Biological Journal of the Linnean Society, 2003, **80**, 353–368. With 2 figures

Evolution of dioecy and sex chromosomes via methylation driving Muller's ratchet

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Received 13 October 2002; accepted for publication 7 April 2003

Why are there two sexes in certain species, instead of one hermaphroditic sex? Why are Y chromosomes shorter than X chromosomes, but only in certain lineages? I propose that differences between sexes are initially determined by differential methylation in nuclear DNA between females and males, driving Muller's ratchet. Methylation of promoters suppresses transcription, including loci coding for gamete production, thereby converting hermaphroditic individuals into females or males. Differential methylation of sex chromosomes suppresses recombination and increases mutation rate, thereby geometrically increasing the speed of Muller's ratchet. Higher mutability of methylated nucleotides plus loss of sex-determining function of previously methylated nucleotides provides selective pressure to excise these loci, resulting in shorter Y or W chromosomes. Derived lineages usually have more methylation than do ancestral ones, and hence have relatively shorter sex chromosomes. Methylation canalizes dioecy and degeneration of sex chromosomes. Latter stages of sex chromosome evolution may have occurred via other mechanisms, for example sexually antagonistic genes or chromosomal rearrangements. A few aberrant derived lineages lost most methylation, and their sex determination and sex chromosomes may have evolved via other means. Differential methylation provides a mechanism for early evolution of dioecy in anisogamous sexual diploid eukaryotes and of sex chromosomes in metazoans.

ADDITIONAL KEYWORDS: canalization – demethylation – differentially methylated region (DMR) – epigenetic – heterochromatin – temperature-dependent sex determination (TSD).

INTRODUCTION

For at least the past 3000 years, people have been speculating on the causes of sex and dioecy (Mittwoch, 2000). However, it took until the advent of modern genetics at the dawn of the 20th century for scientists to connect two distinct sexes with sex chromosomes (McClung, 1902; Stevens, 1905). For the next seven decades, insufficient molecular evidence existed for a more refined or reductionist model of evolution of dioecy. However, in the past quarter century, there has been an almost evangelical search for a single sex-determining locus on sex chromosomes (Wachtel & Tiersch, 1994; Mittwoch, 2000; but see Graves, 2002), ignoring substantial evidence that earlier researchers may have been correct that sex is determined by differential properties of whole sex chromosomes. My approach falls in between these two extremes; it can account for the evolution of dioecy via a single gene and via effects spread out more diffusely over multiple genes.

There is no single unified hypothesis explaining sex determination, not even when confining attention to metazoans or even to vertebrates. The problem is that environmental and chromosomal sex determination are usually considered to be different phenomena. I propose a hypothesis that explains both types of sex determination by focusing on epigenetic patterns across wide swaths of incipient sex chromosomes, rather than genetic patterns of nucleotides on one or a few loci. These epigenetic patterns consist of a binary code of methylation: is each nucleotide locus methylated or not? Methylation is the addition of a methyl group (CH_3) to a DNA nucleotide, usually to the 5′-carbon of cytosine (5-methylcytosine) in cytosine-guanine dinucleotides (CpG). Heterochromatic proteins bind to methylated nucleotides, causing the G-chromosomal banding patterns that are observable

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under a light microscope (Miniou *et al*., 1997; Singer, Yordan & Martienssen, 2001; Richards & Elgin, 2002). Methylation patterns are highly heritable (Holliday, 1988; Vyskot *et al*., 1995). I propose that differential methylation of regulatory genes controlling production of primary sex characteristics, gonads, and/or gametes on a pair of homologous chromosomes provided the origin of two distinct sexes and proto-sex chromosomes.

When biologists think of sex chromosomes, they typically do not think of differential methylation of sex-determining loci. Instead, they think of a pair of chromosomes of unequal length in one of the two sexes. This distinction between dioecy and sex chromosomes is a result of chromosomes of unequal length being readily observable under a light microscope. Differential methylation levels are much less visible, although they are potentially visible as subtle differences in chromosomal banding patterns under a light microscope. Differential methylation of sex-determining loci is merely the first step in shortening of one of the two sex chromosomes (Solari, 1994 and Steinemann & Steinemann, 1998 point out that differential heterochromatin is the first step in forming heteromorphic sex chromosomes in some species, but fail to mention that heterochromatin often contains methylated DNA sequences).

The most widely accepted explanation for the evolution of sex chromosomes of unequal length is Muller's ratchet for the accumulation of deleterious mutations on one of the two sex chromosomes (Muller, 1914; Muller, 1964; Frota-Pessoa & Aratangy, 1968; Nei, 1970; Felsenstein, 1974). The problem with Muller's ratchet and other proposed models is in starting the process of sex chromosome evolution without invoking seemingly ad hoc assumptions, such as a mechanism by which recombination is initially suppressed. I modify this traditional model by using the effects of methylation to adjust two key parameters in Muller's ratchet: recombination and mutation rates. This accelerates the rate of Muller's ratchet, providing a rationale for why there exists only one pair of sex chromosomes, and further explaining the close relationship between evolution of dioecy and sex chromosomes.

There probably exists a plurality of mechanisms for evolution of dioecy and sex determination (West, Lively & Read, 1999). My proposal tries to elucidate the most widespread and likely origin. In plants, where there is much more movement of genes between nuclei and organelles (Palmer *et al*., 2000), there is a much higher likelihood of cytoplasmic sex determination. In those few aberrant and highly derived animal and fungal lineages in which cytosine methylation was lost, dioecy and sex chromosomes may have evolved via mechanisms not associated with methylation. Unfortunately, these aberrant derived lineages contain those taxa that are most often studied to determine the origins of dioecy and sex chromosomes: the yeasts *Saccharomyces cervisiae* and *Schizosaccharomyces pombe*, the fruit fly family Drosophilidae, and the nematode *Caenorhabditis elegans* (Riggs & Pfeifer, 1992; Wolffe & Matzke, 1999; Gowher, Leismann & Jeltsch, 2000; Lyko, Ramsahoye & Jaenisch, 2000). *Drosophila* have highly derived and atypically complicated sex-determining systems (Dübendorfer *et al*., 2002), hence extensive earlier work on the evolution of sex determination and sex chromosomes derived from studies of *Drosophila* are probably not applicable to most other taxa. The evolution of sex determination is also well studied in mammals, but here the sex-determining mechanisms are probably so highly canalized that it is impossible to gain any glimpses of the early evolution of sex determination. Therefore, our best windows into the evolution of dioecy and sex chromosomes come from relatively ancestral animal taxa.

The first half of this paper provides this theory of methylation driving the evolution of dioecy and sex chromosomes, while the second half provides several testable predictions and details regarding testing.

THEORY

Evolution of dioecy

It is generally believed that two distinct sexes evolved via a single sex-determining locus. However, this fails to explain how such a locus could have independently evolved many times, why chromosomal banding patterns differ over large portions of sex chromosomes and not just near the putative sex-determining locus, how recombination between incipient sex chromosomes is initially suppressed, and the mechanisms by which environmental and hormonal sex determination operate. To rectify this, I propose an alternative model of evolution of dioecy via differential methylation of sex-controlling portions of the genome. Differential methylation could be on a single gene, which may then be interpreted as a sex-determining locus, but could just as well occur on multiple unlinked genes.

Methylation (CH_3) is invariably attached symmetrically on double-stranded DNA at specific palindromic nucleotide sequences. This symmetry allows for faithful copying of methylation patterns during chromosomal replication, in which maintenance methylation corrects asymmetries by adding methylation to the unmethylated strand (Woodcock *et al*., 1997). Methyl groups are usually attached to the 5′-carbon of cytosine (5-methylcytosine) in CpG dinucleotides, or less frequently to CpNpG trinucleotides, where N can be any nucleotide (Ramsahoye *et al*., 2000). I disregard the other forms of methylation, N^6-methylade-

nine and N^4-methylcytosine, because they are absent from all meiotic genomes. Methylation patterns are highly heritable and form a code akin to and on top of nucleotide sequences, but with a different alphabet. Methyl groups attach directly to DNA nucleotides, hence they are referred to as epigenetic phenomena ('epi-' means 'on top of').

I hypothesize that dioecy and sex chromosomes originated in ancestral diploid hermaphrodites as a pair of ordinary homologous chromosomes (autosomes) in which one chromosome had more methyl groups attached to a sex-controlling region than did its homologue (see de Almeida-Toledo *et al*., 2001 for a similar notion). The chromosome with the more highly methylated sex-controlling region was the incipient Y or W chromosome. Thus, a second distinct sex was initially determined solely by methylation patterns. Moreover, genomic imprints (i.e. parent-specific 'differentially methylated regions') are the cause rather than effect of having two sexes. Below I show why sex-specific methylation and proto-sex chromosomes were initially confined to one pair of homologous autosomes, consistent with theoretical models and empirical data from all eukaryotes that have multicellular diploid stages (Hurst & Hamilton, 1992).

Methylation blocks binding sites for enzymes that mediate transcription (Iguchi-Ariga & Schaffner, 1989; Tate & Bird, 1993; Henry *et al*., 1999; Yung *et al*., 2001) (Fig. 1) in several interrelated ways (see Gorelick, 2003 and references therein). Transcription is invariably suppressed if promoters are methylated, especially in dense regions of cytosine-guanine dinucleotides (Futscher *et al*., 2002). In some but not all promoters, the degree of suppression of transcription is proportional to the density of cytosines that are methylated (Boyes & Bird, 1992; Tate & Bird, 1993).

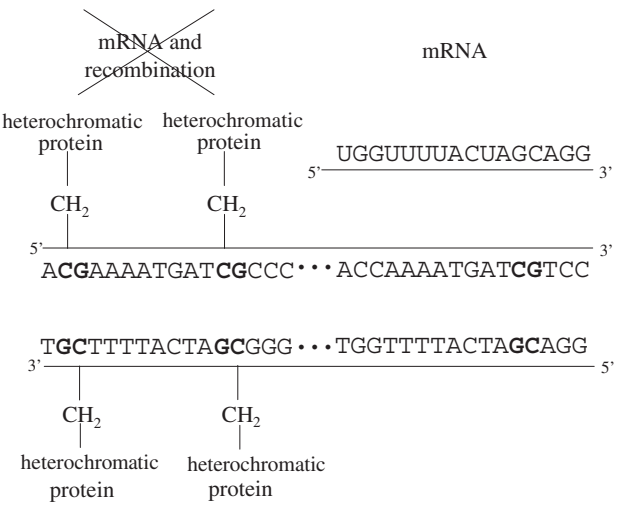

Figure 1. Effects of methylation on recombination and transcription.

In hermaphroditic anisogamous diploid eukaryotes, I hypothesize that differential methylation of a locus whose gene products are responsible for primary sex characteristics or gamete formation caused the origin of a second sex. Methylation regulates production of gene products in a regulatory cascade that produces female or male sexual characteristics (Iannello *et al*., 1997; Grant, 1999; Iannello *et al*., 2000; Griswold & Kim, 2001). Suppression of such gene products could suppress the production of either eggs or sperm (Charlesworth & Charlesworth, 1978). Whichever pair of autosomes this differential methylation first occurred on would become the proto-sex chromosomes. If the differential methylation suppressed production of male (female) sex hormones or some other gene product that contributes to production of sperm (egg) cells, then this would become the proto-Y (W) chromosome. A second pair of autosomes could not subsequently form another set of proto-sex chromosomes because they could only do so by indirectly regulating the production of eggs or sperm, which would either result in (1) no effect if both methylated sites affected production of the same form of gamete or (2) zero fitness if both methylated sites inhibited production of different forms of gametes. Differential methylation could affect regulation of genes on other chromosomes, however, so long as these genes do not affect sex determination and production of gametes. Furthermore, the chromosomes on which these other genes reside will not have loci excised. Below, I further discuss why there can only be one pair of homologous chromosomes of unequal length.

'In principle, one genetic or epigenetic signal . . . would suffice for sexual differentiation' (Dübendorfer *et al*., 2002: 75). Only a small regulatory change, such as differential methylation, would be required to create the first female or male individual in a hermaphroditic species. In hermaphroditic individuals, female (male) functions are suppressed in male (female) sex organs. Therefore, a strictly female individual could be created via co-option (by the male sex organs) of the regulatory machinery that originally suppressed male function in female sex organs of hermaphrodites. For ancestral lineages, sex determination was probably controlled by one or a few loci. However, with repeated duplications of these genes over evolutionary time, derived lineages may have multiple loci involved in sex determination.

If sex is determined by many independent (but possibly paralogous) genes and each locus requires relatively dense promoter methylation to be suppressed, then the old notion of global determination of sex is correct. If sex is determined by one or a few genes whose resulting biochemical pathways are tightly

interlinked, then the modern notion of a single sex-determining locus is correct (e.g. Canning & Lovell-Badge, 2002), especially if these gene products have promoters that are down-regulated by the addition of only one or a few methyl groups.

Evolution of sex chromosomes

Evolution of sex chromosomes is usually explained by a traditional application of Muller's ratchet. Muller's ratchet is a population genetic model of how the minimum number of point mutations (hereinafter mutations) per individual accumulates over evolutionary time in a finite population with reduced recombination, i.e. with reduced crossing over between distinct homologues. The class of individuals with the minimum number of mutations is lost via genetic drift and mutation. The speed of Muller's ratchet is proportional to the per-genome mutation rate (i.e. per-locus mutation rate times genome length) and inversely proportional to the effective population size and recombination rate (Nei, 1970; Haigh, 1978). Muller's ratchet only applies when the haploid stage of the life cycle is immune from selection. Technically, Nei's (1970) model only accounts for a single iteration of the ratchet. An extension of Nei's model to multiple steps of Muller's ratchet does not exist for sexual lineages. I therefore rely on his single step approach, but refer to the output of his model as speed of Muller's ratchet.

Evolution of sex chromosomes has also been explained using other forms of the Fisher–Muller model (Felsenstein, 1988), such as genetic hitchhiking or Hill–Robertson effect, and by retrotransposon traps. None of these models explain how recombination is initially suppressed in the vicinity of putative sex-determining loci, especially in genetically identical sex chromosomes, although this is a prerequisite for each model. Nor do these models explain why derived lineages have more highly degenerated sex chromosomes compared with ancestral lineages nor why derived lineages usually have two genetically distinct sexes.

I propose that methylation suppresses recombination, thereby starting Muller's ratchet, that methylation increases mutation rate and decreases recombination rate, thereby speeding up Muller's ratchet, and that mutation and epimutations of methylated nucleotides provide the selective force for deletion of loci from Y and W chromosomes.

The traditional Muller's ratchet model has recombination rate and per genome point mutation rate given exogenously. Nei (1970) appears to have provided the only mathematical model of Muller's ratchet that explicitly includes recombination rate as a parameter. The speed of Muller's ratchet is an increasing function of per-genome mutation rate and a decreasing function of recombination rate (Nei, 1970). Recombination and mutation rates only appear as products of each other in Nei's formulation of Muller's ratchet. In the remainder of this section, I show that recombination rates are inversely proportional to methylation level and that mutation rates are directly proportional to methylation level. Therefore, an increase in methylation level increases the speed of Muller's ratchet in two different ways: by suppressing recombination and by increasing mutation. Since these two factors appear as products of each other in Nei's formulation, this results in a geometric increase in the speed of Muller's ratchet. Once recombination and mutation rates are considered functions of methylation level, methylation both starts and accelerates Muller's ratchet.

Methylation suppresses recombination (Holliday, 1984; Holliday, 1988; Colot & Rossignol, 1999) in the same way it suppresses transcription. Heterochromatic proteins bind to methylated loci (Miniou *et al*., 1997; Singer *et al*., 2001; Richards & Elgin, 2002), thereby blocking sites for enzymes that mediate recombination (Catcheside, 1986; Hsieh, Meyn & Camerini-Otero, 1986; Rauth *et al*., 1986). It is currently believed that methylation suppresses recombination of any loci that are known recombination sites. What is important here is starting Muller's ratchet, so there simply need to be a few sites that have suppressed recombination, such as promoters that control sex determination.

Contrary to many claims, sexually antagonistic genes (Fisher, 1931; Bull, 1983; Rice, 1987a, 1996) do not in fact provide a mechanism for initially suppressing recombination. They only accelerate already existing local suppression of recombination. The sexually antagonistic genes model contains two assumptions: (1) there is a single or there are a few sex-determining loci and (2) genes that locally suppress recombination are common near those sex-determining loci (Bull, 1983; Rice, 1996). With these two strong assumptions, sexually antagonistic genes can cause the degeneration of Y or W chromosomes and may play a role in the latter stages of sex chromosome evolution, but probably played no role in incipient sex chromosome formation. Differential methylation obviates these two assumptions and does so by providing the molecular mechanisms for initially suppressing recombination, mechanism that the leading supporters of the sexually antagonistic genes hypothesis claim are unknown (e.g. Rice, 1996: 334).

Methylated cytosine mutates to thymine at a much higher rate than does unmethylated cytosine. I review the deamination pathways by which methylated and unmethylated cytosine incur point mutations and then discuss correction mechanisms by which these mutations are often repaired (Fig. 2).

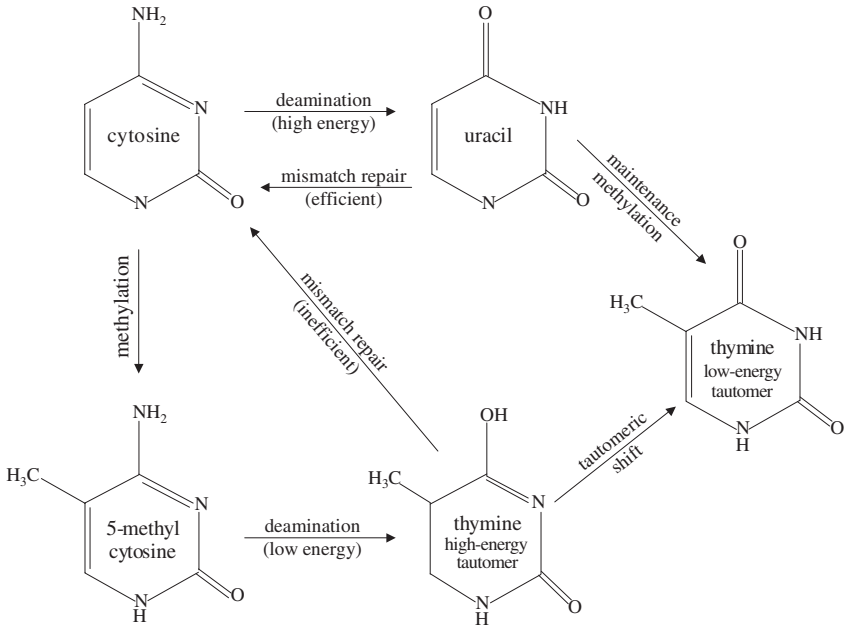

Figure 2. Pathways for methylation, point mutation, and mismatch repair. High-energy tautomer of thymine is 4-hydroxy-5-methyl-2-pyrimidinone. Low-energy tautomer of thymine is 5-methyl-2,4-pyrimidinedione. Methylation of cytosine can be via de novo methylation.

Both methylated and unmethylated cytosine nucleotides incur point mutations (transitions) via deamination. Point mutations of unmethylated cytosine to thymine are via hydrolytic or enzymatic deamination to uracil, which – if not corrected by subsequent mismatch repair – are converted by maintenance methylation to the commonest and lowest energy form of thymine, the dioxo-tautomer, 5-methyl-2,4-pyrimidinedione (Yang, Jones & Shibata, 1996). Point mutations of 5-methylcytosine to thymine are via hydrolytic deamination to a monoxo-tautomeric form of thymine, 4-hydroxy-5-methyl-2-pyrimidinone, which – if not corrected by subsequent mismatch repair – are almost immediately converted to the commonest and lowest energy form of thymine, the dioxo-tautomer, 5-methyl-2,4-pyrimidinedione (Norberg & Vihinen, 2001). Most likely, point mutations of methylated or unmethylated cytosine are spontaneous and methylation of cytosine increases the mutation rate because the attached methyl group lowers the energy required for deamination (Shen, Rideout & Jones, 1994; Yang *et al*., 1996). The energy required for deamination differs if one looks at single oligonucleotides, single-stranded DNA, or double-stranded DNA (Yang *et al*., 1996), indicating that the conformational changes resulting from DNA methylation probably also affect deamination and mutation rates of methylated cytosine. Alternatively, housekeeping enzymes that bind to methylated cytosine, but depend on the structure of DNA molecules, may trigger this deamination (Norberg & Vihinen, 2001). Regardless of the details, methylated cytosine has approximately three times the deamination rate of unmethylated cytosine, and hence a higher mutation rate.

Relative rates of point mutation of methylated and unmethylated cytosine to thymine are also affected by mismatch repair mechanisms that bind to the intermediate products of deamination (Fig. 2). Mismatch repair enzymes appear to detect hydrogen bonding between the incumbent uracil or 4-hydroxy-5-methyl-2-pyrimidinone and the unmethylated or methylated cytosine's original complement of guanine (Barrett *et al*., 1998). Mismatch repair enzymes then convert uracil or 4-hydroxy-5-methyl-2-pyrimidinone to unmethylated cytosine. Mismatch repair does not correct all such point mutations because the mismatch repair enzymes sometimes operate after DNA replication, hence there is no complementary template to use in correcting errors (Brown & Jiricny, 1987; Jones *et al*., 1992). Mismatch repair involves a different, but probably homologous, set of enzymes for point mutations of methylated vs. unmethylated cytosine (Barrett *et al*., 1998). Mismatch repair of hydrogen bonded uracil : guanine pairs is six thousand times more efficient than that of 4-hydroxy-5-methyl-2-pyrimidinone : guanine pairs (Schmutte *et al*., 1995). Heuristically, this is entirely plausible because uracil is recognized as a foreign nucleotide in double-stranded nuclear or mitochondrial DNA (uracil is never used for coding in double-stranded DNA in any

organism), whereas 4-hydroxy-5-methyl-2-pyrimidinone can easily go undetected by mismatch repair enzymes because it is merely a tautomer of thymine. Because it is a low-energy reaction, tautomeric conversion of 4-hydroxy-5-methyl-2-pyrimidinone to 5-methyl-2,4-pyrimidinedione occurs much more rapidly than does maintenance methylation of uracil to thymine, thereby precluding the opportunity for mismatch repair of many deaminated molecules of 5-methylcytosine. Therefore, the ultimate genetic point mutation rate – following deamination, maintenance methylation, and mismatch repair – is almost 20 000 times greater for methylated cytosine . . . so much so that methylated cytosines cause 30–40% of all germ-line point mutations in humans (Jones *et al*., 1992).

If a methylated cytosine is converted to unmethylated cytosine via deamination and mismatch repair, that individual retains an altered methylation signature. From a genetic perspective, this is not a problem because both methylated and unmethylated cytosine are transcribed into identical messenger RNA, if they are transcribed at all (Jones *et al*., 1992). However, from an epigenetic perspective, this can have dire consequences, especially if it occurs at a sex-determining locus. The only opportunity for correcting this loss of methylation is during global demethylation and de novo methylation of the meiotic (nuclear) genome at or immediately following gamete formation and syngamy. However, this is too late for an individual that depends on its methylation to regulate production of primary sexual characters and gametes.

Increased mutation rate of methylated (vs. unmethylated) cytosine into thymine increases the speed of Muller's ratchet. Once genes containing sex-determining methylation patterns contain nucleotides that mutate from methylated cytosine into thymine, there is strong selective pressure to excise these loci, resulting in immediate degeneration of the proto-Y chromosome into a true (i.e. shorter) Y chromosome. Note that the only way in which one of two differentially methylated homologous chromosomes will initially have loci excised is if the differential methylation suppressed production of eggs or sperm. Because it is dire to completely suppress production of both eggs and sperm and of no consequence to suppress two separate parts of the same regulatory pathway that produces either eggs or sperm, only one set of autosomes can have loci initially excised and thereby become sex chromosomes in any given lineage. Existence of only a single pair of sex chromosomes is not predicted by any other theory, including those hypothesizing a single putative sex-determining locus, because such putative loci can be duplicated.

As with all previous theories regarding degeneration of sex chromosomes (e.g. Charlesworth, 1991; Rice, 1994, 1996; Charlesworth & Charlesworth, 2000), I remain mute about the precise mechanism by which mutated and epimutated loci are excised from non-recombining portions of sex chromosomes. Although such loci may be responsible for severe fitness disadvantages, this does not explain how they are excised. Because recombination is locally suppressed in all of these models, excision cannot be due to increased crossing over. However, with methylation driving evolution of sex chromosomes, a lack of crossing over provides an absolute fitness disadvantage insofar as X or Z chromosomes do not accumulate beneficial mutations more rapidly than do Y or W chromosomes, as would be case with background trapping (Rice, 1996).

Once recombination is initially suppressed and Muller's ratchet has started on a pair of proto-sex chromosomes, there is no longer a requirement that the mutations driving the ratchet be associated with sex determination. Mutated loci merely need to be tightly linked to an ancestral sex-determining locus, i.e. be on the same chromosome with suppressed recombination. Thus, ceteris paribus, when the heterogametic proto-sex chromosome has more methylation of any loci, it will have a faster Muller's ratchet.

Although I propose that methylation is largely responsible for the origin of two distinct sexes and sex chromosomes, other mechanisms could be responsible for the latter stages in this evolution. Inversions and translocations are responsible for many recently derived structural differences in sex chromosomes. For example, the relative sizes of sex chromosomes across all mammals and across all species in the lizard genus *Sceloporus* are probably due to chromosomal rearrangements, and not to gradual deterioration of a Y or W chromosome (Sites *et al*., 1992; Graves, 1995b).

Because sex is initially determined by differential methylation and (evolutionarily) later by gene deletions from Y or W chromosomes, differences between proto-sex chromosomes canalize dioecy. Originally, each pair of homologous chromosomes had the same lengths and the same methylation patterns, and these lineages were cosexual. Differential methylation of one pair of homologous chromosomes canalized dioecy (i.e. loss of hermaphrodites) in diploid eukaryote lineages, although these lineages may still have been cosexual as with temperature-dependent sex determination. With sufficient differences in methylation between the two sexes, sex change became unidirectional or nonexistent and heterogameity became canalized. Evolution from X/Y to Z/W, or vice versa, became impossible. Furthermore, dioecy itself became canalized. Charles Darwin was the first to suggest that the sex of animals and plants is canalized from an initial hermaphroditic condition (Darwin, 1873; Stauffer, 1975). However, lack of infor-

mation regarding heredity precluded him from hypothesizing about molecular or physiological causes for this canalization.

The most likely reason why nobody as yet has independently applied methylation to Muller's ratchet for the evolution of sex chromosomes is that virtually all modern biologists working on this problem have used *Drosophila* as their study organism (Charlesworth, 1978; Rice, 1987b; Charlesworth, 1991; Steinemann & Steinemann, 1992; Rice, 1996; Charlesworth & Charlesworth, 2000). The family Drosophilidae and other isolated lineages of classical model organisms, such as the nematode *Caenorhabditis elegans* and the yeast *Saccharomyces cervisiae*, independently lost all or most DNA methylation in the recent evolutionary past, while 'normal' higher levels of methylation were retained in all related lineages (Urieli-Shoval *et al*., 1982; Proffitt *et al*., 1984; Riggs & Pfeifer, 1992; Wolffe & Matzke, 1999; Gowher *et al*., 2000; Lyko *et al*., 2000). This highly derived lack of (most) methylation deprived researchers working with *Drosophila* of the key mechanisms by which the sex of an individual is determined, recombination is suppressed, and mutation rate is increased.

Although the arguments in this section on the evolution of sex chromosomes appear to apply to all eukaryotes, they in fact only apply to metazoans because of an underlying assumption behind Muller's ratchet. One of the predictions below posits that other eukaryotes, such as fungi and plants, are immune from the effects of Muller's ratchet due to their extensive gene expression during haploid stages. In contrast, my arguments regarding evolution of dioecy apply to all anisogamous sexual diploid eukaryotes. Their nuclear genomes can become differentially methylated, even though Muller's ratchet cannot subsequently operate on them.

PREDICTIONS

I provide testable predictions arising from the above theory, including several corollaries (Table 1). I also give details regarding the testing of these predictions.

Dioecious lineages have heteromorphic sex chromosomes

I predict that all dioecious lineages will have at least slightly different sex chromosomes. If the homologous chromosomes have identical nucleotide sequences, then they will have different methylation patterns. These chromosomes will have differences in G-banding patterns because heterochromatic proteins bind to methylated DNA (Schmid & Haaf, 1989).

Only after comprehensive data on methylation levels becomes available will it be possible to test whether dioecious lineages with putative sex chromosomes of equal length have differential methylation levels between the two homologues in the heterogametic sex. The output of this analysis would be a phylogenetic comparison of dioecy/monoecy (or dioecy/cosexual) vs. extent of methylation difference between the sexes or between the two proto-sex chromosomes. Cosexual lineages are defined as those in which there exist some individuals that are capable of producing both eggs and sperm or have environmentally determined sex, i.e. lack of strict dioecy (Lloyd, 1980). Comparisons of methylation differences will be especially important if they are of promoters of genes known to code for production of gametes or primary sexual characters. This analysis may require a separate comparison for each large taxonomic group, such as vertebrates and flowering plants. The most appropriate tool would be bisulphite sequencing of promoters affecting sex determination, especially using tools that assess density of methylation (Galm *et al*., 2002; Gitan *et al*., 2002). A simpler approach would be to use reverse-phase high-performance liquid chromatography (HPLC). However, the results would be equivocal because the relevant promoters undoubtedly occupy a small fraction of the genome, which may not have methylation patterns similar to the rest of the genome.

A corollary is that cosexual lineages will contain a pair of sex chromosomes with virtually the same methylation patterns and the same lengths. A perusal of the literature corroborates this corollary, especially for vertebrates, for which the nature of sex chromosomes has been encyclopedically documented (Solari, 1994), assuming that G-banding reflects relative methylation levels.

Sex change can be induced in lineages with equal length sex chromosomes

I predict that most environmentally and hormonally induced sex changes in flowering plants and vertebrates occur via demethylation and these methylation changes are heritable (LoSchiavo *et al*., 1989; Arnholdt-Schmitt *et al*., 1991; Dorazi, Chesnel & Dournan, 1995; Demeulemeester, Van Stallen & De Proft, 1999; Murphy & Jirtle, 2000; Tatra *et al*., 2000). Recent work indicates that sex hormones determine sex via heritable changes in methylation patterns (McLachlan, 2001). Therefore, in lineages in which sex is solely determined by small differences in methylation between proto-X and Y (or Z and W) chromosomes, I predict that it should be possible to change the sex of an individual by subjecting it to demethylating compounds (Vyskot *et al*., 1995). If this theory is correct, these sex changes should primarily be back to the ancestral hermaphroditic condition.

Testing can be done with any dioecious taxon in

Table 1. Predictions arising from methylation driving evolution of dioecy and sex chromosomes

Prediction	Data required to test	Tentative results
Dioecy yields heteromorphic sex chromosomes	Bisulphite sequences; phylogeny	None
Cosexual lineages have both sex chromosomes with virtually the same methylation patterns and lengths	Bisulphite sequences, HPLC, or G-chromosomal banding	Confirmed, based on G-banding (Solari, 1994)
In lineages with equal length sex chromosomes, sex change can be induced	Results from application of 5-azacytidine, ethionine or 5-methyl-deoxcytidine	None
If sex chromosomes are of different lengths, then sex change cannot be induced by altering methylation levels	Results from application of 5-azacytidine, ethionine or 5-methyl-deoxcytidine	None
Sex change is unidirectional, to sex with less methylation of sex-determining loci	Bisulphite sequences, HPLC, or G-chromosomal banding	None
If both sexes have equal amounts but different distributions of methylation of sex-determining loci, then changes in sex can be in either direction, depending on which methyl groups are removed	Bisulphite sequencing	None
Derived lineages have shorter Y (or W) chromosomes	Genome length, phylogeny	Tentative corroboration in vertebrates (Solari, 1994)
Different length sex chromosomes yields dioecy	Chromosome length	Tentative corroboration (Solari, 1994)
Diploid cosexual lineages cannot be descended from dioecious lineages	Phylogenies accurately showing transitions between cosexuality and dioecy	No known counterexamples
Allopolyploidy provides an escape from canalization of dioecy	Whether polyploidy was a result of alloploidy or autoploidy; gene phylogenies with transitions between cosexuality and dioecy	No known counterexamples, but most data necessary for testing is nonexistent
TSD requires homomorphic sex chromosomes	Chromosome lengths	Tentatively corroborated in vertebrates (Olmo, 1986; Spotila *et al*., 1994)
Highly heteromorphic sex chromosomes imply dioecy, hence GSD	Chromosome lengths	Tentatively corroborated in vertebrates (Solari, 1994)
Degeneration of sex chromosomes will be rapid	Estimates of evolutionary rates of sex chromosome degeneration	None
Plant and fungal gametophytes express most genes expressed by their diploid stages	Microarray comparisons of haploid and diploid stages	None
Substantial genetic drift implies higher likelihood of cosexuality	Sister taxa having planktonic and non-planktonic larvae; sex chromosome lengths	None
Most marine invertebrates have identical sex chromosomes and are thus hermaphroditic	Knowledge of cytology and breeding systems	Seems to be true
Terrestrial and marine invertebrates without ancestral planktonic larvae have distinctive sex chromosomes and are strictly dioecious	Knowledge of cytology and breeding systems	Seems to be true, but this may be biased by insects and benthic nematodes

GSD, genetic sex determination; HPLC, high-performance liquid chromatography; TSD, temperature-dependent sex determination.

which there is no difference in length between sex chromosomes and sex is determined solely by small differences in methylation levels. Dosages of 5-azacytidine or ethionine could be applied so that the resulting methylation level corresponds with that of the less methylated sex (Gorelick & Osborne, 2002), realizing that the 'wrong' methyl groups might get stripped away (Giancotti *et al*., 1995). Application of 5-azacytidine to males of the dioecious flowering plant *Melandrium album* changed some of them into androhermaphroditic indi-

viduals, but it had no effect when applied to female plants (Janousek, Siroky & Vyskot, 1996). This indicates that methylation had a female-suppressing function in male individuals. It may also be possible to add methylation to the less methylated sex by applying 5-methyl-deoxcytidine (Holliday & Ho, 1991; Nyce, 1991; Holliday & Ho, 1995).

A corollary is that if sex chromosomes are of different lengths, then sex change producing sexually fertile individuals cannot be induced by altering methylation levels or by any other method aimed at single genes (Solari, 1994).

Sex change is unidirectional, to the sex with less methylation of sex-determining loci

I predict that sex changes will almost always be in one direction – from the more to the less methylated sex or to a relatively unmethylated hermaphrodite – when methylation changes are only on sex-determining promoters. The motivation behind this prediction is that it is much simpler (i.e. requires much less complicated biochemical machinery) to remove methyl groups than it is to add them (in many of these instances, maintenance methylation following mitosis is precluded, rather than methylation being stripped away). Analogously, it is much simpler to teach someone to erase a chalkboard than to teach them to write something intelligent on one (an entropy argument). It is well known that sex change is usually only in one direction (Nakashima, Kuwamura & Yogo, 1995; Pannell, 1997; Kuwamura & Nakashima, 1998). What is new here is that I propose that the change is almost always to the less methylated sex-determining loci.

It is possible to have differential methylation of females and males with both sexes having equal amounts of methylation of promoters or of any and all portions of their genomes (Pardo-Manuel de Villena, de la Casa-Esperón & Sapienza, 2000). In this case, I predict that changes in sex can be in either direction, simply depending on which methyl groups are removed.

This prediction appears to be corroborated. Sex change is usually only in one direction (Nakashima *et al*., 1995; Pannell, 1997; Kuwamura & Nakashima, 1998). In the rare instances where sex change can occur in both directions in a lineage, it would be helpful to determine whether the methylation levels between the two sexes are virtually identical, at least on those promoters that control for gamete production and primary sexual characters.

Derived lineages have shorter Y (or W) chromosomes

Derived lineages often evolved via duplication of regulatory genes, accumulation of transposons, and/or polyploidy (Ohno, 1970; Cooke *et al*., 1997; Force *et al*., 1999), all of which result in disproportionate methylation (Nagl & Ehrendorfer, 1974; Volpe & Eremenko, 1974; Holliday, 1984; Yoder, Walsh & Bestor, 1997; Matzke & Matzke, 1998; Regev, Lamb & Jablonka, 1998; Colot & Rossignol, 1999; Jones & Takai, 2001; Martienssen & Colot, 2001). This starts and accelerates Muller's ratchet because of the combined effect of longer genome lengths, higher per-locus mutation rates, and lower recombination rates. Therefore, I predict that the ratio of lengths of X and Y (or Z and W) chromosomes in a lineage will be proportional to how derived that lineage is. Derived lineages will have shorter Y or W chromosomes (relative to their respective X or Z chromosomes) compared with ancestral lineages. This prediction is an amalgamation of Muller's ratchet driven by methylation coupled with a generalization of the Steinemanns' retrotransposon trap (Steinemann & Steinemann, 1992, 1998). I generalize their retrotransposon trap by asserting that gene duplications could be of things other than retrotransposons. No other hypothesis predicts that more derived lineages will have shorter sex chromosomes.

This prediction can most easily be tested in amniotic vertebrates. Support comes from snakes, birds, and mammals, which are the only vertebrates with well-differentiated sex chromosomes. Only the most basal lineages of each (boas, ratites and tinamins, monotremes) have poorly differentiated sex chromosomes (Jones & Singh, 1985; Solari, 1994; Graves, 1995a; Pigozzi & Solari, 1999; Pigozzi, 1999). Furthermore, vertebrates with poorly differentiated sex chromosomes have much less banding, indicative of low levels of methylation (Ray-Chaudhuri, Singh & Sharma, 1971; Solari, 1994; Pigozzi & Solari, 1997).

We must also account for genome length in any such analysis because application of the traditional Muller's ratchet without methylation also predicts that derived lineages have shorter Y chromosomes. The speed of Muller's ratchet is proportional to the per-genome point mutation rate, which is itself equal to the average point mutation rate times genome length. Therefore, gene duplications in derived lineages would increase the rate of Muller's ratchet by increasing genome length, even without invoking methylation. To discern whether methylation further increases the speed of Muller's ratchet by also increasing the average point mutation rate and suppressing the recombination rate, one should also estimate genome length at ancestral nodes using phylogenetic comparative methods (Martins & Hansen, 1997; Schluter *et al*., 1997). We could then control for the traditional Muller's ratchet being driven solely by increased genome length.

Vertebrate phylogenies are continually being refined (e.g. Cao *et al*., 2000b; Gissi *et al*., 2000). Data

on vertebrate genome lengths have been compiled in the 'Animal cytogenetics' series (Borganonkar, 1974; Egozcue, 1974; Fregda, 1974; Gustavsson, 1974; Hayman & Martin, 1974; Patton, 1974; Olmo, 1986; Christidis, 1990) and references therein. Phylogenetic comparisons should be especially useful in lineages with a large range of ratios of lengths of their sex chromosomes, such as snakes, iguanids and birds.

Methylation can canalize dioecy

I predict that in lineages in which methylation originally determines the sex of an individual, this methylation also canalizes dioecy. This prediction provides an epigenetic mechanism for inducing canalization.

I predict that lineages with different length sex chromosomes will be dioecious because sex is determined by gene deletions and not just differential methylation. In such individuals, sex changes cannot be induced by altering sex-specific methylation patterns because these patterns disappeared when methylated cytosines mutated to thymines. However, this prediction also arises from other theories.

With equal length sex chromosomes, the prediction that diploid cosexual lineages cannot be descended from dioecious lineages does not arise from any other theory. To test this, correlate dioecy/cosexuality with the proportion of differential chromosomal G-banding, especially where only one sex has heteromorphically banded putative proto-sex chromosomes. Most G-banding seems to be due to heterochromatin, which in turn may be associated with methylation (de Almeida-Toledo *et al*., 1998), so that sex determination is epigenetically controlled (Negrutiu *et al*., 2001). G-banding data (Solari, 1994) and robust phylogenies (Olmo, 1986; Baldauf *et al*., 2000; Cao *et al*., 2000a; Gissi *et al*., 2000) exist, including in lineages for which there is evidence that differential methylation may be the first step in differentiation of sex chromosomes.

It will be valuable to test whether diploid cosexual lineages cannot be descended from dioecious lineages in fish, amphibians, or certain basal reptiles (e.g. boas), and seeing where on the phylogenies these unexpected transitions from dioecy to cosexuality occurred. There are apparently no counterexamples to the prediction that lineages with different length sex chromosomes are dioecious (Solari, 1994).

Although I predict that diploid cosexual lineages cannot be descended from diploid dioecious lineages (Johnston**,** Barnett & Sharpe, 1995), allopolyploidy provides an escape – the only escape – from this canalization of dioecy. Loss of loci from proto-Y or proto-W chromosomes canalizes dioecy in diploid lineages. Allopolyploid progeny, however, can contain chromosomes determining both femaleness and maleness, even though each of their parents could only do one of the two. Flowering plants have much higher incidences of polyploidy than do other seed plants (Otto & Whitton, 2000), hence they should have a higher proportion of cosexual lineages, which would corroborate this prediction. This prediction could be made from several other theories if the diploid dioecious lineage has sex chromosomes of different length. What is unique here is that this prediction cannot be made with other theories if dioecy is solely due to differential methylation of sex-determining genes on one pair of homologous chromosomes, the proto-sex chromosomes, as I believe happens with many plants. A robust test of the prediction that methylation canalizes dioecy is whether allopolyploidy provides an escape from canalization of dioecy.

We could test whether transitions from dioecy to cosexuality always occur along with a polyploid event, particularly in the vast majority of plants in which both sex chromosomes are of equal length. There do not appear to be any counterexamples to the assertion that cosexuality cannot evolve from dioecy unless there is allopolyploidy. This test is vacuous for tetrapods, for which there is no known allopolyploidy (Dowling & Secor, 1997), but should be useful for plants. This test does not suffer from the previously listed problems, but suffers from lack of data on whether chromosomal doubling was due to autopolyploidy or allopolyploidy. However, reversing the logic, this test provides a new means for determining whether allopolyploidy or autopolyploidy was the cause of chromosomal doubling in a lineage.

The escape from canalization test potentially suffers from lack of data on evolutionary transitions from dioecy to cosexuality. It also suffers from the problem that phylogenetic comparative methods all assume no reticulate evolution, a problem that can be sidestepped by using gene phylogenies (which are not reticulate), instead of organism phylogenies.

An important corollary of methylation canalizing dioecy is that temperature-dependent sex determination (TSD) requires homomorphic sex chromosomes. One of the most widely discussed forms of cosexuality in vertebrates is TSD. TSD is a specific form of environmental sex determination (ESD), in which sex determination may be induced by osmotic stress, anomalous levels of oxygen or carbon dioxide, or other environmental factors (Ackerman, 1981; Gutzke & Paukstis, 1983; Spotila**,** Spotila & Kaufer, 1994). TSD and ESD are to be distinguished from genetic sex determination (GSD). I predict that TSD and ESD can only occur in species that have virtually identical sex chromosomes; they must have the same length and have very similar levels of methylation and G-banding. Therefore, small environmental changes can result in alteration of their methylation patterns, thereby determining the sex of each individual. As

soon as sex determination becomes more canalized – either via degenerating length of Y or W chromosomes or via highly disparate differential methylation – then TSD and ESD should not be able to function. Highly heteromorphic sex chromosomes imply that a species must be strictly dioecious, hence have GSD.

A cursory look at reptiles corroborates the prediction that ESD is confined to animals with homomorphic sex chromosomes. The only reptiles with TSD are most turtles, all crocodiles, some geckos, and some agamid lizards (Olmo, 1986; Spotila *et al*., 1994). Each of these seem to have identical or virtually identical sex chromosomes (Olmo, 1986 and references therein). Furthermore, all vertebrates that have Y (or W) chromosomes that are shorter than their X (or Z) chromosomes have GSD (Solari, 1994).

Degeneration of sex chromosomes will be rapid

I predict that degeneration of sex chromosomes will be rapid once loci containing sex-determining methylation patterns incur mutations to (unmethylated) thymine or epimutations to unmethylated cytosine because individuals containing such mutations or epimutations will no longer be recognizable as either sex. Silencing such genes will not help because of their necessary role in sex determination. Degeneration will also be hastened because methylated cytosine has a higher mutation rate. Rapid degeneration of sex chromosomes is contrary to all other models of sex chromosome evolution (Steinemann & Steinemann, 1992; Tucker & Lundrigan, 1995; Rice, 1996; Charlesworth & Charlesworth, 2000).

Searching for evidence of rapid degeneration of sex chromosomes is highly non-trivial, yet it might exist in lineages with incipient sex chromosome formation, such as in some snakes (Jones & Singh, 1985) and fishes (Koehler *et al*., 1995).

I also predict that the rate of degeneration and eventual size of heterogametic sex chromosomes (i.e. Y or W) should be proportional to the total amount of methylation contained on the heterogametic proto-sex chromosome (vice the amount of methylation contained on promoters). Lineages with relatively low overall methylation levels per chromosome will therefore not evolve distinctive sex chromosomes.

Plant and fungal haploid stages will express most of the genes expressed by their diploid stages

Methylation driving Muller's ratchet is the primary cause for evolution of unequal length sex chromosomes in metazoan animals, but not in other sexual diploid eukaryotes, such as plants and fungi. Why? Muller's ratchet simply does not apply to plants and fungi because their haploid genomes are subject to strong selection. The thing that differentiates animals from plants and fungi here is the number of genes expressed in their haploid stages (gametophytes). The haploid stages in two plant species express most of the genes that are expressed by their diploid stages (Willing & Mascarenhas, 1984; Willing, Bashe & Mascarenhas, 1988), thereby nullifying the effects of Muller's ratchet. Similar data do not exist for other plants or fungi. I therefore predict that the haploid stages of most plants and fungi will express most of the genes expressed by their somatic diploid stages (sporophytes). If there are any exceptions to this prediction, they should have sex chromosomes of different lengths.

Microarray analysis, beginning with reverse transcribed mRNA from male and/or female gametophytes and a target treatment with a mixture of sporophyte cells, should readily provide estimates of the percentage of genes expressed in gametophytes (Desprez *et al*., 1998; Schaffer *et al*., 2000). Microarray comparisons of haploid vs. diploid stages have never been done.

Substantial genetic drift implies higher likelihood of cosexuality

I predict that most marine invertebrates with planktonic larvae will have virtually identical sex chromosomes and be hermaphrodites. Many marine invertebrates have much larger effective population sizes than do terrestrial invertebrates, largely due to wide dispersal of larvae on ocean currents. A large effective population size virtually stops Muller's ratchet due to a lack of genetic drift (Nei, 1970; Charlesworth & Charlesworth, 2000). Most marine invertebrates will have virtually identical sex chromosomes and can therefore contain many hermaphroditic species. Terrestrial invertebrates and those marine lineages without ancestral planktonic larvae will have distinctive sex chromosomes and therefore be strictly dioecious. Compounding this predicted effect, most terrestrial invertebrates are evolutionarily derived compared with their marine counterparts. This prediction about relative lengths of sex chromosomes arises from the traditional Muller's ratchet model, except that I have added the prediction about the incidence of hermaphrodites and dioecy.

Testing would require compilation of pairs of closely related species in which one taxon has planktonic larvae and the other has non-planktonic (including terrestrial) larvae and then conducting phylogenetic comparisons of the ratios of sex chromosome lengths with the binary variable of whether the taxa are dioecious or cosexual (Felsenstein, 1985). A cursory glance at marine vs. terrestrial invertebrates seems to indicate a greater incidence of strict dioecy in terres-

trial invertebrates, but this corroboration may be due to the predominance of insects in terrestrial faunas (Ehrlich & Wilson, 1991) and nematodes in deep-sea benthic faunas (Baldwin, Nadler & Hall, 1999). Insects are a highly derived and relatively recently evolved class (Kaesler, 1987), while there are no known nematodes with planktonic larvae (Baldwin *et al*., 1999).

CONCLUSION

Differential methylation of sex chromosomes provides a unifying mechanism by which many patterns of sex determination, sex change, dioecy, and sex chromosome length can be explained. Differential methylation suppresses transcription in a sex-specific manner, causing the origin of two distinct sexes. Methylation suppresses recombination, allowing Muller's ratchet to operate on differentially methylated proto-sex chromosomes. Methylation increases point mutation rates and decreases recombination rates, thereby geometrically accelerating Muller's ratchet. These point mutations (i.e. nucleotide transitions) cause loss of a portion of the methylation that originally determined sex, providing strong selective pressure to excise these mutated and epimutated genes. Duplicated genes, which provide a major evolutionary force, are disproportionally methylated and thereby greatly accelerate Muller's ratchet and the degeneration of heterogametic sex chromosomes.

I have detailed many possible predictions and tests for the hypotheses that methylation was the original determinant of sex and that methylation driving Muller's ratchet was responsible for sex chromosome degeneration. The predictions that derived lineages have smaller ratios of Y to X (or W to Z) chromosome lengths and that methylation canalizes dioecy are readily testable with existing data. The remaining predictions are less readily testable at this juncture because of a current dearth of appropriate data, especially of methylation levels. Nonetheless, there are no technological impediments to conducting these tests, which would then allow robust testing of this theory.

ACKNOWLEDGEMENTS

Thanks to Greg Pollock, Sue Bertram, Roy Osborne, Manfred Laubichler, Alberto Solari, Andrea Case, Jennifer Fewell, Paige Warren, Allen Moore, Todd Katzner, Tom Dowling, Chris Lorson, Carlos Castillo-Chavez, Marty Anderies, Tom Taylor, Michael Berrill, Justen Whittall and two anonymous reviewers for their numerous helpful comments. Thanks to the Arizona State University biology department and graduate college for two summer research fellowships.

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