INTRODUCTION

Sexual reproduction is the rule in the vast majority of animals, and reproduction by sexual recombination results in powerful selective forces acting to optimize male and female phenotypes. Given the ancient origin of the two sexes in the animal kingdom as well as the selection pressures that hone and maintain them, it would seem reasonable to expect the genetic pathways underlying sex determination to be highly conserved, especially given the speed at which deleterious mutations causing intersex or infertile individuals will be removed from populations. Although there are some examples of conserved sex determination mechanisms in animals, such as in birds (Mank and Ellegren 2007; Smith et al. 2009) and therian mammals (Graves 2006), there is overwhelming evidence that these are the exceptions and that sex determination can be regulated by different genes even in relatively closely related species. Thus, although male and female sexual phenotypes are conserved, the regulatory control of sex determination is evolutionarily labile and varies substantially across the animal phylogeny (Gempe and Beye 2010).

Beyond its interest to those studying the evolution of sex, the rapid change in a key developmental pathway makes sex determination an excellent model for studying the...
evolution of genetic networks (Davidson 2006; Wilkins 2002). The strong selection against intersex individuals suggests that selection should have optimized developmental signals regarding sex, canalizing them to be released at the correct developmental time. The outstanding problem is then to understand how changes in the genes underlying maleness and femaleness arise without causing disruptions to the sex determination pathway, as these disruptions would lead to the production of suboptimal male and female phenotypes and purifying selection acting against them acting to preserve the status quo.

Despite the essential nature of sex determination, we understand the genetics of it in surprisingly few animals. Those animals for which the genetics are relatively well characterized tend to rely on a network with a locus of major effect (the “master sex regulator”; some of which are shown in Table 2.1). This also has the interesting consequence that the genetic complement is determined at fertilization and, thus, that sex is often inherited in a simple Mendelian fashion. However, although they are the best characterized, mechanisms of genetic sex determination (GSD) involving a single locus of major effect are by no means predominant in animals. There is increasingly strong evidence for polygenic sex determination in fish (e.g., Bradley et al. 2011; Cnaani et al. 2008; Shirak et al. 2006), many species have environmental sex determination (ESD) (Sarre et al. 2011), some have complex relationships between polygenic and environmental factors (Quinn et al. 2007; Radder et al. 2008), and many others can alter their sex over their lifetime in response to ecological or demographic conditions (Avise and Mank 2009). What little is known about the evolution of sex determination has been reviewed numerous times (recent examples include Gempe and Beye 2010; Graves 2006; Heimpel and de Boer 2008; Schutt and Nothiger 2000; Williams and Carroll 2009), and rather than reiterate this material, we will only briefly paraphrase it and draw attention instead to how transitions in the sex-determining system is expected to be reflected at the level of gene networks, how changes in this network have important evolutionary implications, and how we might best go forward to study these aspects of the evolution of sex-determining systems.

**EVO-DEVO OF SEX DETERMINATION**

Although we think of the sexes as discrete phenotypes, male and female differences emerge from an undifferentiated embryo at some point during development. Additionally, although female and male sex differences are most obvious in adults, these dimorphisms begin as small differences early in development and amplify as the individual matures (Mank et al. 2010). Sex determination is therefore perhaps best defined as the processes that underlie differentiation of key components of the sexual phenotype during ontogeny (Uller and Helantera 2011).

At the most basic level, this differentiation revolves around the formation of testes versus ovaries, but sexual differentiation can also involve somatic tissue into distinct male and female types. Under this definition, a sex-determining system represents a particular structure of the developmental regulation of this process, such as the master trigger system found in most mammals. In therian mammals, sex determination is a highly canalized process where expression of a single genetic element early in gonad differentiation (the SRY gene) is sufficient to cause development of testes (Koopman et al. 1991; Sinclair et al. 1990). In other species where sex is based on genotype, sex-determining systems show little genetic variation and constitute a discrete form of phenotypic plasticity in which environmental conditions experienced at some point during development regulate the expression of maleness or femaleness via a developmental switch.
### TABLE 2.1. Key Positions in Animal Sex-Determining Networks

<table>
<thead>
<tr>
<th>Clade or Species</th>
<th>Sex Determination</th>
<th>Mechanism of Action (Roman Numerals Refer to Figure 2.2, When Details Are Known)</th>
<th>Key Paper or Recent Review</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Drosophila melanogaster</em></td>
<td>Females: XX, Males: XY</td>
<td>X chromosome : autosome ratio (I) initiates sex-specific splicing of Sxl (II) that is maintained via a positive feedback (III)</td>
<td>Bopp et al. (1991)</td>
</tr>
<tr>
<td><em>Apis mellifera</em></td>
<td>Females: Heterozygous at CSD, Males: Homozygous at CSD</td>
<td>Heterozygosity versus homozygosity/hemizygosity at the csd locus (II) results in different expression of downstream elements (III) whose expression is maintained via positive feedback involving alternative splicing.</td>
<td>Gempe et al. (2009); Hasselmann et al. (2008)</td>
</tr>
<tr>
<td><em>Nasonia vitripennis</em></td>
<td>Females: Diploid, with paternal allocation of Nvtra, Males: Haploid, without paternally imprinted allocation</td>
<td>Female-specific splicing of Nvtra (II) is initiated by paternal allocation of Nvtra mRNA in eggs (regulated by unknown genes; I) and expression of zygotic Nvtra regulated by imprinted genes (I), which initiates and maintains downstream sex-specific gene expression through self-regulatory loops (III)</td>
<td>Verhulst et al. (2010)</td>
</tr>
<tr>
<td><em>Caenorhabditis elegans</em></td>
<td>Hermaphrodites: XX, Males: XO</td>
<td>X chromosome: autosome ratio (I) results in dosage-dependent expression of XO-lethal 1 (<em>xol-1</em>). In XX hermaphrodites, <em>xol-1</em> (II) is repressed, leading to expression of <em>tra-1</em> (III). X : A ratio of 0.5 in males leads to expression of <em>xol-1</em>, in turn inhibiting <em>tra-1</em>.</td>
<td>Zarkower (2001)</td>
</tr>
<tr>
<td><em>Musca domestica</em></td>
<td>Variable</td>
<td>Sex-specific splicing of Mdtra occupies a central node (II) in the sex-determining network. Depending on the population or strain, sex-specific Mdtra expression can be initiated by the presence of one or several Y-linked or autosomal genes and maternally transferred Mdtra mRNA (I and II). Once initiated, sex-specific splicing of Mdtra is subject to positive feedback supported by additional genetic elements (III).</td>
<td>Hediger et al. (2010)</td>
</tr>
<tr>
<td>Therian mammals</td>
<td>Females: XX, Males: XY</td>
<td>Autosomal genes (I) trigger expression of a Y-linked element only present in males (<em>Sry</em>, II) that in turn triggers expression of a non-sex-linked gene (* Sox9*, II) whose expression is being maintained via a positive feedback involving several loci (III)</td>
<td>Koopman et al. (1991); Sinclair et al. (1990)</td>
</tr>
<tr>
<td><em>Birds</em></td>
<td>Females: ZW, Males: ZZ</td>
<td>Dosage mechanism on the Z chromosome results in higher expression of <em>Dmrt1</em> (I).</td>
<td>Smith et al. (2009)</td>
</tr>
</tbody>
</table>
Although we understand several GSD networks in detail, we know very little about ESD networks. It is therefore not clear whether GSD and ESD employ the same underlying pathways with different regulatory elements (GSD would be controlled by a constitutive promoter, ESD by an inducible one), or if the underlying network structure for these two types of sex determination are completely different. Given the speed of transitions between ESD and GSD (Bull 1983; Mank et al. 2005; Pokorná and Kratochvíl 2009), and systems involving both genetic and environmental effects in some organisms (Quinn et al. 2007; Radder et al. 2008), it seems unlikely that the entire network differs in fundamental ways between ESD and GSD, as there is simply not enough evolutionary space separating these states in many phylogenies to allow for complete network turnover. However, there is no definitive information to support or refute this.

Some of the key problems for the evo-devo of sex determination is therefore to understand how the developmental networks of sex determination become structured in particular ways in the first place (e.g., the master trigger systems), how novel determinants of sex arise within or outside of existing networks, and the implications this has for the rate and pattern of evolutionary diversification of sex-determining systems.

**THE ORIGIN OF NETWORK NOVELTY**

Before we discuss the evolution of sex determination networks in particular, it is perhaps useful to discuss some basic predictions regarding the evolution of genetic networks in general. Both the elements within the network and the structure of the networks themselves can diverge and change over time (reviewed in Davidson 2006; Gompel and Prud’homme 2009; Stern 2010), and recent comparative evidence suggests that some parts of genetic networks are hotspots for evolutionary change, and are therefore more likely than others to diverge over time.

First, genes with higher mutation rates or with a broader mutational target (i.e., the proportions of mutations that have functional consequences) should more often contribute to the origin of novel variation in networks and therefore form the basis of network change. This prediction is based on the need for genetic variation for evolutionary change. Genes with few tolerable mutations are too constrained to contribute to evolvability, and loci where alleles have little to no phenotypic effect are invisible to selection and are therefore unlikely to contribute to evolutionary change. It is important to note, however, that the effect of a single locus is expected to depend on the genetic background (e.g., Nijhout and Paulsen 1997).

Second, changes in genes that occupy the central nodes in networks, and which control the expression of many downstream genes, can typically generate phenotypic change via fewer mutational steps than changes in structural genes. For example, it has been suggested that the evolutionary lability of the dorsal and ventral trichome pattern in *Drosophila* larvae has been facilitated by the central position of the gene shavenbaby, which acts as a node as it integrates several inputs and triggers expression of a constellation of downstream genes that contribute to cell differentiation (Stern and Orgogozo 2009). Therefore, the network position and connectivity of this gene make it a particularly likely target for evolutionary changes in trichome patterning.

Third, network evolution can operate from changes and refinements of regulatory mechanisms. There are at least two routes to this type of innovation. First, the origin and fixation of fine-scale regulatory patterning via duplication and specialization of *cis*-regulatory elements (Carroll 2008) allows for localized expression of previously broadly expressed genes. Fine-scale expression partitioning due to *cis*-regulatory additions
maintains the network position of a gene while reducing potential negative pleiotropic effects in other parts of the body that would result in loss via purifying selection. Alternatively, gene duplication allows for subfunctionalization or partitioning among the daughter loci of the function once completely controlled by the parental gene. This can resolve pleiotropic constraints, particularly related to sex determination and sexual phenotypes (Gallach and Betran 2011; Gallach et al. 2010).

Beyond these three predictors, evolutionary shifts in the developmental regulation of sex determination will also depend on population-level processes (Snell-Rood et al. 2010; Stern 2010). For example, mutations with more dramatic effects on the phenotype may be more likely to go to fixation in small populations. Small populations are also more subject to drift and have lower number of mutations introduced per generation (Hartl and Clark 1997), which may result in selective fixation of genetic variants that determine sex in a less-than-optimal way. The strength of selection will also contribute to the rate of evolutionary change and may influence the positions in gene networks that are most likely to change; strong selection makes it more likely that genes of major effect go to fixation. Relaxation of selection, on the other hand, allows accumulation of mutations and can facilitate divergence in regulatory networks, perhaps in particular in parts of the network that are subject to low levels of pleiotropy and high rates of mutation (Snell-Rood et al. 2010).

EVOLUTION OF GENOTYPIC SEX DETERMINATION

A series of now classic scientific discoveries in the early 20th century established that sex in some animals, both invertebrate and vertebrate, followed Mendelian inheritance based on presence or absence of particular chromosomes (reviewed in Maienschein 1984). This established sex as a genetically determined character, inherited as a dominant or recessive trait. GSD, often related to sex chromosomes, has remained the dominant model of sex determination, although it is clear that it may not be the most common.

The Developmental Basis of GSD

In line with expectations based on models of adaptive evolution (Box 2.1), GSD often involves a single locus of major effect. The genetic networks that have been described in greatest detail are those of the insect Drosophila melanogaster, the nematode Caenorhabditis elegans, and the mammal Mus musculus, and there are excellent and profuse reviews

Box 2.1. Adaptive Evolution of GSD and ESD

Although it is possible that some sex-determining systems are selectively neutral, the phylogenetic pattern of GSD and ESD suggest a selective explanation for their diversity. Because sex determination has a direct impact on the sex ratio, sex ratio selection is expected to strongly influence evolution of sex-determining systems (review in Uller et al. 2007). For example, frequency-dependent selection against the most common sex will tend to favor evolution of equal sex ratios, more formally expressed as the equal investment in sons and daughters (e.g., Pen and Weissing 2002; West 2009). This could select against environmental effects on sex determination. Furthermore, although polymorphisms can be determined by genetic variation at several different loci, systems with only two morphs tend to favor evolution of a single locus of major effect (Bull 1983; Kopp and Herisson 2006; Rice 1986).

(Continued)
There are a variety of factors that can influence sex determination in animals. One reason for this is that a single-locus genetic architecture allows optimal “matching” of the morphs to the selective conditions that arise from intraspecific competition (Kopp and Hermisson 2006). Thus, although complex multilocus GSD systems have been shown to exist (see Ser et al. 2010; von Hofsten and Olsson 2005), polygenic sex determination may be unstable and evolve toward a system with a gene of major effect, although it does not necessarily predict whether the system will exhibit male or female heterogamety. This locus does not even need to be expressed in the embryo; GSD via maternally expressed genes are also possible (Kozielska et al. 2006; Werren et al. 2002), and the evolutionary outcome may be sensitive to the extent of sexual and parent–offspring conflict (MacCarthy et al. 2010; van Doorn and Kirkpatrick 2007).

Although sex ratio selection means that there will always be an element of frequency-dependent selection, theoretical models have shown that ESD should be favored when one sex benefits more than the other from the environment during development, or if the environment correlates with sex-specific selection at some point later during ontogeny (Shine 1999). The adaptive significance of ESD is easy to understand in the case of social sex determination in some species of invertebrates and fish. For example, size-advantage models may explain the origin of sequential hermaphrodites, where reproductive fitness is greater for larger animals of one sex and smaller animals of the other. For species with indeterminate growth, this suggests that most adult individuals will start out as one sex and reach a size threshold at which reproductive fitness is maximized by changing to the other sex (Charnov 1982). In protandrous species, the cheaper cost of sperm means that an individual’s lifetime fitness is maximized by starting adulthood as a male, and only changing to a female when of sufficient size to bear the cost of egg production. In other species with male competition based on size, male reproductive fitness is minimal until a size threshold is crossed, and it is therefore advantageous to start as a female.

In vertebrates, the most common gonochorist ESD is temperature-dependent sex determination, or TSD (Valenzuela and Lance 2005). The adaptive significance of TSD has been elusive, but studies of fish and lizards suggest that one possibility is that the temperature during development influences developmental time and therefore correlates with the timing of hatching or birth, which may have sex-specific fitness consequences (Conover 1984; Pen et al. 2010; Warner et al. 2009). If timing of hatching is the key fitness characteristic, this suggests that other environmental factors that also affect developmental time may influence sex determination in TSD species. This is consistent with emerging evidence that although temperature has strong effects in the laboratory, its effect can be less straightforward under natural conditions (Warner and Shine 2011). However, a direct link with developmental rate has not yet been demonstrated. Nevertheless, recent theoretical modeling suggest that sex-determining systems that are highly canalized toward being responsive only to temperature may be suboptimal and that mechanisms that integrate several different sources of environmental input into sex determination should be favored by selection (Schwanz et al. 2010).

In *Drosophila*, which is male heterogametic and has a male-limited Y chromosome, sex determination is based on the X:A ratio early in development (Figure 2.1A) before
Figure 2.1. Comparison of sex determination in the Dipterans, *Drosophila melanogaster*, and *Ceratitis capitata*. Panel A: Sex determination in *Drosophila* is based on the ratio of X chromosomes to autosomes. The X chromosome to autosome ratio functions when multiple X-linked transcription factors bind to and activate the sex lethal (*Sxl*) gene, and the activated *Sxl* product then initiates sex-specific splicing of transformer (tra) mRNA, and indirectly of the doublesex gene (*dsx*). This pathway leads to alternative splicing of *dsx*, with the male form (*dsx*<sup>m</sup>) ultimately leading to the development of a testis, and the female variant (*dsx*<sup>f</sup>) leading to an ovary. Both variants of *dsx* involve exons 1, 2 and 3, with *dsx*<sup>m</sup> including exons 5 and 6 and *dsx*<sup>f</sup> ending in exon 4. Panel B: In *Ceratitis*, sex determination is not based on X:A ratio, rather a Y-linked element that represses *tra* and *tra-2*, ultimately leading to sex-specific splicing of *dsx* and male and female phenotypes.
the onset of sex chromosome dosage compensation (Bopp et al. 1991). This dosage-sensitive mechanism functions when multiple X-linked transcription factors bind to and activate the Sxl gene, and the activated Sxl product then initiates sex-specific splicing of transformer (tra) mRNA, and indirectly of the doublesex gene (dsx). Importantly, once this process is initiated, feedback loops ensure continued expression of Sxl and maintains female differentiation. The Drosophila system is therefore composed of a key genetic element, Sxl, that occupy a node in the sex-determining network, but where several X chromosome genes regulate the expression of Sxl itself.

Sex determination in C. elegans shares many elements with Drosophila, both in terms of the X chromosome dosage that initiates regulation of the gene xol-1, as well as conserved genes such as tra (Conradt and Horvitz 1999). However, sex determination in C. elegans does not seem to involve sex-specific splicing, which thus far appears to be limited to the insects. Finally, C. elegans is interesting in that XX individuals develop as hermaphrodites, which sequentially produce first male, then female gametes. XO individuals develop as males. Androdioecious mating systems such as this, though common in plants, are relatively rare in animals, and may prevent the spread of a dominant male-determining factor such as that seen in mice (see later) as it would preclude the development of hermaphrodites. It is worth noting that the androdioecy in C. elegans is likely a recently derived state from within a dioecious clade, where individuals are either male or female and do not switch sex.

Sex determination in mice is not based on dosage; rather, the gene that occupies the central point in the network (Sry) is present only in the male as it located on the Y chromosome. It is not clear whether a single or multiple factors regulate the expression of Sry (Sekido and Lovell-Badge 2008). However, it seems like the direct target of the Sry protein (SRY) is a single gene, Sox9, which itself is located on an autosome. Sox9 is necessary for testes formation in the mouse and, after the initial initiation of its transcription by Sry, its expression is maintained assisted by other genes and feedback loops. Failure to maintain SOX9 can lead to development of ovaries in XY individuals with a functional Sry gene. Indeed, unless SOX9 reaches the threshold that initiates the feedback loop that maintains its expression, accumulation of β-catenin in the genital ridge suppresses SOX9.

This brief overview emphasizes some key aspects of the regulation of genotypic sex determination that are important for understanding evolutionary transitions between sex-determining systems (Figure 2.2). First, all networks have at least one central node, the genetic element often referred to as a master trigger. Second, at least in D. melanogaster and C. elegans, this gene also resides in a convergence point in the gonad differentiation network, where it integrates several different inputs into a single signal that in turn switches on multiple other genes. Third, the gene of major effect may or may not be inherited through only one sex. Fourth, once initiated, maintenance of the expression of key elements requires feedback systems, and the elements involved in this feedback system are crucial for the master trigger to have a major effect on sex determination.

**Evolutionary Transitions between Genotypic Sex-Determining Systems**

Are the comparative patterns of sex determination consistent with the hypotheses for where in genetic networks evolutionary change is most likely to occur? More is known about the diversity of sex determination in insects than in any other taxa, making them the obvious candidates for investigating this question. The comparative evidence so far
suggests that many of the systems of sex determination in insects are based on a similarly shared mechanism of sex-specific splicing of the transformer mRNA (Gempe and Beye 2010), where one form leads to female development and the other to male development. More importantly, sex-specific splicing of a single-gene downstream on the sex determination pathway (tra) is conserved in insects (Gempe et al. 2009; Lagos et al. 2007; Pane et al. 2002; Pomiankowski et al. 2004; Raymond et al. 1998; Verhulst et al. 2010; Williams and Carroll 2009) and is even involved in sex determination in C. elegans (Conradt and Horvitz 1999; Goodwin et al. 1993).

Although other downstream elements are conserved in addition to tra (Marin and Baker 1998), this conservation is not present in the integrative control point or node of the network, and there are many different regulatory mechanisms that initiate the sex-specific splicing that ultimately leads to sex determination. This is consistent with the general prediction that nodes in genetic networks are hotspots for evolutionary innovation because they allow change without disruption of development. However, it does not necessarily imply that sex-determining networks evolve simply by mutations in existing nodes or upstream addition of master triggers. Comparisons between Drosophila and the medfly, Ceratitis capitata, are particular interesting (Figure 2.1) as they show how the downstream mechanisms can be retained despite substantial changes upstream, even involving transitions between dosage effects and Y-linked master triggers. Specifically, in contrast to Drosophila, Sxl is not expressed in a sex-specific manner in medflies. Instead, current evidence points toward regulation of the alternative slicing of the ortholog of tra via a Y-linked element (Saccone et al. 2002). The alternative splicing of tra is maintained via positive feedback once initiated in the early zygote. That dramatic changes at the level of
The evolution of sex determination in animal sex-determining systems can arise from very simple gene regulatory interactions is also supported by theoretical models (MacCarthy et al. 2010).

Although the available evidence supports that evolutionary transitions between GSD systems occur relatively upstream in sex-determining networks, the origin of recruited genes is not well understood. The prediction that genes where mutations are tolerable and which have functional phenotypic consequences (i.e., avoid disruption of development by virtue of low pleiotropy) should be more likely to emerge as master triggers has little direct empirical evidence in terms of sex determination. No one has, to our knowledge, done a study on mutability and phenotypic effects of sex-determining candidate genes in a systematic way that would be required to test this prediction. However, analyses of the transcription network in crosses between mouse strains that differ in their susceptibility to sex reversal (relative to the presence of Sry) have shown that autosomal regulatory elements that are involved in maintaining SOX9 expression or the Wnt/β-catennin signaling could be a rich source of genetic variation for evolutionary shifts in GSD networks (Munger et al. 2009). This means that rather than the novel master trigger being a novel genetic element added upstream to the sex-determining network (Wilkins 1995), such genes of major effect may be recruited from downstream genes involved in maintaining SOX9 expression, resulting in a neo-sex chromosome. Alternatively, Sox9 may show sufficient genetic variation in its sensitivity to X-linked transcription factors (or those factors may show genetic variation themselves) to allow evolution of a dosage system analogous to that of Drosophila. Further studies of rare incidence of evolutionary loss of Sry in mammals (Just et al. 1995) are likely to be informative with respect to the extent of change in sex-determining networks after random or selective loss of existing master triggers.

Evidence from vertebrates suggest that network novelties do not always arise directly from variation in genes within the network, but are instead duplicates of loci already present in the sex determination pathway. An indicative example of this comes from the medaka rice fishes in the genus Oryzias. Within this clade, a duplicate of the Dmrt1 gene, called Dmy, has taken over as the master regulator of male sex determination along one lineage (Matsuda et al. 2002; Nanda et al. 2002). Dmrt1 has a conserved role in sex determination spanning invertebrates and vertebrates (Matsuda et al. 2002; Smith et al. 2009; Yi and Zarkower 1999; Yoshimoto et al. 2008) and therefore Dmy was, at the time of duplication, already well configured to play a role in this pathway. It is yet unknown to what extent such changes in genes of main effect in vertebrates was initiated by the gene duplication and whether additional changes are also involved in the regulation of DMY expression or maintenance of downstream feedback loops.

In conclusion, in both insects and vertebrates, there seems to be substantial evolutionary lability, but primarily at the top of the sex determination pathway. Additionally, in vertebrates at least, this change seems to be due to changes in a limited repertoire of genes that are already implicated in sex determination, with paralogues often emerging as master regulators. This sort of recycling within a framework of conserved downstream genes may explain how transitions among GSD mechanisms occur without disrupting the development of sex-specific phenotypes. Nevertheless, the conclusions that can be drawn from this observation are limited because the majority of studies on nonmodel organisms are limited to the study of homologues of genes found to play a role in sex determination in mice and humans. Indeed, recent evidence from cichlids suggests that novel main elements in sex-determining networks can originate from genes ancestrally involved in differentiation of secondary sexual characters (Roberts et al. 2009; Ser et al. 2010). Disentangling the mechanistic basis of this acquired function will help to test predictions regarding the
origin of network novelty, in particular whether pleiotropy facilitates or constrains evolution of novel sex-determining genes.

**GSD and the Evolution of Sex Chromosomes**

Most of the models for studying sex determination possess highly differentiated sex chromosomes, such as the X and Y chromosomes seen in therian mammals, *Drosophila*, and *C. elegans*, and the Z and W chromosomes in birds. This is not to say that distinct sex chromosomes always, or even usually, co-occur with GSD, but that some systems with GSD may eventually present them. It is important to point out that highly differentiated sex chromosomes are not inevitable, as old but undifferentiated sex chromosomes exist in ratite birds (Mank and Ellegren 2007) and snakes (Matsubara et al. 2006). When they do occur, the evolution of highly differentiated sex chromosomes may act as a brake on further changes to the sex-determining pathway. Circumstantial evidence for this comes from the fact that clades with old heteromorphic sex chromosomes, such as birds, mammals, and *Drosophila*, show little evidence of change in sex determination.

There are several reasons why this might be the case, many relating to the unusual sex-specific selection pressures acting on sex chromosomes. Very briefly, sex chromosome systems come in two major flavors: female heterogamety where females have one Z and one W and males have two Z chromosomes, and male heterogamety where females have two X chromosomes and males one X and one Y. The W and Y chromosomes are roughly analogous to each other in that they are both sex-limited, with the W present only in females and the Y only in males. The type of sex chromosome may depend on the type of sex determination, with recessive male mechanisms or dominant female master regulators resulting in ZZ-ZW systems, and dominant male master regulators and recessive female mechanisms in XX-XY system.

The divergence of X-Y and Z-W sex chromosome pairs is based on a model of sexually antagonistic alleles for genes near the sex-determining locus (Charlesworth 1991; van Doorn and Kirkpatrick 2007) that results in selection for recombination suppression between the sex chromosomes and therefore allowing them to diverge from one another. Sexually antagonistic alleles benefit one sex at a cost to the other, and if sex chromosome differentiation is the result of the accumulation of sexually antagonistic alleles, highly differentiated sex chromosomes should have large numbers of antagonistic loci. Because changes in the master regulator often cause sex chromosome loss or turnover (Kitano et al. 2009; Nanda et al. 2002; Ross et al. 2009), changes in GSD may be difficult for lineages with highly differentiated sex chromosomes because loss of those sex chromosomes will result in the loss of a linkage group that carries several loci with sex-specific effects, thereby resulting in less fit individuals. It is important to point out here that there is no strong empirical evidence for this model of sex chromosome evolution (Ironside 2010). Additionally, aside from sex-determining genes themselves, there are no known examples of sexually antagonistic loci where the cost to one sex and the benefit to the other has been measured. This means that although there are indications that large amounts of intralocus sexual conflict are resident within genomes (Bonduriansky and Chenoweth 2009; Harano et al. 2010; Lewis et al. 2011), no one really knows at the molecular or biochemical level what a sexually antagonistic allele actually looks like.

Even if this model of sex chromosome differentiation is not common, highly differentiated sex chromosomes, particularly the W and Y chromosomes, have important sex-specific functions in fertility and mate choice (Hori et al. 2000; Lange et al. 2009; Lemos et al. 2008; Moghadam et al. 2012; Postma et al. 2011), and this alone may present a
serious barrier to change in GSD as males lacking a Y chromosome or females lacking a W would be infertile or subfertile and therefore removed from the population by negative selection.

**EVOLUTION OF ENVIRONMENT-DEPENDENT SEX DETERMINATION**

Environment-dependent sex determination is a polyphenism, that is, a form of phenotypic plasticity with discrete phenotypes. Polyphenisms are common in nature, in particular in invertebrates. Familiar examples include queen and worker morphs in social insects, density-induced winged morphs in aphids, and seasonal morphs with different colours and patterning in some species of butterflies (reviewed in West-Eberhard 2003; Whitman and Ananthakrishnan 2009; Simpson et al. 2011).

Polyphenism describes the sexes in both gonochorist ESD lineages, where sex is determined by some environmental cue or cues and is maintained throughout the remaining life span, and sequential hermaphrodites that change sex based on some ecological factor. The former are illustrated by the temperature-dependent sex-determining mechanisms in turtles and crocodilians (Valenzuela and Lance 2005), and the latter are exemplified by the protandrous (male then female) or protogynous species of many fish (Avise and Mank 2009).

**The Developmental Basis of ESD**

The developmental basis of ESD is poorly understood compared to that of GSD systems, largely because no model organisms have ESD. The best studied examples so far are temperature-dependent sex determination (TSD) in reptiles and fish (Valenzuela 2008; Valenzuela and Shikano 2007) and the protogynous gobies (Black et al. 2005). Studies of the developmental basis of nonsex polyphenisms have revealed that they typically are hormonally regulated (Nijhout 1999, 2003), suggesting that this may also be the case for sex determination. Indeed, with the exception of placental mammals and birds, both ESD and GSD vertebrates can be “sex-reversed” by application of estrogens or estrogen-inhibitors, showing that some aspect of gonad differentiation is estrogen dependent (e.g., Freedberg et al. 2006; Shine et al. 2007). There is also some evidence that other hormones (androgens and corticosterones) can influence sex determination (Hattori et al. 2009), but whether they form a part of a normal, species-typical, sex-determining developmental network remains unclear. Nevertheless, a hormonal basis for ESD systems is well supported, at least in vertebrates.

Despite the fundamental role of estradiol in sex determination in ESD species, very little is known about the molecular mechanism by which the environment exercises its effects, or how estradiol influences gonad differentiation (e.g., what genes it upregulates). Studies of reptiles and fish have shown that orthologues of many of the genes that are involved in sex determination in mammals and birds are expressed in gonads of TSD species (see table 1 in (Rhen and Schroeder 2010). However, the timing of expression and the extent to which it is sex-specific varies between genes and between species. For example, although SOX9 and DMRT1 are relatively consistently expressed differently in gonads developing at male- and female-producing temperatures, expression of estrogen receptor α and SF-1 are less consistent (Rhen et al. 2011). Only recently have the temperature- and estradiol-sensitivity of those genes started to be elucidated. For example, in painted turtles, estradiol suppresses SOX9 expression during the latter phase of gonad
differentiation (Barske and Capel 2010). This suggests that estradiol primarily functions as part of the feedback loop that maintain SOX9 expression, but that it is of limited importance in the early stages of gonad differentiation. Recent evidence from isolated cultured gonads of the painted turtle also shows that several orthologues of genes involved in sex determination in mammals show temperature-dependence in their expression levels (Shoemaker-Daly et al. 2010). This may point toward a regulatory network in which several different genes show temperature dependence in their expression levels. Evolution of some level of redundancy, with several genes that show the same environment-dependent expression, may also be selectively favored because it increases the potential for divergence of developmental trajectories and reduces the risk of intersexes. If this is the case, then the main effect of temperature is better thought of as a mechanism that sustains differential gene expression that results in cumulative differences in gonad differentiation rather than as a master trigger of sex determination (Uller and Helantera 2011). However, in other species, the window of temperature sensitivity is narrow and occurs very early in development (even before gonad differentiation; reviewed in Baroiller et al. 2009). In these species, temperature may either trigger expression of an upstream gene that subsequently switches on a battery of downstream genes, analogous to the GSD systems described earlier, or trigger epigenetic changes to genes involved in initiation or maintenance of the estrogen-dependent feedback loop that sustains gonad differentiation.

Support for the latter scenario comes from recent research on seabass. In this species, high temperature before the onset of gonad differentiation increases DNA methylation in the promoter of the gonadal aromatase gene (cyp191a), which results in suppression of transcription and hence a reduction in the expression of aromatase in the gonads, leading to the development of testes (Navarro-Martín et al. 2011). Similar mechanisms may be important in other species with narrow, early windows of thermal effects on sex determination, whereas those where sustained temperature during gonad differentiation is needed may have different means of regulation of aromatase activity.

**Evolutionary Transitions between Environment-Dependent Sex-Determining Systems**

The comparative pattern of ESD and its developmental basis is still too poorly understood to address what is the developmental basis of the transition between different mechanisms of ESD systems. However, we can identify two different kinds of transition. First, changes in the genetic regulation of ESD, while retaining sensitivity to the same environmental input (Nahmad et al. 2008; True and Haig 2001) and, second, changes in the use of cues with more or less conserved regulation of the genes involved in sex determination (Schwander and Leimar 2011).

Examples of the first kind could be sought by comparing TSD species with different windows of temperature sensitivity (Valenzuelo 2008, 2010). For example, we predict that in species where sex is sensitive to temperature throughout a substantial part of gonad differentiation, the network will essentially consist of downstream elements (i.e., the feedback loop in Figure 2.2), with the expression of one or more key genes being directly influenced by temperature. Evolutionary shifts to successively earlier and more narrow windows of temperature sensitivity should be accompanied by upstream addition of genetic elements that regulates SOX9 expression or, alternatively, stable epigenetic modification of key elements involved in sustaining, for example, aromatase activity later in the developing gonad.
Promising candidates for studying evolutionary shifts between reliance on different environmental cues are species where the effect of temperature has been shown to vary with other environmental or maternal factors. For example, there is evidence from turtles and lizards that the maternal phenotype influence sex determination in TSD species (e.g., Radder et al. 2009; Schanz et al. 2010; Warner et al. 2007). Although there is little evidence that this is due to maternal allocation of hormones per se (Radder et al. 2009), it could involve other gene products or environmental compounds that promote or interfere with regulation of, for example, aromatase. This may require very limited changes at the level of genetic networks of sex determination in the gonad itself. Indeed, endocrine disruptors show the potential for incorporating novel environmental input in sex determination in both ESD and GSD species.

FROM ESD TO GSD AND BACK AGAIN

The most conspicuous evolutionary changes in sex determination are transitions between ESD and GSD, a transition that is probably quite common. In vertebrates, ESD evolved independently in fish (Mank et al. 2006) and reptiles (Pokorná and Kratochvíl 2009). However, TSD may be ancestral in extant reptiles, suggesting that there are at least six cases of independent evolution of GSD from TSD in turtles, and probably several transitions in both directions in lizards, some of which must be very recent (Organ et al. 2009; Pen et al. 2010; Pokorná and Kratochvíl 2009).

Despite the prevalence of ESD–GSD transitions, the origin and evolution of the regulatory processes that underlie transitions between GSD and ESD are poorly understood both theoretically and empirically. A major problem is that most of what we know about the molecular mechanisms of sex determination comes from studies of humans, mice, and Drosophila, and these organisms may not be representative of clades that show evolutionary transitions between GSD and ESD (e.g., mammals are unusual vertebrates in that sex determination apparently is not hormonally regulated and that almost all species have heteromorphic sex chromosomes). This leaves us far more questions than answers; here we only address two of the most fundamental ones.

First, are the networks underlying ESD and GSD similar, or are they built on entirely different premises? Perhaps the most straightforward way to change a GSD system to an ESD one would be to alter the promoter from constitutive to inducible so that its expression becomes environment-dependent. For example, Dmrt1 is implicated in sex determination in many vertebrates, including species with GSD and TSD. A duplicate of this gene occupies a central node in sex determination in some fish species with an upstream mechanism involving dosage effects (Matsuda et al. 2002; Nanda et al. 2002), suggesting that TSD could originate via mutation in this gene, making its expression temperature-dependent. If TSD is under positive selection, this may subsequently result in evolutionary loss of genetic variation (and hence heterogamety) and evolution of a system with strong environmental effects on sex determination.

Dmrt1 may also be involved in transitions in the opposite direction. In birds, the gene sits on the Z chromosome and is therefore more highly expressed in males (the homogametic sex) and seems to occupy the most upstream position in the sex-determining network (Smith et al. 2009). Consequently, this configuration could be a system derived from a reptile TSD system with Dmrt1 being the main temperature-sensitive gene. However, it is equally possible that the avian sex determination network has evolved from a system where one or several other genes involved in the initiation and maintenance of testes or ovarian
developmental pathways exhibited temperature sensitivity in their expression. Regardless, *Dmrt1* may have been a particularly likely target for mutations of major effect, given its particular function as encoding transcription factors and its evolutionary conservation in gonad differentiation (Graves and Peichel 2010).

Overall, it would seem that the main constraint on evolutionary shifts between GSD and ESD is to maintain the self-sustaining expression of genes to prevent the production of ovotestes. Consequently, we might expect that transitions from GSD to ESD could involve complete loss of upstream master triggers as long as one or several key elements in the downstream feedback loop show consistent expression levels under different environmental conditions. This could involve mutations directly in those genes or in genes originally being outside of the sex determination network. For example, it is possible that changes in rates of cell proliferation in the gonad can shift the time window of hormone exposure to expose environmental effects on sex determination even if variation in cell proliferation does not ancestrally appear to have a causal effect on sex determination (Uller and Helantera 2011). A perspective that emphasizes the many different targets for mutations that can influence sex determination (Sarre et al. 2004, 2011) is consistent with the observation that species with TSD often show substantial genetic variation in the extent to which temperature affects gonad development (e.g., Baroiller et al. 2009; Rhen et al. 2011; Warner et al. 2008).

Alternatively, ESD may originate as epigenetic changes in genes involved in self-sustaining feedback loops, which could subsequently become stabilized and refined via selection on novel or existing genetic variation involved in epigenetic regulation (Uller and Helantera 2011). This also raises the possibility that GSD systems could evolve from ESD systems via the evolution of stronger genetic effects on DNA methylation of key genes in existing sex-determining systems. At this point, however, these scenarios remain speculations, and we do not know if transitions between GSD and ESD involve simple changes in master triggers, or whether the entire network, or some substantial portion of it, is being rewired to accommodate evolutionary shifts between different systems (see also Crews and Bull 2009; Sarre et al. 2004, 2011; Uller and Helantera 2011).

Second, are transitions between ESD and GSD as common as the reverse? Comparative data suggest that transitions from ESD to GSD are far more common than the reverse, although this is confounded by different rates of speciation and extinction (Organ et al. 2009; Pokorná and Kratochvíl 2009). If this pattern is indeed true on a broad scale, it may indicate that the required network changes for the different transitions are not equally easy. Sex chromosomes have been referred to as evolutionary traps (Pokorná and Kratochvíl 2009), suggesting that transitions to GSD may be more common than transitions from it. Aside from the problems described earlier associated with turnover of heteromorphic chromosomes, there are few a priori reasons to assume that GSD based on homomorphic sex chromosomes prevents transitions back to ESD. However, our understanding of these transitions is hampered by the fact that sex-determining mechanisms are often unknown in the most interesting and informative clades. Advances in recording mode of sex determination, as well collecting these records into a comprehensive phylogeny of sex determination, would represent a major advance in understanding rates of transitions between different systems.

**Suggestions for Future Work**

First and foremost, a key component for understanding the evolution of sex determination is to actually understand how sex is determined in a broad array of animals. In this regard,
the model systems for sex determination, namely mice, humans, and Drosophila, may not be the most informative because they are present in clades where sex determination is highly conserved. Only when we understand the networks underlying sex determination can we study how the networks change; therefore, it will be more informative to study clades where sex-determining mechanisms change relatively often.

The phenotypic aspects of sex determination are also of potential interest. For example, in some animals, somatic and gonadal sex has been, to a large extent, decoupled. Clear examples of this exist in fish with multiple male reproductive tactics, where males may mimic females in phenotype to dupe a resident male into giving them access to the nest (Mank and Avise 2006) and the opportunity to steal fertilization events. How do female mimics disassociate gonadal sex determination from somatic sexual phenotype? How have they partitioned the genetic network of sex determination into separate gonadal and somatic forms? In some ways, these female mimics can be thought of as intersex individuals, and this leads to questions regarding the origin of cross-sexual transfer of developmental pathways (West-Eberhard 2003), for example, how can changes in the genes underlying maleness and femaleness arise without causing disruptions in the sex determination pathway and suboptimal male and female phenotypes?

Beyond this call for more data that will enable comparative studies of sex-determining systems in greater detail, there is also a paucity of theoretical studies that explicitly model the evolution of sex-determining regulatory networks (as opposed to the selective advantage of GSD versus ESD). This will not only provide more rigorous assessment of verbal arguments, but could also generate directional predictions that can inform empirical work. Recent explicit focus on the evolution of the regulation of sex determination (rather than the selective context that favor genotypic versus environmental control) provide a promising start for being able to understand and predict patterns of sex determination, both in terms of developmental mechanisms and selection (e.g., (MacCarthy et al. 2010; Pen et al. 2010; Quinn et al. 2011; Uller and Helantera 2011).

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