rise to a localized change in  
11 344 DNA sequence, thereby converting an epigenetic into a genetic change, has been  
12 345 contemplated for some time. Further, it has been argued that this chain of events  
13 346 constitutes a possible route through which the environment might directly influence  
14 347 evolution, provided the induced genetic change has phenotypic effects on which  
15 348 selection can act (Duncan et al., 2014; Turner, 2009). Empirical evidence supporting  
16 349 this possibility is being gathered in recent years.  
17 350

18 351 Danchin et al. (2019) reviewed the current knowledge on the role of epigenetics in  
19 352 favouring evolutionary change and proposed that epigenetics is a sort of hub for  
20 353 evolutionary transitions. The main idea is that when environmental changes remain  
21 354 stable for a sufficient number of generations, information inheritance systems gradually  
22 355 move from ones that are relatively labile to more faithful and persistent ones that, of  
23 356 note, can be set as genetic variants (Danchin et al., 2019). This idea relies mainly on  
24 357 DNA methylation and on the well-known hypermutability of methylated cytosine  
25 358 residues, susceptible to deamination and with a higher rate of change to thymine  
26 359 (epimutations) than non - methylated bases (Danchin et al., 2019). Because of their  
27 360 nature, these ideas are difficult to test and thus alternatives must be explored.  
28 361

29 362 Domestication can be regarded as just one of several driving forces of evolution where  
30 363 human-controlled artificial selection replaces natural selection (Zeller and Göttert,  
31 364 2019). Domesticates, therefore, provide good opportunities to test the possibility that  
32 365 environmentally-induced epimutations may end up as genetic variants. Support towards  
33 366 this possibility is available for domestic mammals (Kukekova et al., 2018), birds  
34 367 (Pértille et al., 2019) and fish (Anastasiadi and Piferrer, 2019). Thus, an increasing  
35 368 number of observations suggest that environmentally-induced epimutations may end  
36 369 up fixed as genetic modifications, provided the environmental change persists across a  
37 370 sufficient number of generations (**figure 2b**). Importantly, in this scenario a given  
38 371 environmental cue can alter the DNA methylation of a given loci *de novo* in each  
39 372 generation in what is termed “epigenetic wash-in” (Burggren, 2015). If these  
40 373 epimutations are persistent enough, and thanks to their ability to induce mutations, these  
41 374 mutations could eventually increase their frequency in the population and constitute the  
42 375 genetic basis for a new phenotype (Danchin et al., 2019).  
43 376

### 44 377 45 378 **4. A proposal: a role for epigenetics in regulating sex fate and plasticity**

46 379  
47 380 Based on accumulating evidence and on what has been discussed in the previous  
48 381 sections, epigenetic mechanisms may have two main roles, at least in vertebrate sex.  
49 382 First, and within a given generation, by contributing to the acquisition and maintenance  
50 383 of a) the male *or* female function once during the lifetime in gonochoristic species, b)  
51 384 the male *and* female function in the same individual, either at the same time in  
52 385 simultaneous hermaphrodites, or first as one sex and then as the other sex in sequential  
53 386 hermaphrodites. Second, if environmental conditions change, epigenetic mechanisms

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3 387 may have also a role by providing the necessary phenotypic plasticity to facilitate the  
4 388 transition a) from one sexual system to another, or b) from one sex determining  
5 389 mechanism to another. Furthermore, if the environmental change lasts enough time and  
6 390 there is transgenerational inheritance, epimutations could facilitate assimilation into  
7 391 genetic changes that stabilize the new sexual system or sex determining mechanism.  
8 392 There would be, therefore, two temporal axes for the action of epigenetic mechanisms  
9 393 regulating sex: one within the same generation, contributing to the acquisition and  
10 394 maintenance of sex; and another across generations (**Figure 3**).  
11 395

12 396 Regarding the main first role, i.e., sex determination and maintenance, epigenetic  
13 397 mechanisms would then contribute, in a given individual and regardless of whether the  
14 398 species to which that individual belongs is gonochoristic or hermaphroditic, to the  
15 399 regulation of the gene expression program necessary for sexual fate commitment.  
16 400 Accumulating evidence supports the involvement in this first role, as outlined above,  
17 401 and to gather additional evidence research in more species should be carried out. Thus,  
18 402 to fulfill key knowledge gaps we have that in species with ESD, there is a testable  
19 403 hypothesis on how the environmental cue can be captured and the signal transduced by  
20 404 cellular signalling pathways and epigenetic processes, the CaRe hypothesis (Castelli et  
21 405 al., 2020). Further, in n both GSD and ESD gonochoristic species, as well as in  
22 406 hermaphroditic species, there is the testable CERS model on the sex-specific relation  
23 407 between epigenetic activation or repression and gene expression concerning both pro-  
24 408 male and pro-female genes of the network (Piferrer et al., 2019). Both models provide  
25 409 a framework to advance our further understanding. Studies aimed at confirming the role  
26 410 of epigenetics in sex acquisition and maintenance should attempt to find: 1)  
27 411 mechanisms linking environmental perturbations to epigenetic changes by the analysis  
28 412 of molecules and signalling pathways, 2) finding functional consequences of epigenetic  
29 413 modifications, i.e., a sex being associated with a given set of epigenetic marks, as has  
30 414 it already been demonstrated in the European sea bass (Anastasiadi et al., 2018b), 3)  
31 415 determine whether epigenetic changes are cause or consequence of a given gene  
32 416 expression program. To this end, help can come from manipulations of the epigenome  
33 417 by the use of DNA methyltransferase inhibitors such as 5-aza-2'-deoxycytidine (5-  
34 418 aza-dC), shown to be able to alter sex ratios in zebrafish, *Danio rerio* (Ribas et al.,  
35 419 2017), or the more recently developed technique to edit the methylome in the  
36 420 mammalian genome (Liu et al., 2016), and 4) dealing with the fact that epigenetic marks  
37 421 mostly are cell specific. Thus, cell-specific analysis approaches, currently in  
38 422 development, are needed. Of note, besides the importance that one may give to the  
39 423 epigenetic regulation sex, it should not be forgotten the crucial role of transcription  
40 424 factors such as Sox9, Foxl2 and Dmrt1 in sex determination and maintenance (Garcia-  
41 425 Moreno et al., 2018). Thus, epigenetic mechanisms contribute and are necessary but  
42 426 not sufficient for the establishment of sex.  
43 427

44 428 Epigenetic differences between sex chromosomes are being found as the number of  
45 429 species being examined increases, as shown above. More research in the role of  
46 430 epigenetics in sex chromosome evolution is needed, and the contribution of DNA  
47 431 methylation in recombination suppression is compatible with the recent view that sex  
48 432 chromosome evolution is not necessarily a simple progression of accumulating  
49 433 divergence (Furman et al., 2020).  
50 434

51 435 Testing the second main role, i.e., the involvement of epigenetics in evolutionary  
52 436 transitions is more challenging and here there is an important knowledge gap.

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3 437 Deciphering the contribution of epigenetics in evolutionary transitions can benefit from  
4 438 ongoing efforts aiming at elucidating whether epigenetic modifications can facilitate  
5 439 the inheritance of novel phenotypic variants that are generated by environmental  
6 440 change, a strategy called ‘heritable bet hedging’ (O’Dea et al., 2016). Of note,  
7 441 environmentally-induced epigenetic changes can also produce heritable maladaptive  
8 442 phenotypes, a phenomenon termed ‘epigenetic traps’ (O’Dea et al., 2016). In this  
9 443 regard, it has been argued that epigenetically-mediated alterations in sex ratios could  
10 444 become an epigenetic trap in ESD species facing rapid climate change by consistently  
11 445 producing heavily skewed sex ratios (Consuegra and Rodríguez López, 2016; but see  
12 446 also Piferrer, 2016).  
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16 448 To test the role of epigenetics in evolutionary transitions between sexual systems, taxa  
17 449 where the sexual system varies by order, family, genus or even species, such as  
18 450 Cnidaria, polychaetes and teleost fish (Leonard, 2018) would be most appropriate. The  
19 451 use of techniques such as WGBS could allow the identification of differentially  
20 452 methylated cytosines (DMCs) or differentially methylated regions (DMRs) in the  
21 453 gonads (provided they are examined at exactly the same developmental time and stage  
22 454 of the reproductive cycle) in key genes involved in sex determination. Closely-related  
23 455 and sympatric species with contrasting sexual systems such as, for example, *Diplodus*  
24 456 *puntazzo* (gonochorist) vs. *D. annularis* (protandrous) (Pla et al., 2020), would be  
25 457 appropriate.  
26  
27 458

28 459 Recently, assessment of patterns in the evolution of sex-determination systems in the  
29 460 diverse vertebrate clades of teleost fish, squamate reptiles and amphibians evidenced  
30 461 not only similar transition rates between homomorphic and heteromorphic sex  
31 462 chromosomes in both fish and amphibians but also to ESD from heteromorphic vs.  
32 463 homomorphic sex chromosome systems in fish (Pennell et al., 2018). These  
33 464 observations would then not support the view that heteromorphic sex chromosomes can  
34 465 be a sort of ‘evolutionary trap’ (Pokorná and Kratochvíl, 2009). Thus, to test the role  
35 466 of epigenetics in evolutionary transitions between sex determining systems, taxa where  
36 467 frequent transitions between sex determining systems occur can be useful.  
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40 469 In this regard, in amphibians the ancestral system is thought to be ZW/ZZ with multiple  
41 470 transitions to XX/XY. Transitions are frequent and can be seen in "real time", as some  
42 471 populations of *Glandirana* (formerly *Rana*) *rugosa* from Japan have a male  
43 472 heterogamety (XX/XY) while others have female heterogamety (ZW/ZZ), with  
44 473 different degrees of transition. It is believed that the transition from the ZW/ZZ system  
45 474 to a XX/XY has occurred at least twice independently (Miura, 2007; Nakamura, 2009)  
46 475 Theoretical models predict homology between the W and X chromosomes and the Y  
47 476 and Z chromosomes and it has been suggested that the dominant master sex-  
48 477 determining gene of one heterogametic system could be the dosage-dependent master  
49 478 gene in the other (Quinn et al., 2011). Analysing DNA methylation in the sex  
50 479 chromosomes and key genes of the sex determination cascade would provide insights  
51 480 on the role of epigenetics in the regulation of gene expression in these contrasting sex  
52 481 determining systems. Not only species with chromosomal sex determination (CSD)  
53 482 would be appropriate. Species with PSD in a group of species with CSD could also be  
54 483 a good model to test evolutionary transitions from one genetic sex determination system  
55 484 to another, where the coexistence of newly emerged and ancestral sex determining  
56 485 genes would be expected. For example, it would be worth exploring whether observed  
57 486 differences in the evolutionary trajectories and expression levels of aromatase genes in



487 African cichlids, (Böhne et al., 2013) are related to changes in epigenetic transcriptional  
488 regulation.

489  
490 Finally, the detection of epigenetically-facilitated mutations is challenging and will  
491 need specific experiments (Danchin et al., 2019). Epigenetic changes must occur in the  
492 germline for any evolutionary significance. Among vertebrates, fish may have an  
493 advantage since they show little or low reprogramming of epigenetic marks during  
494 gametogenesis when compared to mammals (Anastasiadi et al., 2018b; Ortega-Recalde  
495 et al., 2019; Ortega-Recalde and Hore, 2019; Wang and Bhandari, 2020), facilitating  
496 the transmission of epigenetic marks from parents to offspring. Inheritance of of  
497 epigenetic marks across one or more generations in association with the influence of  
498 environmental factors such as hormones (Xu et al., 2014), hypoxia (Wang et al., 2016)  
499 and temperature (Ryu et al., 2018) has been reported. In this regard, it is worth noting  
500 that exposure of zebrafish to elevated temperature is able to induce sex ratio shifts and  
501 alterations in the testicular epigenome of the unexposed offspring (Valdivieso et al.,  
502 2020).

503  
504 The eventual identification of differences in the role of epigenetics in sexual fate,  
505 plasticity and evolution across large taxa perhaps could contribute to shed light on why  
506 certain phyla and classes are quite labile and sexual system varies even within a genus,  
507 in some cases, whereas in others are very rigid with little or no diversity. Thus, the  
508 large-scale distribution of sexual systems is best explained by phylogeny rather than by  
509 sex allocation theory, a situation known as Williams' paradox. (Leonard, 2018, 2013).  
510 Although phylogenetic differences in epigenetic regulation are known (Zemach et al.,  
511 2010) whether this affects sex determination and sexual systems is at present unknown.

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3 **825 Figure legends**

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5 827 **Figure 1.** Diagram representing the integration of sexual systems and mechanisms of  
6 828 sex determination in a common framework. The different states are represented as  
7 829 discrete although in fact there is a continuum gradient of possible, intermediate, states.  
8 830 The idea of the evolutionary transitions along a gradient of phenotypic plasticity was  
9 831 postulated by (Leonard, 2013, 2018). Epigenetic mechanisms underlie and made  
10 832 possible this phenotypic plasticity (see text). Figure redrawn and updated from Piferrer  
11 833 (2019a).  
12 834

13 835 **Figure 2.** Epigenetic variation as a link between genetic variation and environmental  
14 836 variation that ultimately influence phenotypic variation. (a) General model.  
15 837 Environmental variation induces epimutations contributing, in addition of stochastic  
16 838 epimutations, to epigenetic variation. If the direction of environmental change persists  
17 839 in a sufficient number of generations, epimutations may end up assimilated as genetic  
18 840 variants. Both genetic and environmental variation (through epigenetics) influence  
19 841 gene expression and, in turn, phenotypic variation. (b) Mechanistic model by which  
20 842 persisting environmental variation across generations (i, ..., i+n) can alter methylation  
21 843 (lollipop) of CpG loci (left), and actual association of environmentally-induced  
22 844 epimutations (DMCs) with on-the-spot SNPs in two sea bass populations (right). The  
23 845 number of overlaps of the two genomic sites is shown. The shaded grey area shows the  
24 846 number of overlaps of randomized regions with the mean represented by the black bar.  
25 847 The green line represents the actual number of overlaps of SNPs with DMCs and the  
26 848 double arrow its distance from the significance limit in red. The significance of the  
27 849 association is indicated by the z-score and the P value (modified from (Anastasiadi and  
28 850 Piferrer, 2019).  
29 851

30 852 **Figure 3.** Summary of the proposal made in this review: epigenetic mechanisms have  
31 853 two main roles in the regulation of vertebrate sex. First, with one generation and at the  
32 854 individual level, by contributing to the acquisition of the male or female phenotype  
33 855 during sex determination/differentiation in gonochoristic species, or the acquisition of  
34 856 the male and female phenotypes at the same time in simultaneous hermaphrodites or  
35 857 first as one sex and then as the other in sequential hermaphrodites. In the latter, they  
36 858 are involved in the process of sex change and in gonochoristic species in sex reversal  
37 859 if that occurs (e.g., due to environmental perturbations during early sensitive periods).  
38 860 Second, across generations, by promoting the evolutionary transitions between one  
39 861 GSD system to another (GSD-1 to GSD-2) or to ESD (GSD-1 to ESD), and between  
40 862 any of these to the different forms of hermaphroditism. As shown in this review, there  
41 863 is evidence supporting the implication of epigenetics in developmental processes (blue  
42 864 arrows) but further evidence is needed to clearly support evolutionary transitions (red  
43 865 arrows).  
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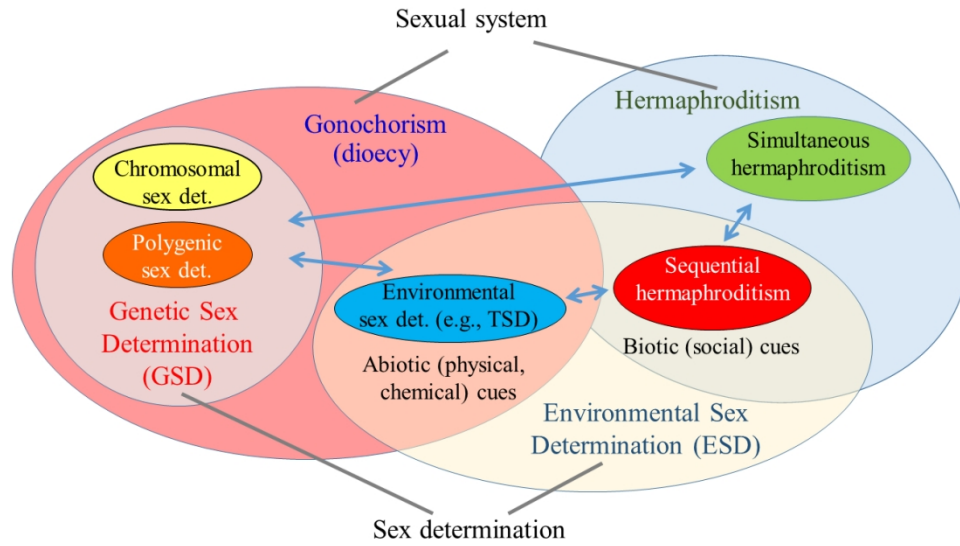


Figure 1

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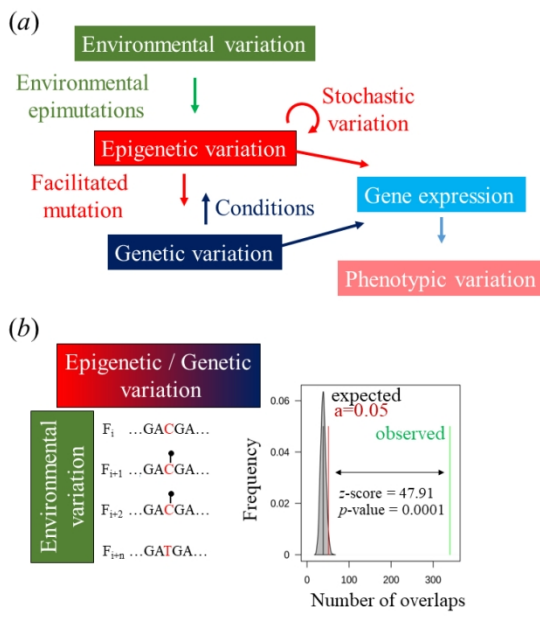


Figure 2

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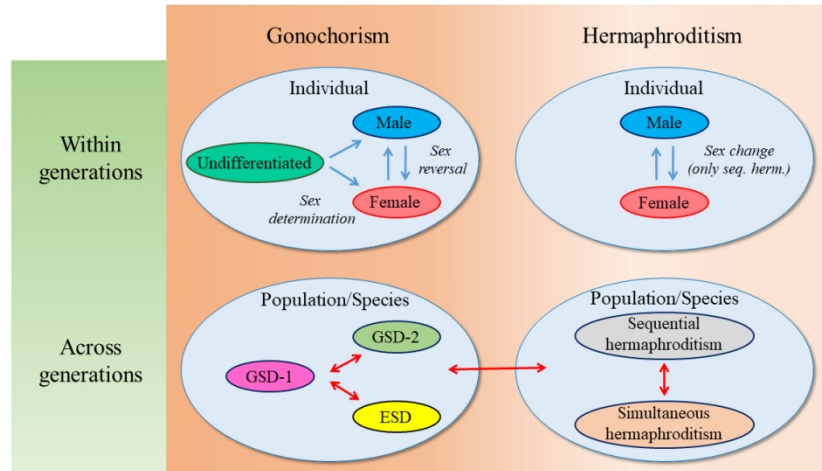


Figure 3

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