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EPIGENETIC STUDIES IN ECOLOGY AND EVOLUTION

Sexually antagonistic epigenetic marks that canalize sexually dimorphic development

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Abstract

The sexes share the same autosomal genomes, yet sexual dimorphism is common due to sex-specific gene expression. When present, XX and XY karyotypes trigger alternate regulatory cascades that determine sex-specific gene expression profiles. In mammals, secretion of testosterone (T) by the testes during foetal development is the master switch influencing the gene expression pathways (male vs. female) that will be followed, but many genes have sex-specific expression prior to T secretion. Environmental factors, like endocrine disruptors and mimics, can interfere with sexual development. However, sex-specific ontogeny can be canalized by the production of epigenetic marks (epimarks) generated during early ontogeny that increase sensitivity of XY embryos to T and decrease sensitivity of XX embryos. Here, we integrate and synthesize the evidence indicating that canalizing epimarks are produced during early ontogeny. We will also describe the evidence that such epimarks sometimes carry over across generations and produce mosaicism in which some traits are discordant with the gonad. Such carryover epimarks are sexually antagonistic because they benefit the individual in which they were formed (via canalization) but harm opposite-sex offspring when they fail to erase across generations and produce gonad-trait discordances. SA-epimarks have the potential to: i) magnify phenotypic variation for many sexually selected traits, ii) generate overlap along many dimensions of the masculinity/femininity spectrum, and iii) influence medically important gonad-trait discordances like cryptorchidism, hypospadias and idiopathic hirsutism.

Keywords: canalization, epigenetics, gonad-trait discordance, sex-specific epimarks, sexual conflict, sexual dimorphism, transgenerational epigenetics

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Introduction

Sexual conflict within the genome is divided into two major categories (Arnqvist and Rowe 2005; Rice & Gavrilets 2014). Intralocus conflict occurs when the optimal allele at a locus differs between the sexes, causing adaptive evolution by one sex to be at the fitness expense of the other sex (Haldane 1926; Mandel 1971). Allelomorphs with opposing effects in the two sexes are referred to as sexually antagonistic alleles (Rice 1984).

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The second category of sexual conflict is called interlocus conflict (Parker 1979; Arnqvist & Rowe 1995; Chapman *et al.* 1995; Rice 1996; Rice & Holland 1997; Gavrilets *et al.* 2001; Rice and Gavrilets 2014). Here, alleles at different gene loci affect a shared interaction trait, with one locus influencing the female component of the trait and the other locus influencing the male component. Males and females usually interact in the context of a dyad to determine the outcome of the interaction trait (e.g., the decisions of whether or not to mate or whether each individual mates monogamously or promiscuously) and the optimal outcome of the interaction differs between the sexes.

Epigenetics creates the opportunity for a new and fundamentally different form of sexual conflict based on epigenetic rather than genetic variation: sexually antagonistic epigenetic conflict (Rice *et al.* 2012). The foundation for this conflict is a sex-specific epigenetic mark that sometimes fails to erase across generations. For the conflict to be manifest, the sex-specific epigenetic marks (i.e., DNA methylation and/or histone tail modification, hereafter called 'epimarks') must be an integral part of a sexually dimorphic ontogenetic process, that function to masculinize or feminize somatic gene expression (and the organismal-level phenotypes that they influence) during ontogeny. Most such epimarks will be absent in the germ line because they first appear too late in ontogeny to be shared between the soma and germ line via a coalescent mitotic lineage, and hence will not be carried-over trans-generationally. However, this barrier is absent for sex-specific epimarks that are produced prior to the separation of cell lineages between the germ line and soma, e.g., in embryonic stem cells (ESCs).

In mammals, the function of these embryonic, sex-specific epimarks is to control sexually dimorphic development prior to the secretion of testosterone (T) by the testes, and/or to canalize (i.e., resist change in response to environmental and genetic perturbations) sex-specific ontogenetic responses to sex steroids. Most sexual dimorphism in mammals, however, is a response to foetal androgen signalling (foetal oestrogen levels have little or no effect on mammalian sexual dimorphism), as well as androgen or oestrogen signalling that commences at puberty (Thornton *et al.* 2009; and more fully described below). Because of this overarching influence of sex hormone signalling in the ontogeny of sexual dimorphism in mammals, we will focus here on sex-specific epimarks in the early embryo that canalize down-stream, sexually dimorphic ontogeny that occurs in response to sex steroid signalling, e.g., those epimarks that reduce the impact of natural variation in circulating sex steroid levels and/or environmental endocrine disruptors and steroid mimics on sexually dimorphic development. Later in the discussion, we will consider SA-epimarks that operate outside the context of sex hormone signalling, as well as sex-specific epimarks that are produced in parallel in both the germ line and the soma during foetal development. Conflict occurs when these epigenetic marks: (i) fail to erase across generations, and (ii) are inherited by opposite-sex offspring and influence their ontogeny. Such an epimark increases Darwinian fitness of the parent where it originated because it canalizes the parent's sexual development, but it reduces fitness in opposite-sex offspring by contributing to discordance between the gonadal sex and a sexually dimorphic trait. We will refer to the epimarks causing this epigenetic conflict as sexually antagonistic epigenetic marks –abbreviated as SA-epimarks (Rice *et al.* 2012, 2013).

The details of the formation, molecular composition, expression and transmission of SA-epimarks are expected to differ between taxonomic groups because the types and relative importance of different kinds of epigenetic marks differ substantially among taxa. Here, we will focus on mammals as a model taxa owing to the extensive epigenetic research that has been done on this group. However, the logic of SA-epigenetic marks that we develop in mammals should be applicable, in a general sense, to other groups.

We have previously detailed the evidence for the existence of SA-epimarks in the context of human sexual orientation (Rice *et al.* 2012) and we have also described a general protocol to screen for the causative epimarks using embryonic and hair-follicle stem cells (Rice *et al.* 2013). Here, we focus on the broader context of SA-epimarks influencing any sexually dimorphic trait. At this time, we have not identified and characterized specific SA-epimarks. Nonetheless, just as Watson & Crick (1953a,b) combined many disparate pieces of information to support a hypothesis for the structure and semiconservative replication of DNA –that motivated pivotal tests of this hypothesis, like that of Meselson & Stahl (1958)– our approach here is to follow the logic of these exemplary studies by integrating the many disparate lines of evidence that SA-epimarks contribute to a new and unappreciated form of sexual conflict.

Requisite conditions for SA-epimarks

The existence of SA-epimarks, that canalize sex-specific ontogeny in response to sex hormone signalling (Fig. 1), is strongly motivated only if a well-defined set of requisite conditions is met, which we enumerate in detail below. In short, sexually dimorphic ontogeny must be canalized, the canalization must be advantageous due to developmental ambiguities, epimarks must contribute to the canalization and sometimes carry over across generations to opposite-sex offspring, and mutations coding for the epimarks must accumulate in response to selection. More specifically:

1 Sexually dimorphic development is canalized. In mammals, this would require evidence that (i) XX foetuses have reduced sensitivity to elevated T, and XY foetuses have increased sensitivity to elevated T, and (ii) starting at puberty, XY males have increased sensitivity to T and decreased sensitivity to oestrogens, *vice versa* for XX females.

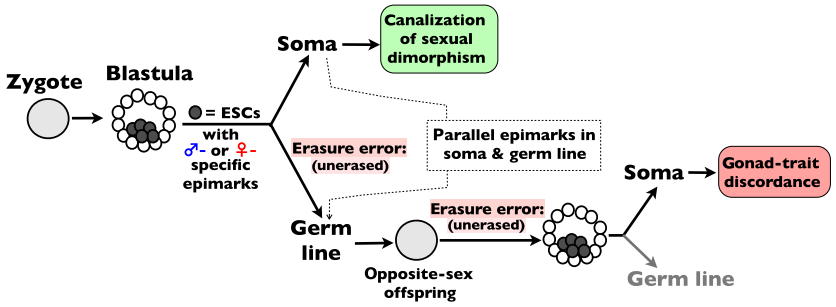

Fig. 1 Sexually antagonistic epigenetic marks (SA-epimarks) occur when a sex-specific epimark –that canalizes sexually dimorphic phenotypes during later ontogeny– is produced in the embryonic stem cell stage (ESC) and fails to erase during early germ line differentiation and also during early embryonic development in opposite-sex offspring. The transgenerationally inherited, sex-specific epimark contributes to gonad-trait discordance in the next generation. SA epimarks can also arise from sex-specific epimarks produced during later development when they are produced in parallel in both the soma and the germ line.

2 There is need (i.e., a fitness advantage) for canalization of sexually dimorphic development. If there is no fitness benefit in the parent that produced them, then carryover epimarks produce no sexual antagonism. In mammals, this would require evidence for sufficient ambiguity in (i) foetal androgen signalling, and/or (ii) androgen or oestrogen signalling after puberty, to make canalizing epimarks advantageous. Sexual ambiguity in sex hormone signalling would be expected to arise due to intrinsic variation in sex-specific levels of sex hormones and/or environmental endocrine disruptors and androgen and oestrogen mimics.
3 Sex-specific epimarks produced during early ontogeny influence down-stream sexually dimorphic development, i.e. there are sex-specific epimarks that are produced sufficiently early in ontogeny that they can canalize sexually dimorphic development later in ontogeny. In mammals, this would require evidence that sex-specific epimarks are produced prior the onset of testicular production of T for traits that become sexually dimorphic during later foetal development.
4 Some epigenetic marks are shared by both the soma and the germ line, i.e., they are mitotically inherited by both cell lineages from a common progenitor cell (s), or they are produced in parallel in both cell lineages. In mammals this would require evidence that sex-specific epimarks are produced in embryonic stem cells before the separation of soma and germ line, and/or that sex-specific epimarks produced later in development are generated in parallel in both the soma and germ line.
5 Epigenetic marks sometimes carry over across generations and influence sexual development of opposite-sex offspring. In mammals this requires evidence that epimarks can escape the nearly genome-wide erasure that occurs while the primordial germ cells migrate to the genital ridge and somatic gonads, as well the nearly genome-wide erasure that occurs during the first few cleavage-stage cell cycles after syngamy.
6 Mutations that cause SA-epimarks can deterministically spread in a population. This requires that feasible selection parameters are within the range supporting the accumulation of new mutations coding for the epimarks.

Below we evaluate these requisite conditions in mammals.

Evidence for requisite conditions

Sexually dimorphic development is canalized

Evidence for the substantial canalization of sexually dimorphic development in mammals can be found in the phenotypes in humans produced by loss-of-function mutations that influence foetal androgen signalling. In human ontogeny, as in other mammals, circulating levels of androgens are the major determinants of sexual dimorphism of the genitals and brain (summarized in Thornton *et al.* 2009): XX females have lower levels of foetal T and XY males have higher levels. The elevated foetal T in males does not occur until week 8 of human foetal development when the testes begin secreting T.

Null mutations at the gene coding for the androgen receptor (AR_{null}) completely block canonical androgen signalling and cause XY foetuses to have nearly completely sex-reversed development, with female-typical genitalia, brains, body proportions and composition, and behaviour –with the exception of the upper third of the vagina which is controlled by the absence of SRY-signalling rather than androgen-signalling (reviewed in Wisniewski *et al.* 2008). Note that the XY individuals (homozygous AR_{Null}) produce substantial amounts of

oestrogen due to the enzymatic conversion of T secreted by their testes. These null mutations demonstrate the overarching influence of foetal androgen signalling on human sexual dimorphism.

In contrast, mutations that strongly lower or elevate T have surprisingly small effects. Null mutations in the CYP21 gene block an intermediate step in the conversion of cholesterol to cortisol. The accumulating intermediates are converted to T causing XX foetuses to experience male-typical levels of T throughout foetal development (Speiser & White 2003; New 2004; Trakakis *et al.* 2009). Despite this male-typical phenotype for circulating foetal T, the external genitalia are only partially masculinized (Hall *et al.* 2004) as is childhood behaviour (Hines 2011). Although population-wide levels of transsexuality and homosexuality are weakly elevated in the subpopulation that is homozygous-null for CYP21, most individuals are female-typical for sexual identity and sexuality (reviewed in Hines 2011). The strongly feminine phenotypes produced by these loss-of-function mutations demonstrate the strong insensitivity (canalization) of XX foetuses to even highly elevated levels of circulating androgens.

Null mutations in the 17β-HSD-3 locus prevent the testes from secreting T during foetal development (reviewed in Rey & Grinspon 2011). Instead, the testes produce the precursor to T in the cholesterol-to-T pathway: the weakly androgenic androstenedione, which has about a hundred-fold weaker affinity for the androgen receptor (Fang *et al.* 2003). Despite this markedly reduced level of androgen signalling, most internal genitalia (epididymis, vas deferens, seminal vesicles and ejaculatory duct) develop normal, or close to normal, male phenotypes. The external genitalia are substantially feminized and most affected newborns are reared as females. But at puberty, when there is a T-surge due to a nontesticular allozyme of 18β-HSD-3 converting circulating, testes-produced androstenedione to T, about half of the affected individuals change their sex to male (Wisniewski *et al.* 2008): the same rate as XY-males with normal circulating T levels throughout development that were reared as girls for other reasons (Reiner & Gearhart 2004). Although the data are limited in number, they indicate that homozygous 18β-HSD-3_{Null} individuals have male-typical heterosexual orientation (Imperato-Mcginley *et al.* 1979; Meyer-Bahlburg 2005). Collectively these data indicate that XY foetuses have strong insensitivity (canalization) to even very low levels of androgen signalling.

The phenotypes of individuals with null mutations at the AR, CPY21 and 17β-HSD-3 loci provide compelling evidence that human male (XY) and female (XX) foetuses are strongly canalized with respect to even extreme sex-atypical levels of foetal androgen signalling.

A need for canalization of sexually dimorphic development

Evidence for a fitness benefit favouring the canalization of androgen signalling described in the above section comes from studies of circulating levels of T in male and female foetuses. If there is substantial overlap in circulating T between male and female foetuses during androgen signalling, then there will be a benefit to canalization of the androgen signal (XX embryos hyposensitive to T, and XY hypersensitive to T) because it reduces ambiguity in the androgen signal, and thereby reduces the expression of intersex phenotypes (quantitative details can be found in Rice *et al.* 2012). Data on humans (Reyes *et al.* 1974; Perera *et al.* 1987) and rats (Weisz & Ward 1980) demonstrate substantial overlap between the sexes in circulating levels of T throughout foetal development. Despite this overlap, including the time when the external sex organs are differentiating, discordance between gonad and genitalia (phallus vs. vulva, including ambiguous genitalia) is rare, in both rats and humans (Ostby *et al.* 1999; Sax 2002; Hotchkiss *et al.* 2007).

Sex-specific epimarks produced in the embryo and early-foetal stages influence down-stream sexually dimorphic development

In mammals, there is epigenetic re-programming across the genome during the embryonic stem cell stage (reviewed in Hemberger *et al.* 2009). Thousands of genes that are expressed only late in development are silenced via DNA methylation of their promotors (Fouse *et al.* 2008). Many hundreds of other genes expressed in later development are bivalently marked with dominant, silencing epimarks (trimethylation of the lysine-27 residue of histone H3, i.e. H3K27me3) and recessive, activating epimarks (trimethylation of the lysine-4 residue of histone H3, i.e., H3K4me3). When released from the suppressing H3K27me3 epimark, there is a strong genome-wide correlation (0.67) between the level of H3K4me3 histone modification and the level of gene expression (Mikkelsen *et al.* 2007).

Evidence for sex-specific epimarks during early ontogeny comes from many sources. Gardner *et al.* (2010) surveyed evidence showing that preimplantation blastocyst embryos are sexually dimorphic far in advance of androgen signalling: including the phenotypes growth rate, metabolic rate and resistance to several environmental stressors. Bermejo-Alvarez *et al.* (2010) estimated that 31% of the transcriptome is sexually dimorphic in

expression level at the blastocysts stage of cattle, and in the mouse, Lowe *et al.* (2015) found 51 genes that were sexually dimorphically expressed in the eight cell cleavage stage embryo (most X-linked) and 566 in the blastula stage (most autosomal). At the gene promotor level, two genes have been identified in the bovine blastocysts in which the promotor was more heavily methylated in one sex compared to the other (Bermejo-Alvarez *et al.* 2008; Gebert *et al.* 2009), and this sex-specific methylation may be influenced by differences in the expression of both DNA and histone tail methyltransferases found in the blastocysts of both cattle (Bermejo-Alvarez *et al.* 2011) and mice (Penaloza *et al.* 2014). Dewing *et al.* (2003) found XX vs. XY differences in gene expression in mouse embryonic brains prior to secretion of T by the foetal testes in XY males (51 genes, most of which were autosomal). Also in the mouse, Penaloza *et al.* (2014) examined the promotors of four genes with sexually dimorphic expression in the embryonic day-10.5 embryo (prior to T secretion by the testes). They found sexually dimorphic methylation of promotor CpG sites at all four genes, and when they reduced this dimorphism with DNA methyltransferase antagonists in cell culture, the sexual dimorphism was markedly reduced. This study also demonstrated that sexually dimorphic CpG methylation prior to androgen signalling was strongly associated with new sexually dimorphic CpG methylation produced in response to androgen signalling. Collectively, these studies indicate that the XX vs. XY karyotype somehow influences (in trans) the expression of many genes during later embryo development (but before the testes start secreting T) in a manner that is independent of androgen signalling, and that sexually dimorphic epimarks produced before androgen signalling can feasibly contribute to new epimarks formation later in development in response to androgen signalling.

How could such strong sexual dimorphism precede T signalling during early ontogeny? Li *et al.* (2014) used genome-wide CHIP/Seq to determine how many DNA locations bind SRY protein (a transcription factor) in early stage embryos (embryonic day-11.5) prior to the start of androgen signalling. They found 3083 unique DNA binding sites. Data from Fiddler *et al.* (1995) in humans, and Silversides *et al.* (2012) in mice, demonstrate that SRY is expressed in preimplantation embryos as well as later stage foetuses (prior to androgen signalling). Data from Banovich *et al.* (2014) and Tsankov *et al.* (2015) indicate that binding to chromatin by transcription factors changes the access of these chromatin regions to both DNA and histone tail methyltransferases. Sekido (2014) has enumerated the diverse ways in which SRY can act to produce XY-specific epigenetic effects including: recruiting enzymes for DNA methylation and histone tail modifications, controlling alternative RNA splicing, regulating sex-specific miRNA levels and acting as an RNA sponge that regulate transcript levels of ncRNAs. Collectively these data suggest that SRY binding may play an important role in the production of sex-specific epigenetic marks in early-stage embryos that originate far in advance of the onset of androgen signalling.

Some epigenetic marks are shared by both the soma and the germ line

In the mouse model system of mammalian development, there is extensive epigenetic reprograming during the embryonic stem cell stage prior to the separation of the germ line and soma (Hemberger *et al.* 2009), and these epimarks can influence gene expression later in ontogeny (Mikkelsen *et al.* 2007). As the primordial germ cells migrate to the genital ridge, these epimarks are erased and then the genome is immediately reprogrammed with new, gamete-specific epimarks (Reik *et al.* 2001; Lees-Murdock & Walsh 2008). One way to have a somatic SA-epimark shared between germ line and soma is a failure of the epimark to erase in the early primordial germ cell stage. Evidence for this lack of erasure comes from studies of epimarks that were environmentally induced (epimutations) in the parent (e.g., by subjecting the parent to stress or endocrine disruptors) and then carried over across one or more generations to offspring and grand-offspring. For example, hundreds of different chemically induced DNA methylation epimarks (and the phenotypes they produce) in the laboratory rat can persist through the patriline across three generations (Manikkam *et al.* 2012; Skinner & Guerrero-Bosagna 2014). To do so, the epimarks must persist through the broad-scale epimark erasure that occurs in the primordial germ cells. These experiments demonstrate that many hundreds of epimarks in embryonic stem cells can be transmitted to both the soma and germ line (and are therefore shared between them), and that they can be transmitted across generations.

Epigenetic marks sometimes carry over across generations and influence sexual development of opposite-sex offspring

An epimark produced in embryonic stem cells that canalizes sexually dimorphic development will carry over transgenerationally only if it can escape erasure: (i) in the early cell divisions of the primordial germ cells that give rise to the germ line, and also (ii) during the nearly global erasure that occurs during the first few cleavage-stage cell divisions of the embryo. In the rodent model system, we now have evidence that it is

not uncommon for methylated CpG sites to evade both erasures: more than 500 hundred chemically induced epimutations have been shown to be transmitted via the patriline for at least three generations (Manikkam *et al.* 2012). But can such epimarks influence sexual development in opposite sex offspring? Recent work in mice supports this outcome. Morgan & Bale (2011) exposed mothers to recurrent unpredictable episodes of mild stress (e.g., leaving the lights on all night, changing the bedding numerous times in a single day, and using damp instead of dry bedding when it was changed) during the first 7 days of foetal development. Sons from these stressed mothers were feminized as measured by ano-genital distance and their sex-reversed response to a stress test. When these sons were mated to unrelated females experiencing no stress during pregnancy, their sons (F_2) also displayed these feminized phenotypes: demonstrating transgenerational inheritance. When the transcriptome of the brains (PNDI region) from of these feminized sons was compared to control F_2 males and females, the sons from *in utero*-stressed fathers showed extensive feminization of their transcriptome –which was associated with feminization of three miRNAs with sexually dimorphic expression. This study clearly demonstrates how transgenerational epigenetic factors can strongly influence (and reverse) sexually dimorphic gene expression and development. It also illustrates the potential for a cascading effect in which a reversal in the expression of a few genes (miRNA in this case) can feasibly lead to much larger sex-reversals in the transcriptome and adult behaviour.

Further evidence that carryover epimarks at one or few genes can have a large effect in reversing sexual dimorphism comes from a recent study in mice (Nugent *et al.* 2015). These researchers found that the preoptic area (POA) in mice becomes sexually dimorphic in response to androgen signalling during the first week of postnatal development. Sexual dimorphism in this brain area influences male mating behaviour in adults. In the neonate, developing sexual dimorphism in the POA is associated with 70 genes with sexually dimorphic transcription rates. Female pups treated with simulated androgen signalling (estradiol [E2], the end-product of androgen signalling in the rodent brain) recapitulated the male phenotype, both in brain structure and adult mating behaviour. Androgen signalling in both males and females resulted in strong down-regulation of DNA methyltransferase (DNMT) activity and reduced the genome-wide number of sites with strongly methylated CpGs. To determine if this epigenetic (DNA methylation) effect mediated the androgen signal, they treated female pups with DNMT antagonists and found strong masculinization of (i) the transcriptome of the POA, the POA cellular structure and adult sexual behaviour. These experiments demonstrate that the organizational effect of androgen signalling on the POA is mediated in large part by epimarks (DNA methylation induced by androgen signalling), and that it is feasible for carryover epimarks influencing one of the DNMT genes to strongly reverse a component of sexual dimorphism (brain structure and mating behaviour) that is controlled by many genes simultaneously.

Mutations that cause SA-epimarks can spread deterministically in a population

SA-epimarks are both beneficial to the parent that produced them and harmful to opposite-sex offspring when the epimarks carry over (un-erased) across generations and contribute to gonad-trait discordance. These costs (C) and benefits (B), however, are expected to be highly asymmetrical. The benefit to the parent producing the epimark is expressed 100% of the time, whereas the detriment is expressed only when (i) the epimark carries over across generations (at rate q) and (ii) it is transmitted to an opposite-sex offspring (at rate 0.5, assuming an even sex ratio). When the trans-generational carryover rate is small, the cost benefit ratio will also be small. Previously we have solved for the conditions under which a new mutation coding for an SA-epimark will accumulate in the gene pool (Rice *et al.* 2012). For an autosomal mutation that epimarks itself or a tightly linked location, the mutation will accumulate when $C/B < 4/q$. So even when transgenerational carryover is substantial ($q = 0.04$), a mutation producing the SA epimark will accumulate whenever $C/B < 100$. For example, a mutation that increased fitness of the parent that produced it by 1% would invade the gene pool even if it lowered the fitness of recipient opposite-sex offspring by 99%. These calculations demonstrate that there is remarkably little selective constraint on the accumulation of mutations coding for new SA-epimarks.

Further details concerning X- and Y-linkage and recombinational distance between the mutation and the epimark, as well as additional evidence for each of the other five requisite conditions, can be found in Rice *et al.* (2012).

Discussion

The SA-epimarks hypothesis is summarized in Fig. 1. The experimental work described in the above sections provides a strong empirical foundation for the existence of SA-epimarks in nature, and the epigenetic sexual conflict they generate. Loss of function mutations in the androgen receptor (AR_{null}, leading to XY sex-reversed

females), and enzymes in steroid biosynthesis pathways (cholesterol-to-cortisol [$CYP21_{null}$, leading to male-typical T in XX foetuses] and cholesterol-to-T [17β-$HSD\text{-}3_{null}$, leading to greatly diminished androgen levels in XY foetuses]) demonstrate the overarching influence of foetal androgen signalling on mammalian sexual dimorphism, and the strong canalization of male and female development (i.e., insensitivity to departures from male- and female-typical levels of foetal androgens). The experiments with cattle using sex-sorted embryos (Bermejo-Alvarez *et al.* 2008; Gebert *et al.* 2009) clearly demonstrate that there are sex-specific epimarks and extensive sexually dimorphic gene expression in the early embryo stage –far in advance of foetal androgen signalling. Experiments on mice reinforce this conclusion in both the preimplantation embryo (Lowe *et al.* 2015) and the early foetal stages (Penaloza *et al.* 2014). Recent experiments with mice further provide evidence for a causative link between sex-specific epimarks and sexually dimorphic gene expression (Penaloza *et al.* 2014; Nugent *et al.* 2015) and adult phenotypes (Nugent *et al.* 2015). The experiments by Skinner and collaborators (e.g., Manikkam *et al.* 2012) on endocrine disruptors demonstrate that it is not uncommon for environmentally induced epimarks (epimutations) to escape cross-generation erasure and carry over across one or more generations. The experiments by Morgan & Bale (2011) demonstrate that stress-induced epimarks can both carry over across generations and generate substantive gonad-trait discordance. Collectively these experimental results make it plausible –we would conclude nearly inevitable– that some newly evolved, sex-specific epimarks will sometimes carry over across generations and contribute to epigenetic sexual conflict via SA-epimarks.

In mammals most sexual dimorphism is controlled by sex hormone signalling with 'organization' influences during the foetal and perinatal stages by androgens and 'activational' influences (that are contingent on the earlier organizational androgen signalling) that commences at puberty and are mediated by both androgens and oestrogens (summarized in Thornton *et al.* 2009). For this reason, the most potent SA-epimarks will be those that differentially influence sex hormone signalling in XX vs. XY individuals. Nonetheless, SA-epimarks can be produced outside this sex hormone signalling context. As described above, mammalian embryos display sexually dimorphic phenotypes far in advance of T production by the XY foetus. Y-linked gene products, like the SRY protein, and the concentration of X-linked gene products from loci that are not dosage compensated, provide an unambiguous signal of gender that is independent of sex hormones. These XX vs. XY signals must somehow contribute to the sex-specific gene expression and phenotypes observed prior to the secretion of T by the foetal testes (as described in the preceding paragraph), and any epimarks they influence would be sexually antagonistic when they are shared between the soma and germ line and also sometimes carry over across generations and lead to discordance between the sex chromosome karyotype and gene expression.

Up to this point, we have focused on SA-epimarks originating during the embryonic stem cell (ESC) stage. The rationale for this focus was the nearly genome-wide epigenetic reprogramming that occurs in ESCs, and the fact that these epimarks – when unerased in early cell cycles of the primordial germ cells– are mitotically inherited by both the soma and the germ line, and hence can be shared by parent and opposite-sex offspring. Franklin *et al.* (2010) exposed male mice (F_1) to stress (prolonged separation from F_0 mothers) over the first 14 days of postpartum development and observed changes in: (i) behaviour (a depressive-like response during a forced-swim assay), and (ii) the methylation status of two genes, *Crhr2* and *Mecp2 (candidate genes influencing depressive-like behaviour)*. The behaviour change was observed in the F_1 males and their F_2 daughters (and their F_3 grandsons). The methylation changes were observed in both the F_1 male's sperm as well the brains of his F_2 female offspring (brains of the F_1 males were, unfortunately, not assayed). Because the stress treatment occurred after the separation of the male's soma and germ line, and because the depressive-like behaviour was observed in both F_1 males and their F_2 daughters, functionally parallel (though not necessarily identical) epigenetic changes were feasibly produced in both the soma and the germ lines and transmitted to the next generation. The transgenerational epigenetic change in the germ line was supported by the parallel methylation changes seen in the sperm of the F_1 males and in the brains of their F_2 daughters. This study supports –but does not conclusively prove– that epigenetic marks can be shared between the soma and germ line when they are not mitotically inherited from the same ancestral cell(s) in which the epimark was initially produced. A possible route to this parallel-epimark-production in soma and germ line is shared miRNAs. Morgan & Bale (2011) found that miRNAs were associated with strong transgenerational feminization of the brains of sons from fathers whose mothers experienced stress during early pregnancy, and Sekido (2014) reviews evidence that the brain and gonad share miRNAs that influence somatic sexual dimorphism. If an epimarking pathway for a miRNA is shared and expressed by the soma and gonad during foetal development, parallel epimarking could lead to shared epimarks without their mitotic coalescence. While this parallel-epimark-production route to feminizing and

masculinizing epimarks can feasibly operate in nature, it is still unclear to us whether or not it has a substantive role in SA-epimark propagation.

SA-epimarks feasibly contribute to many features of the sexual dimorphic phenotype. One feature is the masculinity/femininity spectrum. Although the sex of most individuals is unambiguously dichotomous –male or female– many sexually dimorphic traits are distributed along a continuum between the male and female poles –with substantial overlap between the sexes. For example, human facial features have been extensively quantified by evolutionary biologists because the human face is a strong contributor to sexual attractiveness. Lee *et al.* (2014) used discriminant analysis of human facial landmarks to compare male and female faces. Their discriminant scores were strongly overlapping between the sexes despite a strong difference between the means for each sex. What causes some men to have substantially feminized faces and *vice versa* for women? Environmentally induced stochastic variation may account for some of the overlap, but the high heritability of masculinity/femininity of human faces (Mitchem *et al.* 2014) indicates that deterministic factors play a large role. Sexually antagonistic alleles could contribute to the heritability of the masculinity/femininity of human faces but theory predicts low polymorphism for SA-alleles unless they are X-linked with strong deviations from additivity (Rice 1984) or there is a reversal of dominance between the sexes (Kidwell *et al.* 1977; Fry 2010; but see Arnqvist *et al.* 2014 for the case of polygenic inheritance with epistasis). Alternatively, SA-epimarks could be the major factor contributing to the masculinization of traits like faces of females, and *vice versa* for males. In this case, genetic polymorphism would be unnecessary because masculinization and feminization is produced by carryover epimarks in opposite-sex offspring (even when the mutations coding for the epimarks are fixed) rather than genetic variation (Rice *et al.* 2012).

SA-epimarks may also feasibly contribute substantially to clinically important gonad-trait discordances like hypospadias (urethra length reduced [feminized] in males, causing a subterminal opening of the urethra along the phallus), cryptorchidism (testes position feminized due to their abdominal location), and idiopathic hirsutism (male-like body-hair pattern in women that is unassociated with hormonal imbalances). All of these gonad-trait discordances are surprisingly common. Hypospadias has a prevalence 0.4% to 1% in newborns, and can be as high as 4% when including milder cases (Boisen *et al.* 2005). The prevalence of cryptorchidism ranges between 2 and 9% (Bay *et al.* 2011), and that of idiopathic hirsutism is 6% (Carmina 1998). The high incidence of these gonad-trait discordances is enigmatic because the two male discordances substantially reduce fertility, and the female discordance may also reduce fitness via sexual selection. No major effect genes have been found for any of these conditions despite screens with large sample sizes (see overview in Rice *et al.* 2012), yet recent screens –despite very small sample sizes– have found changes in both the methylome (Choudhry *et al.* 2012) and the transcriptome (Karabulut *et al.* 2013) of hypospadias patients. The observations described in this paragraph collectively motivate the hypothesis that SA-epimarks contribute to clinically important gonad-trait discordances.

Homosexuality represents another gonad-trait discordance. It constitutes a substantial component of humanity's sexuality spectrum, with an estimated prevalence 8% in both sexes in a large study in Australia (with homosexuality being classified as a Kinsey score >0; Bailey *et al.* 2000). Homosexuality in both sexes runs in families and has higher concordance between monozygotic compared to dizygotic twins (reviewed in Ngun *et al.* 2011). Although early GWASs found conflicting results, a recent large GWAS found two QTLs (one X-linked and the other on autosome 8) associated male homosexuality (Sanders *et al.* 2015). There is also robust evidence for a birth order effect, in which males with more older brother are more likely to be homosexual (reviewed in Bogaert & Skorska 2011). In females, null mutations in the CYP21 are associated with a weak but statistically significant increase in female homosexuality (reviewed in Hines 2011). Nonetheless, the birth order effect can explain at most only only one in seven homosexual men (Cantor *et al.* 2002) and no homosexual women, and the two QTLs uncovered by Sanders *et al.* (2015) for male homosexuality have only weak effects, i.e., no genes of major effect on homosexuality have been uncovered despite large sampling effort in males. As we detailed elsewhere, SA-epimarks provide a feasible (Rice *et al.* 2012), and testable (Rice *et al.* 2013) etiology for human homosexuality.

The information we have used to motivate and evaluate the SA-epimarks hypothesis necessarily relies heavily on studies on humans and mammalian model organisms (rodents and primates) because medical research on epigenetics far exceeds basic research with wild, nonmodel organisms. Nonetheless, the logical foundation for SA-epimarks in humans and mammalian model organisms should apply broadly to other taxa. The core features leading to SA-epimarks are (i) sexual dimorphism and a need for its canalization, and (ii) sex-specific epimarks and their transgenerational carryover. When these features are present, the potential for SA-epimarks is manifest even if the specific molecular mechanisms for epigenetic marking differ among taxa. Substantial sexual dimorphism in structure and/or

behaviour is common among many taxa with separate sexes and a broad-scale need for canalization is indicated by the broad range of anthropogenic and naturally occurring sex steroid mimics and disruptors influencing both vertebrates and invertebrates (e.g., see Łebkowska & Załęska-Radziwiłł 2007). Although sex-specific epimarks are only well characterized in humans and laboratory model organisms (e.g., see examples in this paper), transgenerational carryover of epigenetic marks has been documented in taxa as diverse as plants, worms, flies, fish, birds, and mammals (Ho & Burggren 2010). These patterns indicate that SA-epimarks have the potential to contribute to sexual conflict among a wide diversity of plants and animals.

The hypothesis that SA-epimarks exist and contribute to a new and important form of sexual conflict is supported by many lines of indirect evidence (detailed above and in Rice *et al.* 2012) that show that all the requisite conditions exist for their adaptive advantage and their production at nontrivial frequency. Given their high feasibility, the next step will be to screen for SA-epimarks. With the advent of genome-wide screening for histone-tail modifications via CHIP/seq, and for DNA methylation of CpGs (and also CHG and CHH, where H is A, C or T) via genome-wide bisulphate resequencing, it should now be possible to unambiguously screen for transgenerational sex-specific epimarks, determine if they occur at biologically important levels, and statistically evaluate whether they are associated with gonad-trait discordances. Ongoing screens for an epigenetic etiology of clinically important gonad-trait discordances, like cryptorchidism, hypospadias and hirsutism, will probably be the first studies to provide the requisite data in the near future (e.g., like those recently published by Choudhry *et al.* 2012 and Karabulut *et al.* 2013). At present, we already have evidence for methylome and gene expression difference between individuals with and without hypospadias. The critical, next step in testing for SA-epimarks will be determining: (i) when these epimarks are produced (in the embryonic stem cell stage or later in development, and if in later ontogeny, are they produce in parallel in both the germ line and the soma?) and the biological function of the epimarks (do they influence sexually dimorphic development and/or its canalizatiion?). At this point we can conclude that the SA-epimarks hypothesis is plausible and falsifiable, and the data needed to test this hypothesis are accessible with current technology and will be forthcoming on the near horizon.

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