X chromosome evolution in Drosophila

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Abstract

Although the *X* chromosome is usually similar to the autosomes in size, gene density and cytogenetic appearance, theoretical models predict that its hemizygosity in males may cause unusual patterns of evolution. The sequencing of several genomes has indeed revealed differences between the *X* chromosome and the autosomes in the rates of gene divergence, patterns of gene expression and rates of gene movement between chromosomes. In this thesis, I have attempted to investigate some of these patterns and their possible causes.

The first two chapters consist of theoretical and empirical work intended to analyse the rates of evolution of coding sequences of *X*-linked and autosomal loci, with particular emphasis on faster-*X* evolution, the theory that more effective selection on the *X* can lead to higher rates of adaptive evolution on this chromosome. By analyzing *X*-linked and autosomal coding sequence in several species of Drosophila, we found some evidence for more effective selection on the *X*, particularly evident in the higher levels of codon usage bias detected at *X*-linked loci. We argue that this could be due to higher levels of recombination on the *X* chromosome increasing its effective population size (N_{eX}) relative to the autosomal effective population size (N_{eA}). To further investigate this hypothesis, we have modeled the effect of increased N_{eX}/N_{eA} on rates of evolution and confirmed that this can contribute to faster-*X* evolution.

The last two chapters deal with the evolution of sex-biased genes and the possible causes for their differential accumulation on the *X.* We used EST data to create expression profiles for *D. melanogaster* male-, female- and unbiased genes. Our results suggest that the expression levels of sex-biased genes are incompatible with the accepted

model of sex-biased gene evolution. We also show that the deficit of testis-expressed genes that is observed in Drosophila seems to be stronger for highly expressed genes. In fact, for very lowly expressed genes, we observe a small excess of testis-expressed genes on the *X*. We attempt to discuss this pattern in view of what is currently known about the evolution of sex-biased gene expression.

Contents

Declaration

I declare that this thesis has been composed by myself and is entirely my own work.

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Chapter 1: General Introduction

Abstract

Although the *X* chromosome is usually similar to the autosomes in size and cytogenetic appearance, theoretical models predict that its hemizygosity in males may cause unusual patterns of evolution. The sequencing of several genomes has indeed revealed differences between the *X* chromosome and the autosomes in the rates of divergence, patterns of gene expression and rates of gene movement. A better understanding of these patterns should provide valuable information on the evolution of genes located on the *X* chromosome. It may also suggest solutions to more general problems in molecular evolution, such as detecting selection and estimating mutational effects on fitness.

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1.1 Introduction

Sex chromosome systems have evolved independently numerous times, and have attracted much attention from evolutionary geneticists. This work has been mainly focused on the steps leading to the initial evolution of sex chromosomes, and the genetic degeneration of *Y* and *W* chromosomes (e.g. Charlesworth *et al.*, 2005). Here we will discuss the evolution of the *X* chromosome in long-established sex chromosome systems, such those of mammals and *Drosophila* species. The emphasis is on recent molecular evolutionary, genomic and gene expression studies, especially as the whole genome analysis of several *Drosophila* (Richards *et al.*, 2005) and mammalian (The Chimpanzee Sequencing and Analysis Consortium, 2005) species has provided estimates of divergence rates for both coding and non-coding regions of the sex chromosomes and the autosomes. In addition, several studies using microarray technology have revealed that many genes that are expressed exclusively or preferentially in one sex in *Drosophila melanogaster* (Parisi *et al.*, 2003; Ranz *et al.*, 2003), mammals (Lercher *et al.*, 2003; Khil *et al.*, 2004) and *Caenorhabditis elegans* (Reinke *et al.*, 2004).

The evolutionary properties of the *X* chromosome are also relevant to several interesting biological phenomena that occur above the molecular level. In the genus Drosophila, the *X* chromosome appears to be enriched in genes that cause reproductive isolation between species (Tao *et al.*, 2003), helping to explain classic observations such as Haldane's Rule (Coyne and Orr, 2004). Similarly, genes expressed in the brain (Skuse, 2005) and genes controlling fertility (Saifi and Chandra, 1999) appear to be preferentially located on the human *X* chromosome. A better understanding of the

general evolutionary properties of genes located on the *X* chromosome will help to determine the causes of these peculiarities. Furthermore, tests of the predictions of theoretical models of *X* evolution will shed light on the assumptions on which they are based, such as the degree of dominance of mutations or the existence of opposing forces of selection on males and females, leading to better understanding of the forces that shape the evolution of eukaryotic genomes.

We first examine DNA sequence divergence to ask: is the *X* chromosome evolving at a different rate from the autosomes or *Y* chromosome, and what might cause such a difference? Second, we review evidence on the evolution of the expression patterns of *X*-linked genes, in particular discussing why so many of them exhibit sex-biased expression.

In all the clades analysed, the *X* chromosome appears to be under more efficient selection and to accumulate new genes, or genes with new, sex-biased expression patterns, differently from the autosomes. However, differences between the more extensively studied *Drosophila melanogaster* and mammalian *X* chromosomes make it hard to explain all the current data, suggesting that more work is necessary to clarify the processes involved.

1.2 A different mutation rate on the X?

1.2.1 Male-driven evolution and the X chromosome

Most mutational changes in DNA are thought to occur through replication errors during cell division (Drake *et al.*, 1998). Consequently, the mutation rate per generation is expected to increase with the number of divisions in the germline (only mutations in

the germline are transmitted to the next generation) (Keightley and Eyre-Walker, 2000). In species with separate sexes, males and females have different ways of making gametes, which may cause a difference in the number of cell divisions. In mammals, for instance, spermatogenesis requires more cell divisions than oogenesis, so that the mutation rate in the male germline is likely to be higher than that in the female germline (Haldane, 1947; Miyata *et al.*, 1987). This effect is very sensitive to the average ages at reproduction of males and females, since the overall mutation rate for a given sex is the sum over mutations contributed by individuals from all reproductively active ages (Charlesworth, 1994).

Genes on autosomes spend an equal amount of their time in males and females, so that their net mutation rate is the average of the male and female mutation rates. With male heterogamety, *X*-linked genes spend only $^1/_3$ of their time in males and $^2/_3$ of their time in females. If spermatogenesis is more mutagenic than oogenesis, the *X* chromosome is subjected to a lower mutation rate than the autosomes (or the *Y* chromosome) (Haldane, 1947; Miyata *et al.*, 1987). The reverse is true for Z-linked genes in taxa with female heterogamety. This results in corresponding differences in the rate of molecular sequence evolution, since the rate of neutral DNA sequence divergence between species is equal to the mutation rate (Kimura, 1968; Li, 1997).

1.2.2 Use of data on DNA sequence evolution to estimate α

The rate of substitution, *K*, is defined as the number of mutations that become fixed in a population per unit of evolutionary time (Kimura, 1968). This value can be estimated from the degree of DNA sequence divergence between two taxa with a known date of divergence, by dividing the estimated proportion of nucleotide sites for which they differ by the time that separates them (Kimura, 1968). For neutral mutations (i.e. mutations with no effect on fitness), *K* is equal to the mutation rate per site (Kimura, 1968).

Assume that the only factor controlling the relative mutation rates of genes on the X, *Y* and autosomes is the time that they spend in females and males (male heterogamety is assumed). Let the ratio of male mutation rate u_m to female mutation rate u_f be α. Let the substitution rates for autosomal, *X*- chromosome linked and Y- chromosome-linked mutations be K_A, K_X and K_Y, respectively. It is easily shown (Miyata *et al.*, 1987) that:

$$K_A = \frac{(u_f + u_m)}{2} = \frac{(\alpha + 1)\, u_f}{2} \qquad (1.1)$$

$$K_X = \frac{(2u_f + u_m)}{3} = \frac{(\alpha + 2)\, u_f}{3} \qquad (1.2)$$

$$K_Y = u_m = \alpha\, u_f \qquad (1.3)$$

Since u_f is common to all these expressions, it is simple to get two different estimates of α from ratios such as K_A/K_X and K_Y/K_X. Similar expressions can be derived for female heterogamety (Axelsson *et al.*, 2004).

1.2.3 Assessing 'male-driven' evolution in flies

Two complementary approaches have been used to detect such "male-driven" evolution. The first uses comparative data on the numbers of cell divisions required for female and male gametogenesis (Drost and Lee, 1995, 1998). The second estimates between-species divergence levels at silent nucleotide sites for autosomal, *X*- and *Y*-linked sequences; the differences among these yield estimates of α, the ratio of the male to female mutation rates (Miyata *et al.*, 1987). If male-driven evolution is the sole cause of this difference, the estimate of α should be related to the ratio of the numbers of male and female germline divisions required to make a successful gamete, although the sensitivity of net mutation rates to demography (Charlesworth, 1994) means that equality of the two estimates is not necessarily expected. The two approaches have yielded consistent results for *Drosophila melanogaster*: the mean number of divisions is estimated to be 35.5 divisions for spermatogenesis and 34.5 for oogenesis (Drost and Lee, 1998). Although silent divergence among *D. simulans* and *D. melanogaster* is slightly higher for *X*-linked sites, this difference is not significant (i.e. α is approximately 1) (Bauer and Aquadro, 1997; Richards *et al.*, 2005). More recent studies of different Drosophila species have detected some evidence for male-driven evolution in some lineages, but not others (Richards *et al.*, 2005; Begun *et al.*, 2007; Singh *et al.*,

2007). This is further complicated by the high levels of ancestral polymorphisms in Drosophila populations, which can lead to apparent differences in divergence at *X*-, *Y*-linked and autosomal sites. For instance, while a higher rate of substitution of silent mutations has been found on the neo-*Y* chromosome of *D. miranda* compared with the neo-*X* (Bachtrog, 2008), and this has been taken as evidence for male-driven evolution in this lineage, it can be also accounted for by the fixation of ancestral polymorphisms on the neo-*Y*, caused by its greatly reduced effective population size (Bartolomé and Charlesworth, 2006).

1.2.4 Assessing 'male-driven' evolution in mammals

The estimated mean numbers of cell divisions per generation are 401 divisions for human spermatogenesis and 31 for oogenesis (Drost and Lee, 1995). A male-driven evolution effect was detected in a human–chimpanzee sequence comparison (Ebersberger *et al.*, 2002), where α was estimated to be about 3. Overall sequence divergence among humans and chimpanzees estimated from the genome sequences is highest for the *Y* and lowest for the *X* chromosome (The Chimpanzee Sequencing and Analysis Consortium, 2005), yielding an α value of 2–6. This value is much smaller than the estimate from the cell division data. In contrast, a comparison of *X* chromosome and autosomal mouse–rat silent divergence gave a much higher estimate of α than expected (McVean and Hurst, 1997). McVean & Hurst (1997) suggested that the low level of *X*-chromosome divergence was caused by a local reduction in the mutation rate, evolved by selection to avoid the expression of deleterious recessive mutations in

hemizygous males. Their sample of genes was relatively small, however, and subsequent work with larger samples supports male-biased mutation as the main force reducing *X*-chromosome neutral divergence (Malcom *et al.*, 2004). Malcom *et al.* (2004) pointed out that, although there is great variation from chromosome to chromosome in human–mouse and rat–mouse comparisons (Lercher *et al.*, 2001), the *X* chromosome consistently shows the lowest divergence. The shorter generation time of rodents is expected to lead to a smaller α than in primates, making it more difficult to estimate (62 germ cell divisions in males, assuming reproduction at 9 months, compared with 25 in females (Drost and Lee, 1995)).

It has also been argued that there are replication-independent mutational mechanisms, which could explain inconsistencies between the ratio of male to female gametogenesis divisions and α estimates (Huttley *et al.*, 2000). Taylor *et al.* (2005) analysed neutral divergence at *X*-linked and autosomal loci in a human-chimpanzee comparison, but separated mutations at CpG sites from the rest. These sites are known to be hotspots for mutations caused by deamination of methylated cytosines, a process that may be replication-independent. Consistent with this, divergence at non-CpG sites showed a strong male bias, with α corresponding to the ratio of male to female germline divisions, whereas a much smaller effect was observed at CpG sites. Additional support for male-driven evolution in vertebrates comes from sequence comparisons of birds, whose female heterogamety means that genes on the female-limited *W* chromosome should show lower rates of silent evolution than either the Z chromosome or autosomes, as is indeed observed (Montell *et al.*, 2001; Axelsson *et al.*, 2004; Sundstrom *et al.*,

2004). This cannot be explained by the hypothesis of McVean and Hurst (McVean and Hurst, 1997).

In summary, the extent and effects of male-driven neutral evolution depend both on the life history of the species and on the molecular basis of mutation. Current work suggests that the mammalian *X* chromosome and bird *W* chromosomes have lower mutation rates than the autosomes, resulting in lower levels of neutral divergence at *X*- and *W*-chromosome loci. In *D. melanogaster*, on the other hand, no such effect has been detected, as expected from the similar number of cell divisions estimated for male and female gametogenesis, but this needs further investigation in other Drosophila species.

1.3 Is selection more efficient for genes on the X?

1.3.1 The fixation of beneficial and deleterious mutations

In randomly mating populations, newly arisen autosomal mutations are found mostly in heterozygotes, where any recessive effects are masked by the ancestral allele and hence not exposed to selection (Haldane, 1924). If they arise on the *X* (or *Z*) chromosomes, however, their effect on fitness is fully expressed in the hemizygous males (or females). Therefore, selection is expected to fix beneficial recessive, or partially recessive, mutations (and remove deleterious recessive mutations) more efficiently on the *X* or *Z* chromosomes than on the autosomes (Rice, 1984; Charlesworth *et al.*, 1987). Theoretical predictions concerning the rates of molecular evolution for favourable mutations at *X*-linked and autosomal sites are shown below.

Selection on autosomal and X-linked mutations

A simple model of the effects on fitness of a mutation is as follows (Table 1.1), where *s* denotes the homozygous or hemizygous effect of a mutation, A_2, and *h* measures its degree of dominance.

Table 1.1 : The fitness model used in Charlesworth *et al.* (1987).

	Females			**Males**		
Autosomal mutation						
Genotypes	$\mathbf{A_1A_1}$	$\mathbf{A_1A_2}$	$\mathbf{A_2A_2}$	$\mathbf{A_1A_1}$	$\mathbf{A_1A_2}$	$\mathbf{A_2A_2}$
Fitnesses	1	$1+hs_f$	$1+s_f$	1	$1+hs_m$	$1+s_m$
X-linked mutation						
Genotypes	$\mathbf{A_1A_1}$	$\mathbf{A_1A_2}$	$\mathbf{A_2A_2}$	$\mathbf{A_1}$		$\mathbf{A_2}$
Fitnesses	1	$1+hs_f$	$1+s_f$	1		$1+s_m$

The fate of a mutation is mainly determined by its rate of spread when rare, so we show the expressions for gene frequency change when A_2 is at a low frequency, *p*. Provided that selection is weak ($s \ll 1$), the change in frequency per generation of a rare autosomal mutation is (Ewens, 2004):

$$\Delta p \approx \frac{ph(s_f + s_m)}{2} \qquad (1.4)$$

The corresponding expression for an *X*- chromosome-linked mutation is:

$$\Delta p \approx \frac{p(\,2hs_f + s_m)}{3} \qquad (1.5)$$

A mutation will only spread in a very large population if Δp is positive, i.e. there is a net selective advantage to the mutation over wild-type, A_1. In a finite population, it can spread by genetic drift even if $\Delta p < 0$; the probabilities that this happens for autosomal and *X*- chromosome-linked mutations can be calculated (Charlesworth *et al.*, 1987), but will not be given here.

It is also of interest to know the rate of substitution (K) of mutations with fitness effects like A_2, since theoretical values of K can be compared with data on between-species DNA sequence divergence.

K for mutations that arise as unique copies in the population is equal to the expected number of mutations that enter the population, times the probability that a mutation spreads through the population (Kimura, 1968; Charlesworth *et al.*, 1987). The former is given by the product of the mutation rate and the number of gene copies in the population (2 x the population size N for autosomal genes; $1.5N$ for *X*-linked genes). With weak selection, the latter is determined by the ratio $\Delta p/p$.

To simplify the formulae, we express K relative to the product of $2N$ and the mutation rate (Charlesworth *et al.,* 1987). For beneficial autosomal mutations in a large population, we have:

$$K_A \approx 2h(\,s_f + s_m\,) \qquad (1.6)$$

(provided that $s_f + s_m > 0$; otherwise $K_A = 0$).

The corresponding expression for *X*-linked mutations is:

$$K_X \approx (2hs_f + s_m) \tag{1.7}$$

(provided that $2hs_f + s_m > 0$; otherwise $K_X = 0$).

The ratio of *K* for *X*-linked and autosomal mutations (when both are > 0) is thus:

$$R \approx \frac{(2hs_f + s_m)}{2h (s_f + s_m)} \tag{1.8}$$

If there are no sex differences in selection ($s_f = s_m$), $R \approx \{1 + 1/(2h)\}/2$; with selection on males only ($s_f = 0$), $R \approx 1/(2h)$; with selection on females only ($s_m = 0$), $R \approx 1$.

These predictions show that, under certain conditions, the *X* chromosome is expected to accumulate beneficial mutations at a faster rate than the autosomes, whereas weakly deleterious mutations are expected to accumulate by genetic drift at a higher rate on the autosomes (Charlesworth *et al.*, 1987). This effect is especially strong for mutations affecting only males (Charlesworth *et al.*, 1987). Higher male mutation rates, on the other hand, reduce any tendency for faster evolution of beneficial mutations on the *X* chromosome, but have the reverse effect for *Z* chromosomes (Kirkpatrick and Hall, 2004). In addition, if adaptive evolution uses variants that have been maintained in the population by mutation pressure, rather than picking up new mutations, the relative rates

of evolution for the *X* chromosome and autosomes can behave in the opposite way to these predictions (Orr and Betancourt, 2001).

If a substantial fraction of DNA sequence divergence for non-synonymous mutations is driven by the fixation of beneficial mutations by natural selection (positive selection), as has been claimed for mammals (Fay *et al.*, 2001) and some *Drosophila* species (Smith and Eyre-Walker, 2002; Sawyer *et al.*, 2003; Bierne and Eyre-Walker, 2004; Welch, 2006; Andolfatto, 2007), we might see a higher rate of protein sequence evolution for *X*-chromosome-linked versus autosomal mutations. The reverse would be the case if protein evolution largely reflects the fixation of weakly deleterious, at least partly recessive, mutations. The availability of large quantities of sequence data makes it possible to examine this question.

1.3.2 Testing the faster-*X* hypothesis in Drosophila species

Sequence divergence is often studied using K_a and K_s, the rates of evolution at non-synonymous sites (where mutations change the protein sequence of the gene) and synonymous sites (where mutations do not change the protein sequence), respectively. The nature of selection that has shaped the between-species sequence divergence of a gene affects its K_a/K_s ratio. If positive selection is more effective at *X*-linked loci, these should have higher K_a/K_s ratios than autosomal loci; the reverse would the case if purifying selection against deleterious mutations is more effective. One way to test for this is to estimate average K_a and K_s values over large numbers of genes on the *X* chromosome and the autosomes. Betancourt *et al.* (2002) found no difference between 51 *X*- chromosome-linked and 202 autosomal loci in the *D. melanogaster /D. simulans*

comparison. An even larger sample was provided by the release of the *D. pseudoobscura* genome (Richards *et al.*, 2005)**.** The values of K_a and K_s for alignable genes in this pair of species are similar for *X*-linked and autosomal loci (Richards *et al.*, 2005). Thornton and Long (2002), on the other hand, studied duplicate gene pairs in the *D. melanogaster* genome, and observed that K_a/K_s values were significantly higher when both copies were located on the *X* chromosome than when one or both were located on an autosome. Subsequent population genetics work detected more positive selection on *X*-linked duplicates (Thornton and Long, 2005).

These comparisons suffer from several problems, especially the fact that different sets of genes are often being compared, which may differ for reasons other than chromosomal location. This can be avoided by asking if the *same* gene evolves faster when it is on the *X* chromosome than when it is on an autosome. In the *D. pseudoobscura* group, an autosomal arm (3L in *D. melanogaster*) has fused to the *X* chromosome. Counterman *et al*. (2004) argued that, if there is a faster-*X* effect, then the genes on this new *X* chromosome arm will evolve faster than their autosomal homologues. They compared rates of evolution in the *D. pseudoobscura* group and the *D. melanogaster* group and found that, for 3L/XR genes, there is an excess of genes evolving faster in the *D. pseudoobscura* group (where they are *X*- chromosome linked) than in the *D. melanogaster* group, in agreement with the faster-*X* hypothesis. However, a recent study where the same approach was applied to a larger sample of genes suggested similar rates of evolution for *X*-linked and autosomal protein sequences (Thornton *et al.*, 2006).

These mixed results suggest that either some of the assumptions on which the model is based are incorrect, or that the fraction of mutations fixed by positive selection has been overestimated. There seems to be some evidence for the latter. Most of the studies that detected a faster-*X* effect in *Drosophila* were biased towards fast evolving genes. Counterman *et al.* (2004) obtained part of their sample from a male-specific EST screen, thereby selecting genes that might be under stronger positive selection than is typical (Zhang *et al.*, 2004). Similarly, newly duplicated genes (Thornton and Long, 2002) are likely to evolve under strong positive selection or to decay into pseudogenes.

1.3.3 Testing the faster-X hypothesis in mammals

Recent studies also provide some indication of faster-*X* effects in mammals. Human-chimpanzee K_a and K_s values for many genes have been estimated (The Chimpanzee Sequencing and Analysis Consortium, 2005; Lu and Wu, 2005), showing that *X*-chromosome genes have a statistically significantly higher mean K_a/K_s than autosomal genes. The values for *X*-linked genes are skewed towards the two extremes, giving further support to the idea that *X*-linked genes evolving mainly under negative selection are evolving more slowly, whereas genes subject to positive selection are evolving faster. Several studies have suggested that sperm proteins are under strong positive selection, and might therefore be a good target for faster-*X* evolution (Torgerson *et al.*, 2002; Swanson *et al.*, 2003). Furthermore, they are only expressed in males, which would enhance this effect. In accordance with this prediction, *X*-linked sperm proteins in mammals evolve significantly faster than autosomal ones (Torgerson and Singh, 2003; Torgerson and Singh, 2006). Similarly, Khaitovich *et al*. (2005) analysed a

large dataset of tissue-specific genes and found that only testis-expressed *X*-linked genes have a higher K_a/K_i (K_i is the divergence for non-coding sequences).

1.3.4 Excess of codon bias on the X

Recent studies of codon bias suggest that purifying selection may be more efficient on the *X* chromosome. Although synonymous codons are often assumed to evolve neutrally, in several organisms there is evidence for selection favouring preferred codons (Powell and Moriyama, 1997). Hambuch and Parsch (2005) and Singh *et al.* (2005) estimated the levels of codon bias for *X*-linked and autosomal genes in Drosophila and *C. elegans* and found a stronger bias on the *X* chromosome. Lu and Wu (2005) found a lower value of K_s for synonymous sites on the *X* chromosome in the human–chimpanzee genome sequence comparison. This pattern suggests more effective weak purifying selection on the *X* chromosome, possibly indicating that mutations affecting codon usage have partially recessive deleterious fitness effects (McVean and Charlesworth, 1999).

1.3.5 *X* chromosomal divergence within species

We have so far discussed the divergence of the *X* chromosome between species, but the same processes apply within a species. Both positive selection on new beneficial mutations and the continual removal of deleterious mutations reduce polymorphism levels at sites linked to the genes in question (Gordo and Charlesworth, 2001). If positive selection is more efficient on the *X* chromosome, we expect it to harbour less variability than the autosomes (Betancourt *et al.*, 2004). Although this pattern is not observed in African populations of *D. melanogaster* and *D. simulans*, the *X* chromosome

is indeed less variable than the autosomes in non-African populations (Begun and Whitley, 2000; Andolfatto, 2001; Kauer *et al.*, 2002; Mousset and Derome, 2004; Schofl and Schlotterer, 2004). Because these species have recently spread from Africa into Europe and North America, they might have experienced new selection pressures, so that the lower levels of polymorphism on the *X* chromosome reflect a higher frequency of recent fixations of favourable mutations on this chromosome than on the autosomes. However, other demographic scenarios could account for this pattern (Charlesworth, 2001), and more work is necessary to determine how much of it is caused by selection (Haddrill *et al.*, 2005).

Similarly, Wang *et al.* (2006) have detected an excess of linkage disequilibrium for *X*-linked loci in a large human polymorphism dataset. This result may be caused either by reduced recombination or increased selection. Although the human *X* chromosome appears to have a lower recombination rate than the autosomes, it seems likely that the 2-fold difference in linkage disequilibrium is at least partially caused by more effective selection on *X*-linked loci (Wang *et al.*, 2006).

1.3.6 Summary: is there really a faster-X effect?

Theoretical models predict that if mutations are on average recessive, then selection will be more efficient on the *X* chromosome. Between- and within-species DNA divergence data are sometimes consistent with this prediction, both in *Drosophila* species and in mammals. Whether this corresponds to a faster or slower evolution of *X*-linked sites, however, depends on how much of the divergence is fixed by positive selection versus genetic drift. The fact that whole genome comparisons among

Drosophila species mostly yield similar rates of divergence for *X* and autosomes, whereas studies that focus on genes under strong positive selection find a higher K_a/K_s at *X*-linked sites, suggests that positive selection is rarer than previously estimated (Smith and Eyre-Walker, 2002; Bierne and Eyre-Walker, 2004). In human–chimpanzee comparisons, higher K_a/K_s is consistently observed for *X*-linked loci. However, faster or slower *X*-evolution can arise in other ways, for example, if mutations have effects of opposite sign on the fitnesses of males and females, i.e. they are sexually antagonistic (see next section). This means that no unambiguous conclusions concerning causality can be drawn simply from differences among *X* chromosome and autosomes in the distribution of K_a/K_s values.

1.4 Accumulation of sex-biased genes on the *X* chromosome versus autosomes

1.4.1 The accumulation of antagonistic mutations

The occurrence of sexual antagonism also implies that the *X* chromosome may preferentially accumulate genes with sex-biased fitness effects (Rice, 1984). If an autosomal mutation with a significant heterozygous fitness effect is beneficial for females but deleterious for males, it will increase in frequency under positive selection only if the advantage to females is greater than the disadvantage to males (Rice, 1984). If a similar mutation occurs on the *X* chromosome, it will be subject to negative selection only 1/3 of the time, and thus has a higher probability of becoming fixed in the population. Similar predictions to those of Rice (1984) can be made by rewriting Equations 1.4 and 1.5, but using opposite signs for s_f and s_m:

a. Male advantage, female disadvantage:

Let $s_m > 0$, $s_f = -k\ s_m$. For autosomal inheritance, a mutation will spread in a large population if $k < 1$. For *X*-linked inheritance, it will spread if $k < 1/(2h)$. The ratio of substitution rates for *X*-linked versus autosomal mutations (when both rates are > 0) is:

$$R \approx \frac{(1 - 2hk)}{2h(1 - k)} \qquad (1.9)$$

$R > 1$ if $h < 0.5$, and approaches infinity as h tends to zero.

The conclusion is that some degree of *recessivity* ($h < 0.5$) of favourable fitness effects in males tends to leads to a higher rate of fixation of mutations on the X; *dominance* ($h > 0.5$) leads to a higher rate for the autosomes. This is true even if there are no deleterious effects in females ($k = 0$), but the effect increases with the value of k.

b. Female advantage, male disadvantage:

Let $s_f > 0$, $s_m = -k\ s_f$. For autosomal inheritance, a mutation will spread in a large population if $k < 1$; for *X*-linked inheritance, if $k < 2h$. The ratio of *X* to autosome rates (when both are > 0) is:

$$R \approx \frac{(2h - k)}{2h\ (1 - k)} \qquad (1.10)$$

$R \geq 1$ if $h > k/2$, and approaches infinity as k tends to 1.

With favourable fitness effects in females, sexual antagonism leads to a higher rate of fixation of mutations on the *X* if there is some degree of dominance, and to a higher rate on the autosomes with recessivity; again, this effect increases with *k*.

1.4.2 The accumulation of sex-biased genes

Rice's (1984) model of the fixation of sexually antagonistic mutations relies on modifiers that inhibit the expression of sexually antagonistic mutations in the harmed sex, so that the mutation becomes unconditionally beneficial and is consequently driven to fixation. The gene involved therefore becomes sex-biased, that is, primarily expressed in one of the sexes.

If the accumulation of antagonistic mutations leads to the creation of sex-biased genes, the *X* chromosome is likely to accumulate genes that are expressed in females rather than males, at a faster rate than the autosomes (when the initial sexually antagonistic mutation is dominant). But sexual antagonism involving alleles with recessive fitness effects predicts an accumulation of male-biased genes on the *X* chromosome rather than the autosomes (Rice, 1984): New *X*-linked recessive mutations that are beneficial for males and deleterious for females can spread, since their beneficial effects are expressed in males, whereas at low frequencies their deleterious effects on females are masked. Depending on the level of dominance of the fitness effects of mutations, accumulation of either male- or female-biased genes on the *X* chromosome relative to the autosomes can occur.

1.4.3 Results for Drosophila and C. elegans

Microarray datasets can be used to determine the patterns of expression of genes in relation to sex, allowing the distribution of female- and male-biased genes in the genome to be determined. Using this approach, an excess of female-biased genes on the *X* chromosome has been found in both Drosophila species and *C. elegans* (Parisi *et al.*, 2003; Ranz *et al.*, 2003; Reinke *et al.*, 2004; Table 1.2), whereas genes with male-biased expression are under-represented on the *X* chromosome. Genes expressed in the gonads seem to show a particularly strong effect of this kind (Parisi *et al.*, 2003).

Table 1.2: Summary of the studies on the genomic distribution of sex-biased genes. (Lercher *et al.*, 2003; Parisi *et al.*, 2003; Khil *et al.*, 2004; Reinke *et al.*, 2004; Kaiser and Ellegren, 2006). A plus sign is used to mark an excess of genes on the *X* chromosome, whereas a minus sign denotes a deficit. NA stands for not applicable. To disentangle the effects of meiotic inactivation and sexual antagonism in the distribution of male-biased genes in the mouse genome, Khil *et al.* (Khil *et al.*, 2004) focused on genes involved in early spermatogenesis, before the *X* chromosome has been inactivated. To do so they analysed testis expression data from young mice, as developing testes contain a higher proportion of cells in early spermatogenesis, and spoII-/- mice, whose spermatogenesis is blocked in early meiosis.

		Genes on the *X* chromosome	
Organism	**Tissue/Function**	**female**	**Male**
Drosophila melanogaster	Gonads	+	-
	Whole adults	No effect	-
	Adult soma	No effect	-
Caenorhabditis elegans	Gametogenesis	-	-
	Soma	+	No effect
Mouse	Gonads	+	-
	Testis, SpoII-/-	NA	+
	Young testis	NA	+
Human	Prostate	NA	+
	Ovary+mammary gland	No effect	NA
Chicken (females *ZW*)	Brain	-	+
	Gonads	-	No effect

1.4.4 Different results for mammals

There has been some debate about whether there is evidence for an excess of female-biased genes on the *X* chromosome in mammals (Lercher *et al.*, 2003), but a recent study suggests that there is such an effect (Khil *et al.*, 2004). Initial reports in rodents suggested that the *X* chromosome had an excess of male-biased genes (Wang *et al.*, 2001). The *X* chromosome is inactivated during meiosis in the male germline, so that genes whose expression is required late in spermatogenesis must be located on the autosomes or *Y* chromosome (Lifschytz and Lindsley, 1972). This would prevent any accumulation of members of this subset of male-biased genes on the *X* chromosome. It has accordingly been suggested that the differences between the mouse and *C. elegans*/ Drosophila results were mainly due to experimental design, since early spermatocytes were used in the rodent study. If this were the case, then the mammalian *X* chromosome should also show a deficit of late spermatogenesis genes, and the male-biased gene deficit on the *Drosophila*/*C. elegans X* chromosomes should be confined to spermatogenesis-related genes. The first prediction was confirmed by Khil *et al.* (2004), who found that the rodent *X* chromosome was deficient in male-biased genes from mature testis arrays (consisting mostly of mature spermatocytes), but enriched in male-biased genes from immature testis (where mature spermatocytes, with an inactive *X* chromosome, are absent or rare).

Oliver and Parisi (2004) pointed out that somatically expressed male-biased genes in *Drosophila melanogaster* are also scarce on the *X* chromosome, so that the second prediction is falsified. In particular, the accessory gland proteins are fertility-enhancing proteins produced by Drosophila males and transferred to females during

mating. These are not expressed in spermatocytes, but are also present more rarely than expected on the *X* chromosome (Mueller *et al.*, 2005), suggesting that the deficit of this class of male-biased genes on the *X* chromosome is caused by evolutionary forces other than avoidance of *X*-inactivation.

1.4.5 Why the difference?

There thus seems to be a real difference between the *Drosophila* species and mammalian results, once the effect of *X*-inactivation in spermatogenesis is removed. There is, however, no obvious reason why the dominance of the fitness effects of favourable mutations should be consistently different between these groups. Without direct evidence of the dominance effects of favourable mutations, it will be challenging to resolve this difficulty, and the interpretation of the patterns we have discussed remains speculative. One possibility is that differences in the mechanisms of dosage compensation could influence the evolution of the expression pattern at *X* -linked loci. In flies, nematodes and mammals, mechanisms are in place to ensure that haploid males and diploid females produce similar amounts of *X*-derived mRNAs (Gupta *et al.*, 2006). In *Drosophila melanogaster***,** this involves increasing the rate of expression of genes on the male *X* chromosome. It has been suggested (Connallon and Knowles, 2005) that male-biased genes evolve mostly by increases in the level of expression of existing genes in males; if this is the case then higher expression levels could be harder to achieve on the already hyperactive *X* chromosome than on the autosomes, if the rate of mRNA transcription is limited.

It is interesting to note that a study of the distribution of sex-biased genes in the chicken genome has recently been completed (Kaiser and Ellegren, 2006). The results are similar to the *Drosophila* and *C. elegans* results, with a deficit of female brain and ovary genes on the *Z* chromosome, and an excess of male brain genes (Table 1.2). Studies in birds, where the female is heterogametic, are useful, since they decouple the effects of sex and heterogamety. On the other hand, not much is known about the biology of the *Z* chromosome, making it difficult to evaluate the influence of other factors, such as dosage compensation, on its evolution.

It is important to note that the gene content of the *X* chromosome is very stable in both *Drosophila* species and mammals (Brudno *et al.*, 2004), so that the patterns we have described must overwhelmingly reflect evolutionary shifts in gene expression, not physical movements of genes on and off the *X* chromosome. This casts doubt on the SAXI hypothesis (Wu and Xu, 2003), the idea that the *X*-chromosome has a deficit of male-biased genes because there is a selective pressure for genes involved in spermatogenesis to be duplicated onto autosomes (followed by the loss of the original copy or of the male-biased function of the original *X*-linked copy) in order to avoid *X* meiotic inactivation.

1.5 What have we learnt from these patterns?

Although they have evolved independently, the sex chromosomes of mammals and *Drosophila* species are quite similar in their general properties, and their evolution appears to be shaped by similar evolutionary forces. However, we have highlighted

several differences between them, which probably result from differences in the biology of insects and mammals.

The number of cell divisions is higher for spermatogenesis than for oogenesis in mammals, but not in *D. melanogaster*. Probably as a result of this difference, silent site divergence for *X*-linked loci is lower than for autosomes in mammals, but is usually similar in Drosophila species. Recombination is lower for the *X* chromosome than the autosomes in humans, but higher in Drosophila.

The evidence on rates of protein sequence evolution and codon usage bias from both Drosophila species and mammals suggests that both positive and negative selection act more efficiently at *X*-linked loci. The classic explanation for faster protein sequence evolution on the *X* chromosome invokes the faster accumulation of favourable recessive mutations (Charlesworth *et al.*, 1987). As noted above, there are other possible causes of this pattern. It will probably be necessary to relate differences in patterns of gene expression between the sexes to differences in evolutionary rates between *X*-linked and autosomal genes to answer questions of causation: for instance, genes which have been expressed only in one sex for a long period of evolutionary time are not likely to be subject to sexual antagonism.

The recessivity of beneficial mutations suggested above is contrary to the expression data in Drosophila, for which the patterns of sex-biased genes are consistent with predictions for dominant mutations, with an accumulation of female-biased and a deficit of male-biased genes on the *X* (Parisi *et al., 2003)*. Since an excess of male-biased genes is observed for mammals (Khil *et al.*, 2004), it is possible that other biological causes, such as differences in dosage compensation mechanisms, are

preventing male-biased expression patterns evolving on the Drosophila *X* chromosome, but this needs to be further studied. A study of the evolution of patterns of gene expression in species such as *D. pseudoobscura*, in which a former autosome has been attached to the *X* chromosome for a long period of evolutionary time (Counterman *et al.*, 2004), would be illuminating in this regard.

Finally, both the faster-*X* effect and the accumulation of sex-biased genes on the *X* due to sexual antagonism can account for the excess of brain- and testis-expressed genes detected on the human *X* chromosome, without involving female choice of more intelligent males as proposed by Zechner *et al.* (2001). Cognitive function and fertility are probably critical for the evolution of mammalian lineages (Wilda *et al.*, 2000), and it is possible that genes that influence them are especially subject to positive selection. *X*-linked loci in mammals might thus have accumulated an excess of mutations that enhance these characteristics, making them more prone to mutations that impair them. Furthermore, behavioural patterns differ in the two sexes, and this might lead brain-expressed genes to accumulate on the *X* chromosome through sexually antagonistic effects (Arnold, 2004). This is consistent with the higher expression level of *X*-chromosome versus autosomal genes detected in the brain (Nguyen and Disteche, 2006) (but not in other tissues), if sexual antagonism results in increased gene expression in the beneficiary sex (Connallon and Knowles, 2005). Analyses of gene expression in different mammalian tissues have shown that there is a correlation between testis and the brain in patterns of gene expression, so that brain-expressed genes are to a certain extent also testis-expressed genes (Guo *et al.*, 2003; Son *et al.*, 2005), which may further enhance their accumulation on the *X* chromosome.

1.6 Aims of this thesis

This chapter summarizes important results that have come out of recent analyses of *X*-linked and autosomal divergence, polymorphism, and expression. There are several inconsistencies, both between different studies of the same organism and between different organisms, that still need to be accounted for.

The initial focus of this thesis was faster-*X* evolution in Drosophila. We used two different approaches that can improve our understanding of the processes leading to presence or absence of faster-*X* evolution:

-Empirical approach (Chapter 2): Estimating K_a, K_s and K_a/K_s for a set of genes that are *X*-linked in some species of Drosophila but autosomal in others can highlight differences in evolutionary rates that are caused solely by being located on the *X*, and not by other factors that could differ between chromosomes. This was used by some previous studies to test for faster-*X* evolution (see section 1.3). For this type of analysis, the species chosen to estimate K_a, K_s and K_a/K_s are crucial: if the species are too close, differences in rates of evolution between chromosomes can be the result of different levels of ancestral polymorphism; if the species are too distant, more sites are saturated, which makes estimates of rates of evolution (in particular of K_s) unreliable. We have chosen two species pairs that are nearly ideal for this purpose: *D. melanogaster-D. yakuba* and *D. pseudoobscura-D. affinis*. We compare rates of evolution of genes when they are autosomal, in *D. melanogaster-D. yakuba*, with the respective rates when they are *X*-linked, in *D. pseudoobscura-D. affinis*.

-Theoretical approach (Chapter 3): Current models of *X*-linked and autosomal evolutionary rates often assume that the effective population size of the *X* chromosome

is equal to ¾ of the autosomal population size. However, polymorphism studies in *D. melanogaster* and *D. simulans* suggest that there are often significant deviations from this value. We have used a FORTRAN program to compute fixation rates of beneficial and deleterious mutations at *X*-linked and autosomal sites when this occurs.

The second part of the thesis is dedicated to the evolution of sex-biased genes, as many patterns concerning these genes remain to be understood (section 1.4). We were interested in two main questions:

-What are the steps that lead to the creation of sex-biased genes, and how do they relate to Rice's (1984) model for the accumulation of sexually antagonistic mutations? To investigate this, we used EST data to compare expression profiles of genes that are sex-biased in *D. melanogaster*, but not in *D. simulans*, with the expression profiles of non-biased genes (Chapter 4).

-The results presented in Chapter 4 suggest that male-biased genes arise through a large increase of expression in the testis. Can this provide an alternative explanation for the deficit of male-biased genes on the *X* chromosome, observed in Drosophila? Using EST and microarray data, we investigate the possibility that the high levels of testis expression observed for male-biased genes in Drosophila may be harder to achieve on the single male *X* chromosome, if there is an upper limit to the amount of mRNA that can be produced from one copy of each gene (Chapter 5).

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Chapter 2: Faster-X evolution in Drosophila

Abstract

Population genetics models show that, given certain conditions, the *X* chromosome is expected to be under more efficient selection than the autosomes. This could lead to "faster-*X* evolution", if a large proportion of mutations are fixed by positive selection, as suggested by recent studies in Drosophila and mammals. We used a multi-species approach to test this: Muller's element *D*, an autosomal arm, is fused to the ancestral *X* chromosome in *Drosophila pseudoobscura* and its sister species, *D. affinis*. We tested whether the same set of genes had higher rates of non-synonymous evolution when they were *X*-linked (in the *D. pseudoobscura-D.affinis* comparison) than when they were autosomal (in *D. melanogaster-D. yakuba*). Our results suggest this may be the case, but only for genes under particularly strong positive selection/weak purifying selection. They also suggest that genes that have become *X*-linked have higher levels of codon bias and slower synonymous site evolution, consistent with more effective selection on codon usage at *X*-linked sites. We also analyzed published *D. melanogaster* polymorphism data to investigate why selection is more effective on the *X*. We find that this is at least partly due to different rates of recombination for *X*-linked and autosomal sites.

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2.1 Introduction

2.1.1 Faster-*X* evolution

Positive selection may be more effective on the *X* chromosome compared with the autosomes, because the impact of recessive mutations is never masked in males, which could lead to a higher number of beneficial mutations being fixed at *X*-linked loci. Charlesworth *et al*. (1987) modelled the rates of evolution of sex-linked and autosomal loci, assuming that this occurs by fixation of new unique mutations. When selection acts equally on both sexes (the selection coefficient, *s*, is the same in males and females) and there is dosage compensation, the ratio of the rates of fixation of advantageous mutations at autosomal sites to *X*-linked sites, in a very large population, is given by:

$$R_x \approx 4h/(2h + 1) \text{ (where } h \text{ is the dominance coefficient)} \qquad (2.1)$$

This implies that, if most beneficial mutations are at least partially recessive, they will accumulate faster on the *X* chromosome. If selection is acting on males only (e.g. for genes that are only expressed in males), the effect is even stronger.

On the other hand, under the same conditions (selection on both sexes with dosage compensation), the ratio of the rates of fixation of slightly deleterious mutations is such that if most deleterious mutations are at least partially recessive, they will accumulate faster on the autosomes. This occurs because the deleterious effect of an *X*-linked mutation will be immediately expressed, and selected against, in the hemizygous males, whereas an autosomal equivalent would not be selected against until it reaches a significant frequency in the population.

Ultimately, *X*-linked loci will evolve faster or slower than autosomal ones depending on the fractions of mutations that are fixed by positive selection versus genetic drift. Studies in

mammals suggest that only a small fraction of non-synonymous divergence is fixed by positive selection in this group (Fay *et al.*, 2001; Zhang and Li, 2005; Eyre-Walker, 2006; Studer *et al*., 2008). In Drosophila, on the other hand, recent studies have suggested that 25 to 50% of the divergent non-synonymous sites in Drosophila (Smith and Eyre-Walker, 2002; Bierne and Eyre-Walker, 2004; Welch, 2006; Andolfatto, 2007) were fixed by positive selection. In view of this considerable amount of adaptive divergence, we might expect to observe a faster-*X* evolution in Drosophila.

This will, however, only occur if divergence comes from the fixation of new mutations and not from standing variation. Orr and Betancourt (2001) modelled the fixation of polymorphic alleles and concluded that evolution by fixation of alleles initially present at frequencies expected under mutation-selection balance always proceeds more slowly at *X*-linked than autosomal genes (Orr and Betancourt, 2001).

Kirkpatrick and Hall (2004) further extended Charlesworth *et al*.'s (1987) model to investigate the effect of higher male mutation rates on the evolution rates of *X*-linked and autosomal loci (Kirkpatrick and Hall, 2004). Spermatogenesis often involves a higher number of cell divisions than oogenesis and, as mutations often occur as a result of replication mistakes during cell division, this can lead to a higher mutation rate in males than in females (reviewed in Vicoso and Charlesworth, 2006). Since the *X* chromosome only spends 1/3 of its time in males, its overall mutation rate is equal to:

$$u_X = {}^1/_3\, u_m + {}^2/_3\, u_f \qquad (2.2)$$

where u_m is the male mutation rate, and u_f the female mutation rate.

The autosomal mutation rate is :

$$u_A = {}^1/_2\, u_m + {}^1/_2\, u_f \qquad (2.3)$$

since autosomes spend $^1/_2$ of their time in males.

This means that a higher male mutation rate will affect the autosomal mutation rate more strongly than the mutation rate at *X*-linked sites (Miyata *et al*., 1987). Since the rate of divergence is affected by the mutation rate, this can, in principle, counteract the faster-*X* effect. Kirkpatrick and Hall (2004) showed that, in this case, *X*-linked genes only evolve faster when mutations are quite recessive (e.g. $h < 0.32$ for $\alpha = 5$, where α is the ratio of male to female mutation rates). Although this result should carefully be taken into account when analysing the evolution of the mammalian *X*, it is unlikely to be of major importance in Drosophila, where there is no evidence for a higher male mutation rate in most species (Bauer and Aquadro, 1997, Richards *et al*., 2005). Much lower rates of silent and synonymous evolution were detected on the *X* chromosome than on the *Y* in a study of *D. miranda* (Bachtrog, 2008), and this was taken as evidence for male-driven evolution in this species, but, as pointed out by Bartolomé and Charlesworth (2006), it can also be accounted for by differences in *X*-linked and autosomal ancestral polymorphism levels.

2.1.2 Evidence for faster-*X* evolution in Drosophila

2.1.2.1 Average rates of evolution at *X*-linked and autosomal sites

The current availability of large DNA sequence datasets has made possible extensive analyses of *X* chromosome molecular evolution. In particular, by using between-species comparisons, we can now study separately K_s, the rate of synonymous divergence (the accumulation of mutations that do not affect the amino-acid sequence), and K_a, the rate of non-synonymous divergence (the accumulation of new amino-acid sequence differences). It is commonly assumed that K_s reflects nearly neutral evolution, and the ratio K_a/K_s is used to

estimate the effect of selective forces: neutral or nearly neutral sequences evolve at $K_a/K_s \approx 1$. Negative (purifying) selection decreases this ratio whereas recurrent positive selection increases it. If positive selection is more efficient on the *X* chromosome, we expect *X*-linked sites to show higher K_a/K_s values than autosomal sites (reviewed in Hurst, 2002).

Thornton and Long (2002) studied the molecular evolution of 1841 duplicated gene pairs in the *Drosophila melanogaster* genome. They observed that K_a/K_s values were significantly higher when both duplicates were located on the *X* than when one or both were located on an autosome. They eliminated the possibility that this could be caused by an excess of pseudogenes on the *X* by using only gene pairs with K_a/K_s smaller than 0.5 (if one of the two genes is constrained but the other is a pseudogene evolving neutrally, K_a/K_s has a minimum of 0.5). Although the possibility of an accelerated rate of divergence for *X*-linked genes by relaxation of negative selective pressure could not be rejected, this would require the unlikely condition that most of the deleterious mutations were dominant. Increased positive selection on recessive advantageous mutations seemed to provide the best explanation.

To further explore this hypothesis, they followed this work with a second study in which they performed a population genetic analysis for some of their fast evolving duplicates, as well as others collected from the literature (Thornton and Long, 2005). By comparing levels of polymorphism and divergence, it is possible to detect positive and negative selection: under a purely neutral scenario, divergence is the consequence of the fixation of segregating mutations by drift, and the rate of divergence is proportional to the level of polymorphism. Sites under positive selection, on the other hand, will be quickly

swept through the population, and will not contribute significantly to polymorphism levels: this will cause an excess of fixed to segregating mutations. Sites under negative selection will have the opposite effect, as they are unlikely to be fixed but can segregate at low frequencies. This forms the basis of the McDonald-Kreitman test (McDonald and Kreitman, 1991), which uses synonymous variation as a neutral control to evaluate the selective forces acting on non-synonymous sites. Using a variant of this test, Thornton and Long (2005) detected a significant excess of amino-acid fixations for *X*-linked loci and a deficit of fixations for autosomal loci, giving further support to the hypothesis that more efficient positive selection on *X*-linked duplicates is causing them to diverge faster.

Betancourt *et al*. (2002), on the other hand, evaluated K_a and K_s values in the *D. melanogaster /D. simulans* species pair for 51 *X*-linked and 202 autosomal loci, and detected no difference in K_a/K_s values between them. In fact, the average K_a/K_s value was higher for autosomal loci, though not significantly. An even larger sample was provided by the release of the *Drosophila pseudoobscura* genome (Richards *et al*., 2005). Although the *X* was the chromosome with the lowest fraction of alignable sequence with the *D. melanogaster* genome, which is pointed at by the authors as potential evidence for faster-*X* evolution, the values of K_a and K_s for alignable genes in this pair of species are actually similar for *X*-linked and autosomal loci.

Finally, a large amount of work has been done on the evolution of proteins involved in male reproduction. Previous studies have suggested that sperm proteins are under strong positive selection (Torgerson, *et al*. 2002; Swanson *et al*. 2003). Furthermore, they are only expressed in males, which could enhance an existing faster-*X* effect (Charlesworth *et al*., 1987). Sperm proteins therefore provide the ideal sample to detect faster-*X* evolution.

Torgerson *et al*. (2003) analyzed the molecular evolution of 33 mammalian sperm proteins. They found that *X*-linked sperm proteins evolve significantly faster than autosomal ones. Other tissue-specific proteins did not display the same pattern.

2.1.2.2 Estimates of positive selection on the *X* chromosome and the autosomes

Instead of simply looking at the average rates of evolution, another common approach has been to estimate the fraction of genes that are likely to have diverged under positive selection, to see if this proportion varies between the *X* and the autosomes. Results have been mixed: in an analysis of 12 different Drosophila genomes, the authors found that the set of genes that had been singled out as having been under positive selection was enriched for *X*-linked genes, but only marginally (Drosophila 12 Genomes Consortium, 2007).

Combining polymorphism and divergence data is a more powerful method to detect selection, through derivations of the McDonald and Kreitman (MK) test (McDonald and Kreitman, 1991). Connallon (2007) examined divergence rates between *D. melanogaster* and *D. simulans* and compared them to polymorphism levels in *D. melanogaster*, and found no evidence for increased positive selection on the X.

The recent sequencing of 6 lines of *D. simulans* has allowed for the first true "population genomics" study, and the first comparison of polymorphism and divergence for whole chromosomes (Begun *et al*., 2007). Although they found that the *X* chromosome was evolving faster, at both coding and non-coding sites, they could not find evidence for more positive selection at *X*-linked sites when they compared polymorphism and divergence (in fact, there was a significantly higher number of MK tests significant for positive selection for the autosomal genes). This study suffered from several drawbacks: the average cover

was actually only 3.9, and the alleles were sampled both from ancestral (African) and derived (cosmopolitan) populations, so that the results are likely to influenced by strong demographic effects, and need further investigation.

2.1.2.3 Paired comparisons

The *X* chromosome can differ in its gene content from the autosomes. In *Drosophila melanogaster*, for instance, male-biased genes are rarely found on the *X* (Parisi *et al*., 2003). This could cause systematic biases in the mean sex-specificity of selection coefficients of *X*-linked and autosomal mutations. Since the value of K_a/K_s depends on the selection coefficients affecting the genes, variation in these coefficients could be masking an existing faster-*X* effect in some of the previous studies. If genes with similar functions have similar selection coefficients, then focusing on gene groups with related functions could expose a hidden faster-*X* evolution, as in the case of the sperm proteins. However, an even better approach would consist of studying the same group of genes in an autosomal and an *X*-linked context.

Drosophila species vary both in the number and in the organisation of their chromosomes. It was, however, noted early on that chromosomal arms seemed to be homologous, as genes linked in one species also appeared to be linked in others, and all the described karyotypic differences could be explained by rearrangements of the six basic arms (Muller, 1940). Muller summarized the correspondence between the chromosomes of several species of Drosophila (Muller, 1940), and the chromosomal arms have become known as Muller's elements A to F. The comparative analysis of the Drosophila genomes has confirmed that, despite extensive within-arm rearrangements, only small fragments of

DNA have been translocated between arms (Richards *et al*, 2005, 12 Genome Consortium, 2007).

After splitting from the *D. melanogaster* group, species in the *D. pseudoobscura* subgroup accumulated one rearrangement that has made them exceptionally useful for studying the evolution of the *X* chromosome: Muller's element D (the autosomal 3L arm of *D. melanogaster*) fused to the *D. melanogaster X* chromosome (element A) to form respectively the R and L arms of the *D. pseudoobscura X* (Figure 2.1). Therefore, whatever forces are shaping the evolution of the *X* chromosome should also be acting on this new R arm of the *D. pseudoobscura X* chromosome.

Counterman *et al*. (2004) used a multi-species comparison to test for faster-*X* evolution, by examining whether a given set of genes evolves faster when it is located on the *X*, in the *D. pseudoobscura* subgroup, than it does when it is autosomal, in the *D. melanogaster* subgroup. First, they compared the K_a/K_s values of genes on the element D for *D. pseudoobscura-D. melanogaster* (the genes are autosomal in one species, but *X*-linked in the other) with *D. simulans-D. melanogaster* (where the genes are autosomal for both species). Consistent with the faster-*X* evolution predictions, they found a significant excess of genes with higher K_a/K_s for *D. pseudoobcura-D. melanogaster*.

Their second approach was to use two pairs of species, *D. melanogaster-D. simulans* and *D. pseudoobscura-D. miranda*. *D. miranda* is a close relative of *D. pseudoobscura* and also shares the new *X*R (Muller, 1940). For 15 genes on the *3*L-*X*R arm and 15 genes on the *X*-XL arm, they evaluated the K_a/K_s for each species pair and found that the percentage of genes with higher K_a/K_s in the *D. pseudoobscura-D. miranda* pair was higher, though not significantly, for *3*L-*X*R genes. Thornton *et al*. (2006) and Musters *et al*. (2006) used the

same approach but increased the sample to over 200 DNA fragments for the four-species comparison (Thornton *et al*, 2006), and whole-genomes for the three-species comparisons (Musters *et al*., 2006; Thornton *et al*., 2006). Unlike Counterman *et al*. (2004), they found no evidence for faster-*X* evolution. The lack of statistical significance in the previous four-species comparisons could be due to the species pairs used; these show very low levels of divergence (about 4% for *D. pseudoobscura/D. miranda* synonymous sites in Bartolomé *et al.*'s (2005) analysis of 32 genes). As such they may not be ideal for sequence comparisons, especially as some apparent differences may reflect polymorphisms within species (Bartolomé and Charlesworth, 2006). Similarly, the evidence for faster-*X* found in a whole genome comparison using *D. melanogaster-D. simulans* and *D. pseudoobscura-D. persimilis* (Singh*, et al.*, 2008) should be interpreted with caution, as *D. persimilis* is even closer to *D. pseudoobscura* than *D. miranda*, and higher rates of evolution on the X could reflect higher levels of ancestral polymorphism on this chromosome, as is currently observed in African populations of *D. melanogaster* and *D. simulans.*

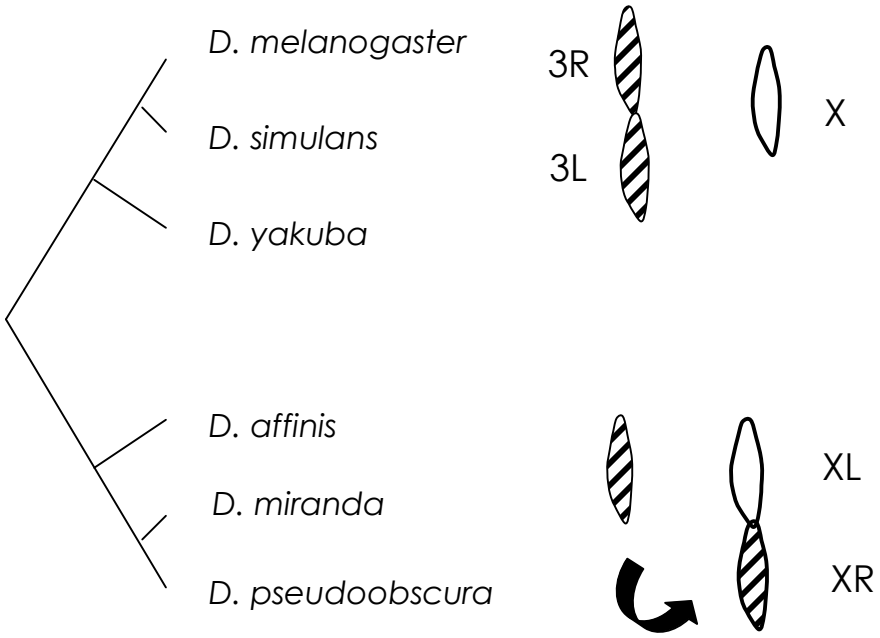

Figure 2.1: In *Drosophila pseudoobscura* and its sister species *D. affinis* and *D. miranda*, Muller's element D (the autosomal 3L arm in *D. melanogaster*) is fused to the *X* chromosome.

2.1.3 Our project

Our project follows the rationale behind Counterman *et al*.'s second approach (Counterman *et al*, 2004), with alterations to increase its statistical power. We use comparisons between more divergent pairs of species, as K_a and K_s estimates from very close species may be imprecise and are likely to be influenced by ancestral polymorphisms (Charlesworth, *et al*., 2005). *D. yakuba* is thought to have split from *D. melanogaster* over 12 million years ago (Tamura *et al*., 2004) and the synonymous divergence between them is about 29% (Zhang *et al*, 2004), making them ideal for K_a and K_s estimation. Genome sequences for both species have been released. In the pseudoobscura subgroup, we evaluated K_a and K_s for *D. pseudoobscura/D. affinis*, as these have similar levels of divergence as the previous pair (about 23% at synonymous sites in Bartolomé *et al*, 2005) and *D. affinis* shares the XR rearrangement with *D. pseudoobscura* (Muller, 1940).

Our project used the following:

-selecting 69 annotated genes from the *3*L arm of *D. melanogaster* and 67 genes from other chromosomal arms (27 *X*-linked, and 40 autosomal) (49 of the genes came from Bartolomé *et al*., 2005 and Bartolomé and Charlesworth, 2006).

-for each gene, identifying its orthologue in the genomes of *D. yakuba* and *D. pseudoobscura*.

-using the *D. pseudoobscura* sequence to design primers, amplifying and sequencing the homologues in *D. affinis* (*D. affinis* gene sequences were available for the genes provided by C. Bartolomé).

-estimating K_a and K_s for the species pairs *D.melanogaster*-*D. yakuba* and *D. affinis*-*D. pseudoobscura*. We expected to find no systematic difference for non-*3*L-*X*R genes, and

used these genes as our control. We could then test if genes on the *3*L-*X*R arm showed an increased K_a/K_s in the *D. affinis/D. pseudoobscura* pair.

2.2 Materials and methods

2.2.1 Selection of the genes

D. melanogaster protein coding genes were downloaded from the Flybase website (http://www.flybase.org). To avoid a possible influence of Hill-Robertson effects due to close linkage to genes under selection, they were all chosen from regions of normal recombination in *D. melanogaster* (cytological region 62A12-71A1 for the *3*L arm and 3C3-15F3 for the *X* chromosome, as described in Charlesworth, 1996). For each gene, we recovered all the corresponding mRNAs in the NCBI database with the Megablast algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) and verified that they had a size between 1000 and 3000 base pairs, with at least 1000 base pairs without introns. We identified the *D. yakuba* homologue through the UCSC BLAT server (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start) and the *D. pseudoobscura* homologue through the NCBI Blast, and kept only genes whose location was syntenic for all three species.

2.2.3 *D. affinis* DNA extraction

DNA was extracted from males of a *D. affinis* line originally from Nebraska (no. 0141.2; Drosophila Species Resource Center), using a Qiagen DNA extraction kit (Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ, United Kingdom).

2.2.2 Sequencing of the genes

Primers were designed using the DNAstar package and the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), using the *D. pseudoobscura*

sequence to amplify 1000-1300bps of the gene in *D. affinis* (the list of primers used is given in Appendix A2.1). Additional internal primers were designed for sequencing. Since the *D. affinis* sequences of the 39 autosomal genes we used were provided by Carolina Bartolomé (Bartolomé*, et al.*, 2005; Bartolomé & Charlesworth, 2006), all the genes we sequenced were on the *D. affinis/D. pseudoobscura X* chromosome (66 on the *3*L-*X*R arm and 20 on the *X*-*X*L arm). PCR products were therefore directly sequenced on both strands using the BigDye (version 3) sequencing kit and run on an ABI 3730 Genetic Analyser (Applied Biosystems, Foster City, CA) by the sequencing service of the School of Biological Sciences, University of Edinburgh.

2.2.3 Evaluation of K_a and K_s

All sequences were translated and virtual protein sequences were aligned with the European Bioinformatics Institute ClustalW interface (http://www.ebi.ac.uk/Tools/clustalw/index.html). The resulting alignment was used to align the DNA sequences with Tranalign (http://phytophthora.vbi.vt.edu/cgi-bin/emboss.pl?_action=input&_app=tranalign), which aligns coding DNA according to a protein alignment. The rates of synonymous (K_s) and non-synonymous (K_a) divergence were calculated using Nei and Gojobori's (1986) model of substitution (Nei & Gojobori, 1986), implemented in DnaSP version 4.50 (Rozas & Rozas, 1995; http://www.ub.es/dnasp/), with the Jukes-Cantor correction for multiple hits. Since several models of substitution can lead to artifactual biases in K_s when there are differences in codon usage bias (Bierne & Eyre-Walker, 2003)**,** we also analysed the data using the Goldman and Yang (1994) model of substitution (using the PAML software package:

http://abacus.gene.ucl.ac.uk/software/paml.html). The list of all the genes analysed, and their respective K_a, K_s and K_a/K_s values, is given in Appendix A2.2.

2.2.4 Codon usage

The alignments obtained for the K_a and K_s analyses were used to estimate the frequency of optimal codons, *Fop*, with CodonW (Peden, 1997). We used the *D. melanogaster* table of preferred codons (Shields *et al*., 1988), as patterns of codon usage have been shown to be well conserved in Drosophila (Powell and Moriyama, 1997).

2.2.5 Statistical analysis

The descriptive statistics, Mann-Whitney tests and Wilcoxon signed rank tests were performed with the Statview software (version 4.5).

2.2.6 Polymorphism

2.2.6.1 Datasets

We have used sequences published in two previous studies (Andolfatto, 2005; Shapiro *et al*., 2007) to compare polymorphism levels at *X*-linked and autosomal coding sites. Coding sequences for the *X*-linked loci were downloaded from Peter Andolfatto's website (http://www.biology.ucsd.edu/labs/andolfatto/link_nature2005.html). The autosomal sequences from Shapiro *et al*. (2007) were retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov/), by searching the nucleotide database for "Shapiro AND adaptive".

2.2.6.2 Aligning the coding sequence

The Andolfatto (2005) dataset consisted of 31 coding sequence files, each containing one sequence from *D. simulans* and 12 sequences from African populations of *D.*

melanogaster. We removed the *D. simulans* sequence and aligned the others with ClustalW (Chenna *et al*., 2003; ftp://ftp.ebi.ac.uk/pub/software/unix/Tools/clustalw/).

The Fasta file containing all the autosomal genes from Shapiro *et al*. (2007) included *D. melanogaster* African lines, but also non-African *D. melanogaster* and *D. simulans* sequences. Since we were interested in comparing *X*-linked and autosomal sequences from African *D. melanogaster* (as African populations for this species are thought to be closer to equilibrium than European or American populations (Haddrill *et al*., 2005)), a Perl script (described in Appendix A2.3) was used to discard all the sequences from cosmopolitan lines or other species, and create, for each gene, a file containing the African sequences.

The protein sequence of each gene was retrieved from the Flybase batch download website (http://flybase.org/static_pages/downloads/ID.html). The corresponding cDNA was extracted from the African sequences using the Genewise software (http://www.ebi.ac.uk/Wise2/doc_wise2.html).

A second Perl script (see Appendix A2.3) extracted the cDNA sequences from the Genewise output into a Fasta file, but only if the score of the alignment was larger than 100 (this value was chosen arbitrarily to remove non-specific alignments). If the initial and the final number of sequences for a gene were the same (suggesting non-specific alignments had been properly removed, and true alignments had not been lost), the sequences were aligned with ClustalW (376 genes). The other 36 genes were reprocessed by hand and then aligned with ClustalW.

2.2.6.3 Analysis

The π_A and π_S values were obtained for all the alignments with DnaSP (Rozas and Rozas, 1995). We checked the alignments manually with Se-Al

(http://tree.bio.ed.ac.uk/software/seal/) and removed visible errors (these came mostly from intronic sequence being extracted as coding sequence by Genewise). The π_A and π_S values were re-evaluated for the 33 alignments that were changed. This did not alter our results.

2.3 Results

2.3.1 Within-clade comparisons

We obtained and aligned sequences for 69 *3*L-*X*R and 66 non-*3*L-*X*R genes (27 from the *X*-*X*L arm, 39 autosomal in both clades) in the *D. melanogaster/D. yakuba* and *D. affinis/D. pseudoobscura* pairs. The average K_a, K_s, and K_a/K_s values are shown in Table 2.1 and Figure 2.2 (the values for individual genes are given in Appendix A2.2). As a first analysis, we can compare K_a, K_s and K_a/K_s values between chromosomes within the two groups (*D. melanogaster/D. yakuba* and *D. pseudoobscura/D. affinis*). Overall, the mean values are in agreement with the faster-*X* predictions: K_a/K_s values are higher for *X*-linked chromosomal arms in both the *D. pseudoobscura* and *D. melanogaster* groups.

Table 2.1: Average rates of evolution. The average K_a/K_s is the ratio of the averages of *Ka* and *Ks*. SE is the standard error.

	D. pseudoobscura/D. affinis			*D. melanogaster/D. yakuba*		
	K_a	K_s	K_a/K_s	K_a	K_s	K_a/K_s
*3*L-*X*R	0.036	0.253	0.138	0.031	0.323	0.096
(SE)	(0.004)	(0.008)	(0.015)	(0.003)	(0.011)	(0.01)
Autosomal	0.020	0.251	0.080	0.018	0.269	0.074
(SE)	(0.003)	(0.01)	(0.013)	(0.003)	(0.015)	(0.014)
X-*XL*	0.037	0.263	0.126	0.038	0.298	0.115
(SE)	(0.007)	(0.017)	(0.019)	(0.008)	(0.023)	(0.021)

The average K_a/K_s is estimated from the ratio of the averages of K_a and K_s. SE is the standard error.

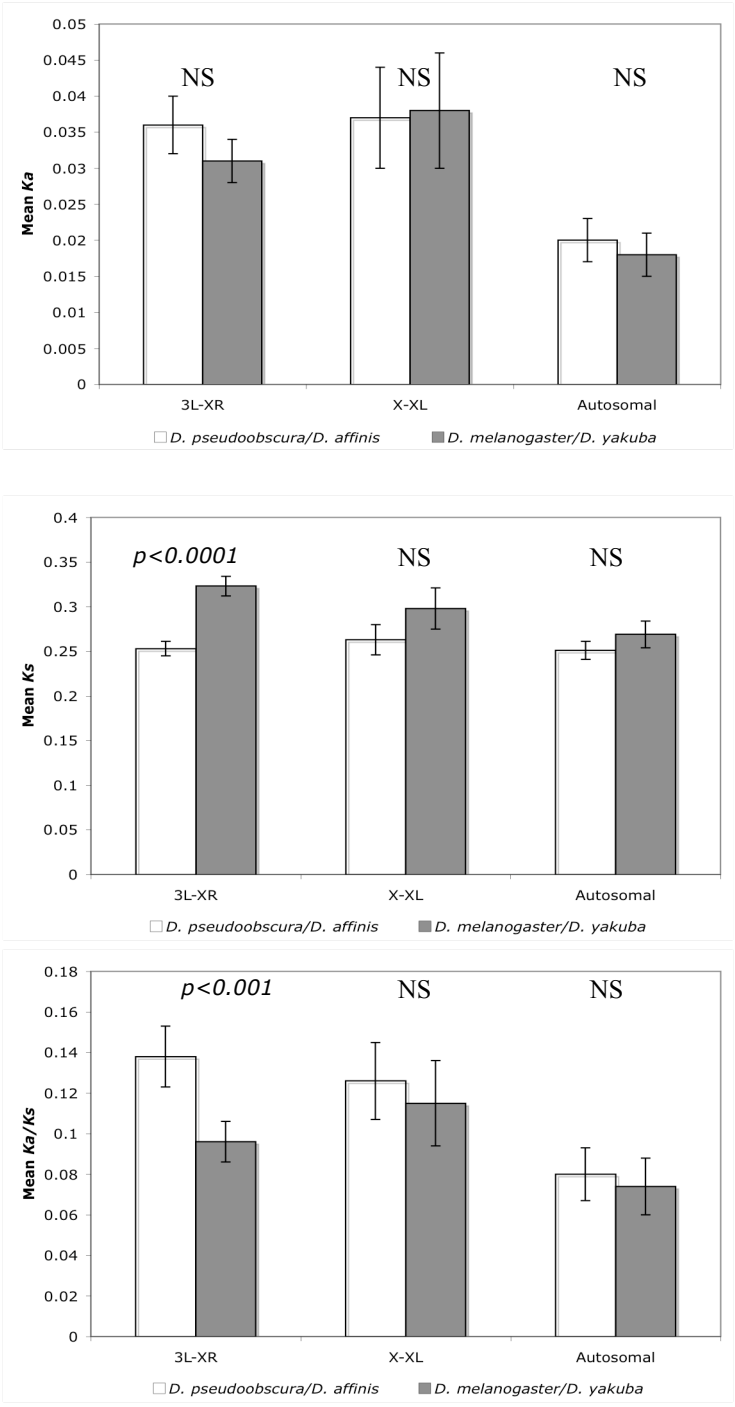

Figure 2.2: The mean and standard error of K_a, K_s and K_a/K_s for all classes of genes. On the X-axis, the Autosomal class includes all the genes that are autosomal in both groups. The *D. pseudoobscura/D. affinis* and *D. melanogaster/D. yakuba* values were compared using Wilcoxon Signed Rank tests, and the significant p-values are shown (NS stands for non significant).

It should however be noted that, whilst the higher K_a/K_s of the *3*L-*X*R genes in the *D. pseudoobscura* group is in agreement with the faster-*X* hypothesis, these genes also exhibit particularly high K_a/K_s values in the *D. melanogaster* group when compared to the rest of the autosomes (Table 2.1 and Table 2.2). This is likely to be caused by a sampling bias, as most of the autosomal genes were previously sequenced by Carolina Bartolomé, and consisted of long, well studied, genes. These differ from the genes we selected (most of the *3*L-*X*R sample), which correspond to small, unnamed (mostly unstudied) transcripts. Genes with no annotated function have been shown to be less constrained than genes with known functions (Drosophila 12 Genomes Consortium, 2007). Consistently, Mann-Whitney tests (Table 2.2) show that the autosomal sample does have significantly lower K_a and lower K_a/K_s than the *3*L-*X*R sample in both *D. melanogaster*-*D. yakuba* and *D. pseudoobscura*-*D. affinis*. Although these differences are not significant after correcting for multiple comparisons, they suggest that direct comparisons of K_a and K_s values between different chromosomal arms may not be reliable. This should, however, not affect the comparison of rates of evolution of the same chromosomal arm between the two clades, since we have the same set of genes in all the four species.

Table 2.2: Within-species comparisons show heterogeneity in our sample. The *p*-values were obtained with a two-tailed t-test. Significant values are in bold.

	Mann-Whitney test *p*-value		
	D. pseudoobscura-D. affinis		
	Ka	*Ks*	*Ka/Ks*
*3*L-*X*R, *X*L	0.938	0.594	0.791
*3*L-*X*R, auto	**0.004**	0.941	**0.006**
X-*X*L, auto	0.099	0.588	0.065
	D. melanogaster-D. yakuba		
	Ka	*Ks*	*Ka/Ks*
*3*L-*X*R, *X*L	0.968	0.359	0.651
*3*L-*X*R, auto	**0.005**	**0.009**	**0.036**
X-*X*L, auto	0.077	0.306	0.069

Significant *p*-values are denoted in bold.

2.3.2 Lower K_s for *X*-linked genes

Further examination of Table 2.1 and Figure 2.2 shows that the most striking pattern is the consistent reduction in K_s for the *3*L-*X*R genes in the *Drosophila pseudoobscura* group. A similar phenomenon was described by Lu and Wu (2005), who found that, in a human-chimpanzee comparison, *X*-linked genes had significantly lower K_s values than autosomal genes.

Two types of explanation have been put forward to account for this:

-Neutral: In several species, a higher mutation rate in males than in females leads to a lower mutation rate on the *X* chromosome, because the *X* is transmitted by females $^2/_3$ of the time, whereas autosomes are transmitted by females only ½ of the time (Miyata *et al.*, 1987).

Whilst in mammals this effect appears to be solidly established (Ebersberger *et al*., 2002), no evidence has been found for such an effect in *D. melanogaster, D. yakuba* or *D. pseudoobscura* (Bauer and Aquadro, 1997, Richards *et al*., 2005). While a higher rate of substitution of silent mutations has been found on the neo-*Y* chromosome of *D. miranda* compared with the neo-*X*, this can be accounted for by the fixation of ancestral polymorphisms on the neo-*Y*, caused by its greatly reduced effective population size (Bartolomé and Charlesworth, 2006; Bachtrog, 2008). It therefore seems unlikely that a male-female mutation rate difference could account for our observations on K_s.

-Selective explanation: although synonymous substitutions are often used as a neutral control, there is ample evidence that synonymous codons appear in the genome at different frequencies, possibly because "preferred" codons increase the efficiency of translation (Powell and Moriyama, 1987). This selective pressure is stronger for highly expressed genes, and these do indeed show higher levels of codon usage bias (Duret and Mouchiroud, 1999).

McVean and Charlesworth (1999) investigated, theoretically, the influence of *X*-chromosome linkage on codon bias and found that, if unpreferred codons are on average recessive in their effect on fitness, they will be selected out of the population more efficiently when they are on the X, thereby reducing the rate of *X*-linked synonymous evolution and increasing the level of codon bias of the *X* chromosome. Singh *et al*. (2005) estimated codon bias levels in *D. melanogaster, D. pseudoobscura* and *Caenorhabditis elegans* and found that, as expected, these were higher on the *X* chromosome in all three species. They excluded differences in expression as the cause of this, because their analysis of an EST dataset showed that, in *D. melanogaster* and *C. elegans*, the *X* has lower levels

of expression than the autosomes. Other factors that are correlated with codon usage bias, such as gene length, recombination rate, gene density, and protein evolution, were excluded as possible causes for the *X*-autosomes difference, suggesting that more efficient selection on the hemizygous male *X* is the main cause of increased codon usage bias on the X. More recently, the analysis of twelve Drosophila genomes confirmed that the *X* chromosome consistently harbours higher levels of codon usage bias (Drosophila 12 Genomes Consortium, 2007).

We evaluated the frequency of preferred codons (*Fop*), a measure of codon usage bias, for all the genes in our sample (Table 2.3). Although *X*-*X*L genes have the highest levels of codon bias in each species, *3*L-*X*R genes have similar levels of *Fop* as the autosomes in *D. pseudoobscura* and *D. affinis*. This might be simply reflecting a sampling bias, since in *D. melanogaster* and *D. yakuba* 3L genes have lower levels of *Fop* than other autosomal genes, which suggests that direct comparisons between different chromosomal arms are, once again, unreliable.

More interesting insights come from the comparisons between the same chromosomal arm in the two clades. *D. melanogaster* is known to have experienced a reduction in codon usage bias, thought to be due to a reduction in effective population size resulting in less efficient selection on this lineage (Akashi, 1995, 1996). We find, in agreement with previous studies, that *D. melanogaster* has significantly reduced levels of codon usage for all the chromosomes compared to *D. yakuba* (not shown). We therefore used *D. yakuba*-*D. pseudoobscura* to compare the Fop values in the two clades (using *D. yakuba*-*D.affinis* yields similar results). Whilst *Fop* values are similar in the two groups for our control genes (Table 2.3), they are significantly higher for *X*R in *D. pseudoobscura* pair than for *3*L in *D.*

yakuba ($p < 0.001$), consistent with the hypothesis that selection to maintain optimal codon usage is more efficient when loci are *X*-linked than when they are autosomal.

Table 2.3: Average values of *Fop* (frequency of optimal codons) for *3*L-*X*R, *X*-XL and autosomal genes.

	D. affinis	*D. pseudoobscura*	*D. melanogaster*	*D. yakuba*
*3*L-*X*R	**0.559**	**0.568**	0.506	0.527
(SE)	**(0.01)**	**(0.01)**	(0.009)	(0.009)
			$p = 0.0001$	
X-*X*L	**0.589**	**0.596**	**0.557**	**0.579**
(SE)	**(0.016)**	**(0.015)**	**(0.019)**	**(0.015)**
			$p = 0.1301$	
Autosomes	0.563	0.562	0.540	0.553
(SE)	(0.017)	(0.017)	(0.019)	(0.019)
			$p = 0.7219$	

Bold values indicate *X*-linked genes. SE is the standard error. Since *D. melanogaster* has significantly reduced levels of codon usage bias for all the chromosomes compared to *D. yakuba* (not shown), we used *D. yakuba*-*D. pseudoobscura* to compare the *Fop* values in the two clades (using *D. yakuba*-*D.affinis* yields similar results). The p-values of the *D. yakuba*-*D. pseudoobscura* comparison were obtained using Wilcoxon Signed Rank Tests.

2.3.3 Higher K_a/K_s for 3L-XR genes in *D. pseudoobscura/D. affinis*

The K_a/K_s values are in agreement with the faster-*X* hypothesis, as pointed out previously: whilst autosomal and *X*-linked loci have similar K_a/K_s values in *D. melanogaster/D. yakuba* and *D. pseudoobscura/D. affinis*, the mean K_a/K_s for *3*L-*X*R genes is significantly higher in the *D. pseudoobscura* group, where they are *X*-linked, than in the

D. melanogaster group. However, this is mostly caused by the reduction in K_s observed for this chromosomal arm in the *D. pseudoobscura* group. Since the corresponding K_a values do not differ, this comparison does not support a faster-*X* effect due to more efficient positive selection at *X*-linked coding sites. Unfortunately, since we are not using any control for the neutral processes, such as differences in the mutation rate that could affect K_a, this analysis is not conclusive.

2.3.4 Pairwise comparisons

In order to test whether the behaviour of the *3*L-*X*R genes is different from that of the control genes, we can, instead of looking at the average values, look at the proportion of genes that are evolving faster (genes that have higher K_a/K_s) in the *D. affinis/D. pseudoobscura* pair than in *D. melanogaster/D. yakuba*. In the absence of a faster-*X* effect, this value will be similar for *3*L-*X*R and non-*3*L-*X*R genes. If, on the other hand, there is faster-*X* evolution, this proportion will be higher for *3*L-*X*R genes (and this prediction is easily testable with a Chi-square test). The values are presented in Table 2.4. Unlike previous observations, there is no detectable faster-*X* effect (although the proportion of genes with higher K_a/K_s in the pseudoobscura group is slightly higher — 70% versus 61% — for *3*L-*X*R genes, this is not significant, and the opposite is observed for the K_a values). Once again, the decrease in K_s for *3*L-*X*R genes in *D. pseudoobscura/D. affinis* is the only significant pattern.

Several models of substitution can lead to an artifactual negative correlation between codon usage bias and K_s (Bierne and Eyre-Walker, 2003), which could account for our reduction in K_s for *X*-linked genes, as these also have (slightly) increased codon usage bias.

The Goldman-Yang (1994) model of substitution, implemented in the PAML package, can be used to bypass this issue. We repeated the analysis using D_N and D_S, estimated with the Goldman and Yang (1994) model of substitution. Although the results differ numerically, the reduction in synonymous divergence observed for *X*-linked genes remains significant when we use D_S instead of K_s (Table 2.4). This is still observable when we use four-fold degenerate sites (D_4 in Table 2.4), but the pattern is not significant.

Table 2.4: Proportion of genes with higher rates of evolution (K_a, K_s or K_a/K_s, D_N, D_S, D_N/D_S or D_4) in the *D. pseudoobscura/D. affinis* pair. The p value was obtained with a Fisher exact test.

	Proportion of genes evolving faster *in D. pseudoobscura/D. affinis*		
	*3*L-*X*R	Control	*p*
K_a	54%	59%	0.38
K_s	19%	45%	**0.002**
K_a/K_s	70%	61%	0.45
D_N	54%	56%	0.86
D_S	35%	61%	**0.003**
D_N/D_S	65%	55%	0.37
D_4	40%	52%	0.23

One of the drawbacks of this Chi-square comparison is that it does not take into account the magnitude of the differences between the two clades. We used a second method to perform

this pairwise comparison. Using the average K_a, K_s and K_a/K_s for the two clades, we calculated :

$$Z = \left(\frac{K_a(pse - 3L - XR)}{K_a(mel - 3L - XR)} \right) \Big/ \left(\frac{K_a(pse - auto)}{K_a(mel - auto)} \right)$$

A Z value larger than 1 reflects faster-X evolution, whereas slower-X evolution would lead to Z being lower than 1. We bootstrapped the data to derive a confidence interval for the above statistic in order to test the significance of observed patterns. Although the values of Z in the table below confirm the patterns that we observed previously (faster-X evolution, when we look at K_a, D_N, K_a/K_s and D_N/D_S, but lower K_s and D_S for X-linked genes), only the decreased Ks for *3*L-*X*R genes in *D. pseudoobscura/D. affinis* is significant.

Table 2.5: Z-values for K_a, K_s, k_a/K_s, D_N, D_S, D_N/D_S and D_4. CI_{05}, CI_{95}, CI_{01} and CI_{99} are the 5%, 95%, 1% and 99% values of the confidence intervals obtained by bootstrapping the data.

	Z	**CI_{05}**	**CI_{95}**	**CI_{01}**	**CI_{99}**
K_a	1.17	0.74	1.76	0.63	2.04
K_s	**0.86**	**0.76**	**0.97**	0.73	1.02
K_a/K_s	1.35	0.90	1.89	0.79	2.25
D_N	1.14	0.70	1.70	0.61	2.11
D_S	0.89	0.72	1.08	0.65	1.18
D_N/D_S	1.20	0.79	1.77	0.69	2.05
D_4	0.93	0.75	1.14	0.69	1.22

We can also test whether the lower K_s on the *D. pseudoobscura/D. affinis* *3*L-*X*R arm is accompanied by an increase in codon usage bias by estimating the proportion of genes that have higher *Fop* values in the *D. pseudoobscura* group for *3*L-*X*R and control genes, and testing if it differs (Table 2.5). Although there is a higher proportion of *3*L-*X*R genes than control genes that have a higher *Fop* in the pseudoobscura group (68% versus 55%), this trend is not significant. Singh *et al*. (2005) followed the same approach using the whole chromosome arms and found a significant increase in codon bias for *X*R genes compared to their autosomal counterpart, suggesting that increased selection on codon usage at *X*-linked sites is indeed the cause of this reduction in K_s.

Table 2.5: Number of genes that show higher frequencies of preferred codons (*Fop*) in the pseudoobscura and melanogaster groups. To evaluate this, we used, for each gene, the mean *Fop* of *D. affinis* and *D. pseudoobscura* (PA) and *D. melanogaster* and *D. yakuba* (MY). The *p*-value was obtained with a two-tailed Fisher exact test.

	Number of genes with higher *Fop* in:		
	PA	MY	Total
*3*L-*X*R	47	22	69
Control (X+A)	36	29	65
***p*-value**	0.156		

2.3.5 Is there any evidence for faster-*X* effect in fast evolving genes?

Many factors could be causing this absence of faster-*X* evolution: it is possible that most beneficial mutations are not partially recessive or that beneficial alleles are fixed in

Drosophila from the array of standing variation (Thornton *et al.*, 2006; Betancourt *et al.*, 2004). A slower-*X* evolution is also expected if most fixed mutations are either neutral or slightly deleterious (Charlesworth *et al.*, 1987). If a higher K_a/K_s reflects at least partially the action of stronger positive selection, then genes with high K_a/K_s will be the ones prone to faster-*X* evolution. Genes with low K_a/K_s, on the other hand, will be expected to have a slower-*X* evolution. To test for this we divided our sample into fast, medium and slow evolving genes in the following way: we ordered our *3*L-*X*R genes according to their K_a/K_s in the *D. melanogaster/D. yakuba* pair and classified the first 23 as slow-evolving genes, the next 23 as medium, and the last 23 as fast-evolving genes. We then repeated the analysis for these three classes, using as a control the non-*3*L-*X*R genes that had K_a/K_s values, in *D. melanogaster/D. yakuba,* in the same range as the *3*L-*X*R genes. The resulting sample contains 39 fast evolving genes (23 *3*L-*X*R, 16 non-*3*L-*X*R), 46 medium (23 *3*L-*X*R, 23 non-*3*L-*X*R) and 50 slow evolving genes (23 *3*L-*X*R, 27 non *3*L-*X*R). The proportion of genes with higher K_a, K_s and K_a/K_s in the *D. affinis/D. pseudoobscura* pair is shown in Table 2.6 (the detailed number of genes for each class is given in Appendix A2.4).

Although our sample size is now drastically reduced, the proportion of genes evolving faster in the *D. affinis/D. pseudoobscura* pair is behaving in the predicted direction (for K_a/K_s values): for fast evolving genes, only 44% of the non-*3L-XR* loci are evolving faster in *D. affinis/D. pseudoobscura*, compared with 61% of the *3*L-*X*R loci, which is in agreement with the faster-*X* hypothesis. Slow-evolving genes, on the other hand, do indeed show a moderate "slower-*X*" effect (70% of *3*L-*X*R genes evolving faster in the pseudoobscura group, versus 78% for the control genes).

Table 2.6: Proportion of slow and fast evolving genes with increased K_a/K_s, K_a and K_s in the *D. affinis/D. pseudoobscura* pair.

Ka/Ks	*3*L-*X*R	Non-*3*L-*X*R
Fast ($p = 0.34$)	61%	44%
Medium ($p = 0.12$)	78%	52%
Slow ($p = 0.2$)	70%	78%
Ka	*3*L-*X*R	Non-*3*L-*X*R
Fast ($p = 0.52$)	52%	38%
Medium ($p = 0.77$)	48%	57%
Slow ($p = 0.11$)	61%	74%
Ks	*3*L-*X*R	Non-*3*L-*X*R
Fast ($p = 0.006$)	17%	63%
Medium ($p = 0.33$)	22%	39%
Slow ($p = 0.07$)	17%	41%

The *p*-values were obtained using two-tailed Fisher tests.

This trend is partially caused by differences in K_s (*3*L-*X*R fast evolving genes have the strongest K_s reduction in the *D. pseudoobscura* group), but the values for K_a are also consistent with the faster-*X* hypothesis. For fast evolving genes, the *3*L-*X*R arm has a higher proportion of genes with higher K_a in the *D. pseudoobscura* group than the control. For slow evolving genes, we observe the opposite.

Although they are not conclusive, these results are of interest in view of the contradictory results obtained by previous studies on faster-*X* evolution, as they suggest that such an effect can only be observed for genes that are under particularly strong positive

selection and / or relaxed negative selection. In fact, most of the studies that detected faster-*X* evolution in *Drosophila* were in some way biased towards fast evolving genes. Counterman *et al*. (2004) obtained part of their sample from a male-specific EST screen (Swanson *et al*. 2001). Male-specific genes are not only expected to show an enhanced faster-*X* evolution, but it has also been claimed that they evolve faster than non-sex-biased genes in Drosophila, possibly as a consequence of increased positive selection (Zhang *et al*. 2004). Consistent with this, several studies of male-biased or male reproductive genes have detected faster rates of evolution on the *X* (Torgerson, 2003; Wang, 2004). Thornton *et al*. (2006), whilst following a similar approach, chose their genes randomly, and observed no effect.

Although K_a/K_s values were significantly higher for *X*-linked duplicates in Thornton and Lang's study (2002), one of their results was puzzling: according to the faster-*X* hypothesis, the average K_a/K_s should be highest when both duplicates are on the *X*, lowest when they are both autosomal and intermediate when one gene is *X*-linked and its pair autosomal. In fact, the values are similar for *X*-linked/autosomal and autosomal/autosomal pairs (0.26 and 0.27, respectively). They suggest this occurs because the direction of duplication is not random: there is an excess of duplications from the *X* onto the autosomes (Betran, 2002). Therefore, for most *X*-linked/autosomal pairs, the gene on the *X* is the ancestral one. In a pair of newly duplicated genes, for the newborn duplicate to acquire a new function, the ancestral gene has to maintain its original function: only the new gene is expected to show increased rates of evolution. The value of K_a/K_s does not depend on whether the ancestral gene is on the *X* or on an autosome, reinforcing the idea that faster *X* evolution can only be detected for genes under strong positive selection (or weaker negative selection).

2.4 Discussion

2.4.1 Is selection more efficient on the *X* chromosome?

We have obtained several lines of evidence that suggest that selection is more efficient at *X*-linked loci than at autosomal loci:

-X-linked genes have, on average, higher *Fop* values, and lower K_s. Whilst neutral processes could explain both the lower K_s (if mutation was lower on the X, which seems unlikely in Drosophila) and the higher *Fop* values (if these are caused by an increase in GC content), more efficient selection against unpreferred codons can account for both (McVean and Charlesworth, 1999).

-Overall, *3*L-*X*R genes do not differ from our control genes as far as K_a values are concerned. However, a closer look at the data shows that fast-evolving genes appear to have higher K_a in *D. pseudoobscura/D. affinis*, where they are *X*-linked, than in *D. melanogaster/D. yakuba*, whereas slow-evolving genes show the opposite pattern (Table 2.6). Whilst these patterns are not significant, they are in the expected direction, if selection is indeed more efficient on the *X*.

Our discussion has focused mostly on the faster-*X* hypothesis, the idea that partially or completely recessive mutations on the *X* will be instantly expressed in the haploid males and therefore more efficiently selected than autosomal ones. However, another factor that could cause selection to be more efficient on the *X* is a difference in effective population size at *X*-linked loci. Below, we discuss why this could occur and how it would affect the evolution of the *X* chromosome.

2.4.2 The effective population size of the *X* chromosome and the autosomes

The proportion of neutral mutations and mutations under selection was the subject of heated discussions for decades in evolutionary biology (Nei, 2006). Ohta (1973) showed that the behaviour of mutations depends not only on their selection coefficient *s*, but also on the effective population size (N_e) since, when $N_e s < 1$, their fate is mostly controlled by drift and they behave as effectively neutral mutations. Therefore differences in *X*-linked and autosomal population sizes could, in principle, also explain the more efficient selection observed at *X*-linked sites.

Males have only one copy of the *X* chromosome. This implies that, all other things being equal, the effective population size of the *X* chromosome is only ¾ of the autosomal population size, and selection is expected to be less efficient at *X*-linked sites (Hedrick, 2007). However, it has been shown that differences in male and female reproduction variance can distort this ratio (Charlesworth, 2001; Laporte and Charlesworth, 2002). If, for instance, males have a high variance in reproductive success, only a small proportion will contribute to the next generation, and therefore the male effective population will be smaller than the female population. Since $^2/_3$ of the *X* chromosomes are in females (assuming equal sex ratio), their effective population size will be less affected by the reduction in male N_e than the autosomal N_e. If the difference between male and female variance is large enough, this can result in a higher effective population size for the *X* than the autosomes. Higher female than male reproductive variance results in an N_e ratio lower than 3/4.

One way to assess if the effective population size of the *X* chromosome is higher or lower than the autosomal one is to compare neutral polymorphism at *X*-linked and autosomal sites, since neutral polymorphism levels are proportional to the effective population size (π, the pairwise average divergence, is equal to $4N_e\mu$, where μ is the neutral mutation rate). Whilst not being strictly neutral (see previous section on codon usage bias), synonymous sites have been shown to be under lower selective constraints than either non-synonymous or non-coding sites in Drosophila species (a large proportion of the latter is probably involved in transcription regulation (Halligan and Keightley, 2006; Begun *et al*., 2007)). We have compared *X*-linked and autosomal synonymous polymorphism data from African populations of *D. melanogaster* collected by Andolfatto (2005) and Shapiro *et al*. (2007) (Table 2.7).

At first glance, differences in N_e seem to account for the increase in selection efficiency at *X*-linked sites, since, at synonymous sites, π_X is much higher than π_A. A similar result was recently described by Hutter *et al*. (2007), who found that, in African populations of *D. melanogaster*, *X*-linked sites have higher levels of non-coding polymorphism than autosomal sites.

Table 2.7: Synonymous polymorphism at *X*-linked and autosomal genes (data from Shapiro *et al*., 2007 and Andolfatto, 2006).

	N	Average π_s	Standard deviation	t-test P-value	Mean recombination rate (cM/Mbp)
X	31	0.029	0.009	NA	3.932
Autosomes	407	0.018	0.015	0.000	2.274
Autosomes (high recombination only)	148	0.023	0.016	0.043	3.266

Autosomal genes were classified as being in a region of "high recombination" when the estimate of the recombination was equal or larger than 3.08, the smallest value for the *X*-linked genes. The p-values refer to the comparison of diversity levels between X-linked and autosomal loci.

One caveat of our comparison, however, is that Andolfatto (2005) focused his sampling efforts on regions of high recombination of the *X* chromosome, whereas Shapiro *et al*. (2007) aimed for a more homogeneous coverage of mostly the third chromosome. Since low recombination can also decrease neutral polymorphism levels (see next section), we had to verify that the difference between the *X* chromosome and the autosomes was not due to higher levels of recombination. We used Singh and Lipatov's online recombination estimator (http://cgi.stanford.edu/~lipatov/recombination/recombination-rates.txt) to obtain recombination rates for all our loci, and plotted π_s as a function of recombination rate for each locus (Figure 2.3).

Figure 2.3 clearly shows that our *X*-linked sample consists of high recombination loci, and that, when we focus on regions of high recombination only (lower panel), synonymous polymorphism levels are more similar in our two samples (the effect of linkage was not completely eliminated, as the mean recombination rate was still higher for *X*-linked genes than for high recombination autosomal genes). We can therefore not exclude the possibility

that higher recombination at the *X*-linked loci is the cause of the observed higher synonymous polymorphism levels.

In Hutter *et al*. (2007), recombination rates for *X*-linked and autosomal loci were estimated and found to have very similar means. Consistent with this, silent polymorphism seems to be, overall, higher at *X*-linked sites for all recombination levels (Figure 2.4, upper panel). When we use Singh *et al*.'s (2005) estimates of recombination rates, however, this effect disappears (Figure 2.4, lower panel), and we cannot exclude differences in recombination as the cause of the increased polymorphism levels in their sample. How, then, can we explain that this effect has been observed repeatedly in African populations of *D. melanogaster* (Andolfatto, 2001; Kauer *et al*, 2002, Mousset and Derome, 2004)? One possibility is that higher rates of recombination on the *X*, and not just demographic history, are causing the increase in the effective population size of the *X* chromosome.

2.4.3 Is there a higher recombination rate on the Drosophila *X*?

The effective population size was defined as the population size that would be required for the same amount of genetic diversity to be accumulated by drift as in the observed population, under an ideal random mating model (Wright, 1931). Reduced recombination can increase this amount, by dragging linked variation to fixation when beneficial mutations are swept to fixation, or by removing variation linked to deleterious mutations. This is thought to account for the observation, in several species, that recombination rates and neutral polymorphism levels are positively correlated (Betancourt and Presgraves, 2002). We therefore expect the effective population size, and the efficiency of selection, to be higher for chromosomes with higher rates of recombination.

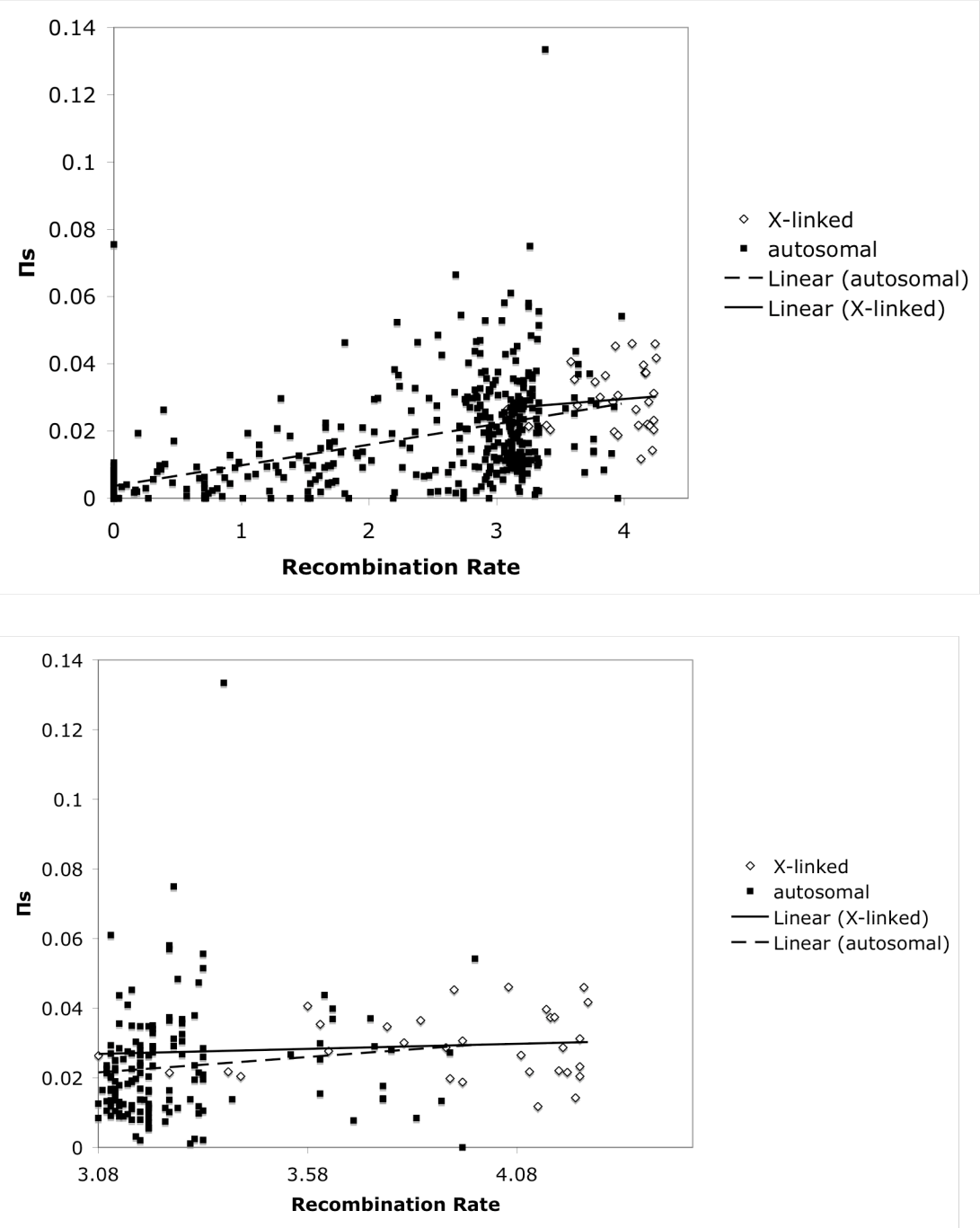

Figure 2.3: The data from Shapiro *et al.* and Andolfatto was used to compare synonymous average pairwise diversity (π_s) at *X*-linked and autosomal loci for all loci (upper panel) and high recombination regions only (lower panel).

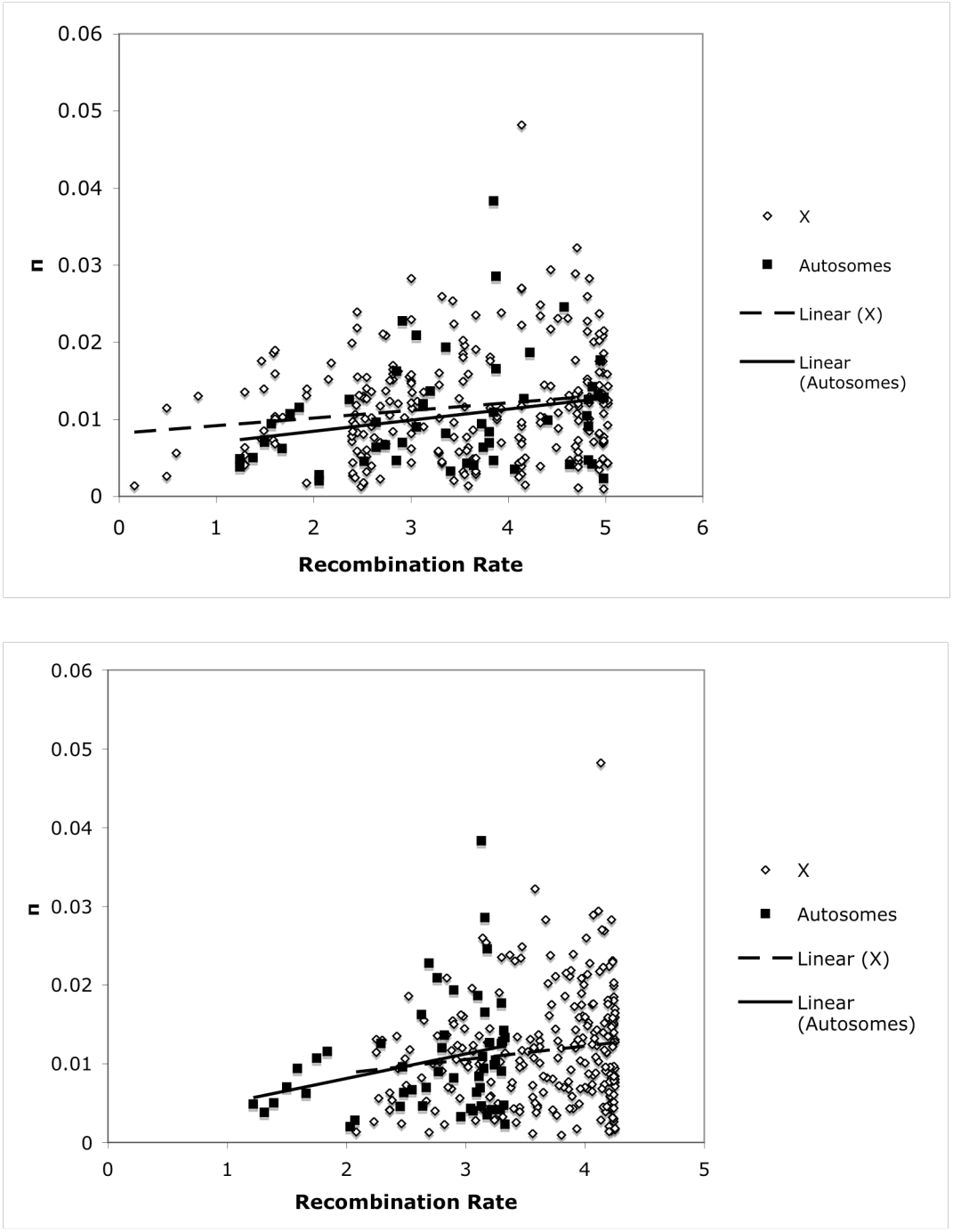

Figure 2.4: Non-coding polymorphism levels (π) at *X*-linked and autosomal loci, from Hutter et al. (2007), using the recombination estimates they provided (upper panel) and the estimates of Singh *et al.* (2005, lower panel).

In Drosophila, recombination only occurs in females. Since the *X* chromosome is transmitted $^{2}/_{3}$ of the time by females, as opposed to only $^{1}/_{2}$ of the time for autosomes, it is expected to have higher recombination levels than the autosomes ($^{4}/_{3}$ as high). Connallon (2007) analysed rates of evolution at *X*-linked sites in order to test the faster-*X* hypothesis. For this purpose, he used several methods to estimate the average rates of recombination at *X*-linked and autosomal sites and found a much lower value for the autosomes with all the methods (the A:X ratio estimates ranged from 0.45 to 0.66). Bachtrog and Andolfatto (2006) analysed linkage disequilibrium in *D. miranda*, a sister species of *D. pseudoobscura*, and found a similar effect, with autosomes harbouring higher levels of linkage disequilibrium, compatible with a lower recombination rate, than the *X* chromosome. Ortiz-Barrientos *et al*. (2006) estimated mean rates of recombination for the two *X*-linked chromosomal arms and two autosomal arms in *D. pseudoobscura* and obtained similar results (121kb/cM and 105kb/cM for the *X*-linked arms, versus 148kb/cM and 232kb/cM for the autosomal arms, where kb/cM represents kilobases per centimorgan). This higher rate of recombination on the *X* is therefore not specific to *D. melanogaster*, and is likely to be contributing to the differences we observe between *X* and autosomal rates of evolution and codon usage bias. The extent to which this occurs will become clearer once more precise estimates of recombination rates on the *D. pseudoobscura X* become available.

2.4.4 The dominance coefficient of new mutations

Whilst a large amount of research has been dedicated to the estimation of the mean dominance coefficient of deleterious mutations, the dominance level of beneficial

mutations remains elusive. Anecdotal evidence from resistance genes in plants has mostly supported the dominance of beneficial mutations, whereas in insects there is less consistency (Bourguet and Raymond, 1998; Kacser and Burns, 1981). Furthermore, the hypothesis that new beneficial mutations are on average partially recessive is somewhat counterintuitive, as some of these are likely to confer a gain of function. However, the only systematic analysis of the dominance of beneficial mutations comes from mutation-accumulation studies in *Saccharomyces cerevisiae* and does not support this (Zeyl *et al*., 2003; Anderson *et al*., 2004).

Since *S. cerevisiae* can exist in both a haploid and diploid state, it is an excellent model to estimate dominance coefficients of artificially selected beneficial mutations: recessive mutations do not affect diploid fitness, whereas dominant mutations have the same effect on both haploid and heterozygous fitness (intermediary dominant coefficients cause a larger increase of haploid fitness than diploid fitness). These studies show that selection on diploids leads to the accumulation of partially dominant beneficial mutations (which is expected, since in diploids recessive mutations are more often lost by drift). Selection on haploids, on the other hand, leads to the accumulation of more recessive mutations: one study found that 17 out of 29 mutations accumulated were recessive (59%) (Anderson *et al*., 2004), the other estimated that the mean dominance coefficient of the beneficial mutations fixed in a haploid population was 0.25 (Zeyl *et al*., 2003), suggesting that, in yeast, beneficial mutations are on average partially recessive. It is possible that this is also the case in multi-cellular organisms and that the observed beneficial mutations are dominant because they were selected in diploids.

Comparisons of rates of evolution at *X*-linked and autosomal sites offer a promising approach to this problem, as predictions differ depending on the dominance coefficient of new beneficial mutations (Charlesworth *et al*., 1987). Two recent studies on faster-*X* evolution have concluded that new beneficial mutations were likely to be, on average, partially dominant (Thornton *et al*., 2006; Connallon, 2007). This conclusion was derived from the fact that no detectable difference in adaptive rates of evolution was found between the *X* and the autosomes. Our results differ slightly from Connallon's (2007) and support the view that, whilst it is possible that there is more efficient positive selection at *X*-linked loci, this is only detectable for genes which evolve predominantly under positive selection or are under weak negative selection (further support for this comes from studies of *X*-linked reproductive or sex-biased genes that consistently find a faster-*X* effect). This is not incompatible with dominant mutations, if an increased rate of recombination is sufficient to account for the faster-*X* effect.

2.5 Conclusions

By using a multi-species model, we were able to examine directly the consequences being on the *X* chromosome on the evolution of coding sequence. Our main result was an increase in codon usage bias on the *3*L-*X*R arm (and decrease in synonymous divergence) in *D. pseudoobscura/D. affinis*, possibly due to more efficient selection against unpreferred codons at *X*-linked loci.

We did not observe a significant increase in K_a for *X*-linked sites. K_a/K_s values were significantly higher, but this effect comes mostly from differences in K_s. In fast-evolving genes, there seemed to be an increase in K_a at *X*-linked loci, and the opposite was observed

for slow-evolving genes (though none of these patterns were significant). This suggests that further studies on faster-*X* evolution should focus on fast-evolving genes.

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Chapter 3: Effective population sizes and substitution rates of the X chromosome and the autosomes

Abstract

Current models of *X*-linked and autosomal evolutionary rates often assume that the effective population size of the *X* chromosome (N_{eX}) is equal to $^3/_4$ of the autosomal population size (N_{eA}). However, polymorphism studies in *D. melanogaster* and *D. simulans* suggest that there are often significant deviations from this value. We have used a program to compute fixation rates of beneficial and deleterious mutations at *X*-linked and autosomal sites when this occurs. We find that N_{eX}/N_{eA} is a crucial parameter for the rates of evolution of *X*-linked sites compared to autosomal sites.

We also tested different parameters that are known to influence the rates of evolution at *X*-linked and autosomal sites, such as different mutation rates in males and females and mutations that are sexually antagonistic, to determine which cases can lead to faster-*X* evolution.

3.1 Introduction

As discussed in Chapter 2, Charlesworth *et al.* (1987) modelled rates of evolution on the *X* chromosome and the autosomes and showed that, under certain conditions, the *X* chromosome is expected to accumulate beneficial mutations at a higher rate than the autosomes. This happens because selection on recessive or partially recessive mutations is more efficient on the *X*, as the effect of these mutations is not masked by the ancestral allele in males (Charlesworth *et al.*, 1987, Vicoso and Charlesworth, 2006).

Another factor that affects the efficacy of selection is the effective population size (N_e), as in smaller populations a larger fraction of mutations fall within the zone of near-neutrality (Ohta, 1973). Since males have only one copy of the *X* chromosome, the effective population size of the *X* (N_{eX}) equals $^3/_4$ of the autosomal effective population size (N_{eA}), when offspring number for both females and males follow a Poisson distribution, as assumed by Charlesworth *et al.* (1987). However, it has also been shown that differences in variance of reproductive success between males and females could invalidate this assumption (Caballero, 1995; Charlesworth, 2001; Laporte and Charlesworth, 2002). If male reproductive success has a higher variance than female reproductive success, then the male effective population size will be smaller than the female effective population size, as only a few males will effectively contribute to the next generations. Since the *X* chromosome only spends $^1/_3$ of its time in males, N_{eX} will be less affected by this reduction in male effective population size than N_{eA} (as the autosomes spend ½ of the time in males). Consequently, N_{eX} will be larger than ¾ of N_{eA}. The opposite is expected if females have a higher variance of reproductive success than males.

In Drosophila, synonymous and silent polymorphism levels are not consistently lower on the *X* chromosome. In fact, in African populations of *D. melanogaster*, the pairwise diversity is higher on the *X* chromosome than on the autosomes, suggesting that N_{eX} could be larger than N_{eA} (Andolfatto, 2001; Kauer *et al*, 2002; reviewed in Mousset and Derome, 2004). It therefore seemed of interest to modify Charlesworth *et al.*'s (1987) model, to examine the effects of variance in reproductive success on the rates of evolution of the *X* chromosome and the autosomes. It should be noted that non-African populations of *D. melanogaster* have lower polymorphism levels on the *X* chromosome than on the autosomes (Mousset and Derome, 2004). However, these populations are thought to have recently expanded from ancestral African populations. The bottlenecks and/or different selective pressures deriving from expanding into a new environment affect the diversity of the *X* chromosome and of the autosomes to different extents, so that these newly expanded populations are unlikely to represent the ancestral effective population sizes of the *X* and the autosomes (Mousset and Derome, 2004; Haddrill *et al*., 2005; Pool and Nielsen, 2007).

A related question has been addressed by Singh *et al.* (2005) and Lu and Wu (2005), who examined the expected rates of synonymous evolution (Lu and Wu, 2005) and the expected levels of codon usage bias on the *X* chromosome (Singh *et al*., 2005) when the sex-ratio is biased; this can also increase N_{eX}/N_{eA}, if the sex-ratio is male-biased. However, the effect of varying N_{eX}/N_{eA} through other mechanisms has so far not been the focus of any work. We have written a FORTRAN program that extends the model of Charlesworth *et al*. (1987) to compare the rates of evolution at *X*-linked and autosomal sites under different scenarios for the variance in male and female reproductive success

(and consequent differences in N_{eX}/N_{eA}). By varying the strength and direction of selection in males and females, we have produced a more exhaustive description of possible scenarios that can lead to faster-*X* (or slower-*X*) evolution.

Finally, we have examined the effect of different mutation rates in males and females on the rates of evolution at *X*-linked and autosomal sites. This has been shown to occur in some organisms (Ebersberger *et al.*, 2002; Axelsson *et al*., 2004**)**, and can lead to different mutation rates on the *X* and the autosomes, as these spend different amounts of time in males and females (Miyata *et al.*, 1987; reviewed in Vicoso and Charlesworth, 2006). Since the rate of neutral evolution is equal to the effective mutation rate, this difference will be reflected in the rates of neutral evolution at *X*-linked and autosomal sites. In mammals, for instance, males have higher mutation rates than females, possibly because the male germline undergoes more cell divisions to form gametes and this leads to lower rates of neutral rates of evolution at *X*-linked sites (Ebersberger *et al.*, 2002, Taylor *et al.*, 2005). Kirkpatrick and Hall (2004) investigated the effect of different mutation rates in males and females on the rates of fixation of mutations under positive selection, and how this affected faster-*X* evolution. They found that, when the rate of mutation was higher in males (and, consequently, so was the mutation rate on the autosomes), this could counteract the faster-*X* effect, whereas the opposite was expected, when the mutation rate was higher in females. We have extended this analysis to the fixation rate of deleterious mutations, as well as the normalized rates of evolution of beneficial mutations.

3.2 Methods

3.2.1 The diffusion approximation

The probability of fixation (U) of a beneficial mutation with an initial frequency p can be calculated by using the following approximation (Ewens, 2004):

$$U(p) = \frac{\int_0^p G(x)dx}{\int_0^1 G(x)dx} \tag{3.1}$$

where:

$$G(x) = \exp\left(-2\int_0^x \frac{M_{\delta y}}{V_{\delta y}}dy\right) \tag{3.2}$$

$M_{\delta x}$ describes the expected change in frequency of an allele with frequency x and $V_{\delta x}$ is the variance of the change in frequency due to finite population size (we are considering the case of a biallelic locus, with alleles A_1 and A_2 at initial frequencies 1-x and x, respectively (Table 3.1). The equations for $M_{\delta x}$ are given below).

U(p) is a double integral and cannot be solved analytically for all cases of interest. We have written a FORTRAN 77 program that estimates the probability of fixation of a new mutation numerically, for given values of s, the selection coefficient, h, the dominance coefficient, and N_{eA} and N_{eX} (More specific details on the program are given in Appendix A3.1).

Table 3.1: The fitness model used in our computations. A_1 is the ancestral allele, A_2 the new mutation, s_m and s_f are the selection coefficient in males and females, respectively, and h is the dominance coefficient of the new mutation.

Autosomal case					
	Females			Males	
$A_1 A_1$	$A_1 A_2$	$A_2 A_2$	$A_1 A_1$	$A_1 A_2$	$A_2 A_2$
1	$1+hs_f$	$1+s_f$	1	$1+hs_m$	$1+s_m$

X-linked case				
	Females		Males	
$A_1 A_1$	$A_1 A_2$	$A_2 A_2$	A_1	A_2
1	$1+hs_f$	$1+s_f$	1	$1+s_m$

3.2.2 Determining $M_{\delta x}$

Autosomal locus, with different selection coefficients for males (s_m) and females (s_f)

We are using the same fitness model as Charlesworth *et al.* (1987, Table 3.1). The deterministic change in the frequency of a mutation is given in standard textbooks (e.g. Crow and Kimura, 1970; Ewens, 2004). For the autosomal case, when selection is weak,

$$M_{\delta x} = \Delta x \approx x(1-x)\left[W_{A2.} - W_{A1.}\right] \tag{3.3}$$

where the marginal fitness of the new beneficial mutation is:

$$W_{A2.} = \frac{x}{2}\left[1 + s_f + 1 + s_m\right] + \frac{1-x}{2}\left[1 + hs_f + 1 + hs_m\right] \tag{3.4}$$

and the marginal fitness of the ancestral allele is:

$$W_{A1.} = \frac{1-x}{2}\left[1 + 1\right] + \frac{x}{2}\left[1 + hs_f + 1 + hs_m\right] \tag{3.5}$$

If we replace the marginal fitnesses in Equation 3.3 by these formulae, we have:

$$\Delta x \approx x(1-x)\left[\frac{x}{2}(2+s_f+s_m)+\frac{1-x}{2}(2+h(s_f+s_m))-(1-x+\frac{x}{2}(2+h(s_f+s_m))\right]$$

which can be simplified to:

$$\Delta x \approx \frac{x(1-x)(s_f+s_m)\left[x(1-2h)+h\right]}{2} \tag{3.6}$$

In the diffusion approximation, we use $M_{\delta x}/V_{\delta x}$. $V_{\delta x}$ is (Crow and Kimura, 1970; Ewens, 2004):

$$V_{\delta x} = \frac{x(1-x)}{2N_{eA}} \tag{3.7}$$

for the autosomal case, we thus have:

$$\frac{M_{\delta x}}{V_{\delta x}} = N_{eA}(s_m+s_f)\left[x(1-2h)+h\right] \tag{3.8}$$

X-linked locus, with different selection coefficients for males and females

Equation (3.3) can be adjusted to take into account the fact that the X chromosome spends $^2/_3$ of the time in females, and $^1/_3$ of the time in males. This time, we have, for the case of weak selection:

$$M_{\delta x} = \Delta x \approx \frac{x(1-x)}{3}\left[2(W_{2.f}-W_{1.f})+(W_{2.m}-W_{1.m})\right] \tag{3.9}$$

where $W_{2.f}$ and $W_{2.m}$ are the marginal fitnesses of the new mutation in females and males, and $W_{1.f}$ and $W_{1.m}$ are the marginal fitnesses of the ancestral allele in females and males.

If we replace them by:

$$W_{2.f} = 1+(1-x)hs_f+xhs_f$$

$$W_{2.m} = 1 + s_m$$

$$W_{1.f} = 1 + xhs_f$$

$$W_{1.m} = 1$$

we now have:

$$\Delta x \approx \frac{x(1-x)}{3}\left[2\left(1 + (1-x)hs_f + xs_f - 1 - xhs_f\right) + (1 + s_m - 1)\right]$$

which can be simplified to give:

$$\Delta x \approx \frac{x(1-x)}{3}\left[s_f(2h + 2x(1-2h)) + s_m\right] \tag{3.10}$$

$V_{\delta x}$ is:

$$V_{\delta x} = \frac{x(1-x)}{2N_{eX}} \tag{3.11}$$

so that, for the *X*-linked case:

$$\frac{M_{\delta x}}{V_{\delta x}} = \frac{2N_{eX}}{3}\left[s_f(2h + 2x(1-2h)) + s_m\right] \tag{3.12}$$

3.2.3 Accounting for the time spent by autosomes and the X chromosome in males and females

We have adjusted the formulae to account for the fact that the *X* chromosome spends $^2/_3$ of the time in females, whereas autosomes are only in females ½ of the time, by calculating the probabilities of fixation in males, U_m, and females, U_f, separately:

$$U_f(p_f) = \frac{\int_0^{p_f} G(x)dx}{\int_0^1 G(x)dx} \tag{3.13}$$

And:

$$U_m(p_m) = \frac{\int_0^{p_m} G(x)dx}{\int_0^1 G(x)dx} \tag{3.14}$$

where p_m is the initial frequency of a mutation in males and p_f its initial frequency in females. Following the approach of Charlesworth (1994), we have used $p_{mA}=N_m/4$, $p_{fA}=N_f/4_f$, $p_{mX=}=N_m/3$, $p_{fX}=N_f/3$, where N_f and N_m are the number of males and females in the breeding population, respectively.

3.2.4 Calculating the substitution rate

We use the formulae of Charlesworth (1994) to calculate the rate of substitution of mutations under selection at *X*-linked and autosomal sites:

$$K_{aA} = (\mu + \alpha\mu)\left[N_f U_f + N_m U_m\right] \tag{3.15}$$

$$K_{aX} = (\mu + \alpha\mu)N_f U_f + \mu N_m U_m \tag{3.16}$$

where μ and $\alpha\mu$ are the female and male mutation rates, respectively (α is a constant).

These are easier to interpret once they are normalized by dividing them by the neutral rate of substitution at *X*-linked (K_{sX}) and autosomal (K_{sA}) sites, as this is an approximation of the often used K_a/K_s. The neutral rate of substitution is simply the effective mutation rate that autosomes and the *X*-chromosome are subject to. In the case of autosomes, it is the average of the male and female mutation rates ($\alpha\mu$ and μ), since autosomes spend half of their time in females and half of their time in males. In the case of the *X*-chromosome, this has to be adjusted to

$$K_{sX} = (2\mu + \alpha\mu)/3 \tag{3.17}$$

as the *X* chromosome spends 2/3 of the time in females and only 1/3 in males.

3.2.5 Modifying the effective population size

Whilst silent polymorphism levels strongly suggest that N_{eX} is larger than ¾ of N_{eA} in *D. melanogaster*, the reasons for this deviation remain unclear (Mousset and Derome, 2004). Female-biased sex-ratio, population expansion, increased recombination on the *X* chromosome and increased male variance in number of offspring have all been suggested as possible causes (Andolfatto, 2001; Kauer *et al*, 2002; Mousset and Derome, 2004; Hutter *et al*., 2007). We focus on the last hypothesis, by estimating N_{eX} and N_{eA}, when the male variance in number of offspring (*Vm*) differs from the value expected with a Poisson distribution, as it is of interest to determine if this can cause deviations in N_{eX}/N_{eA} large enough to skew polymorphism levels on the *X* to higher levels than the autosomal ones, when biologically plausible scenarios are considered. It should however be noted that the results obtained with these values of N_{eX} and N_{eA} for the rates of evolution on the *X* and the autosomes apply when any of the above theories is considered, except for male-biased sex-ratio, as we are merely using differences in *Vm* to vary N_{eX}/N_{eA} without affecting the sex-ratio.

Laporte and Charlesworth (2001) provided formulae to estimate the effective population size of the *X* chromosome and the autosomes, taking into account the variance of male and female reproductive success:

$$\frac{1}{N_{eA}} \approx \frac{(1+F)}{4}\left\{\frac{1}{N_f} + \frac{1}{Nm} + \frac{(1-c)^2\Delta Vf}{Nf} + \frac{c^2\Delta Vm}{Nm}\right\} \tag{3.18}$$

$$\frac{1}{N_{eX}} \approx \frac{1}{9}\left\{\frac{4(1+F)}{N_f} + \frac{2}{N_m} + \frac{4(1+F)(1-c)^2\Delta V_f}{N_f} + \frac{2c^2\Delta V_m}{N_m}\right\} \quad (3.19)$$

where F is the inbreeding coefficient (we assume F=0), N_m the number of males, N_f the number of females, V_m the variance in male reproductive success, V_f the variance in female reproductive success. ΔV_m and ΔVf are the male and female deviations from a Poisson offspring number distribution and c is the proportion of males in the breeding population ($c = N_m/(N_m + N_f)$).

We have created a subroutine (EFFPOP) in our programs to estimate N_{eX} and N_{eA}, for given values of N_m, N_f, ΔV_m and ΔVf, using these formulae. The resulting values are then used in the diffusion approximations for the fixation probabilities (Equations (3.13) and (3.14)). The range of ΔV_m and ΔVf and the associated N_{eX}/N_{eA} that we used are shown in Figure 3.1. Although for lower values of ΔV_m and ΔVf (up to ΔV=20), increasing ΔV strongly affects N_{eX}/N_{eA}, increasing it further only causes a marginal change.

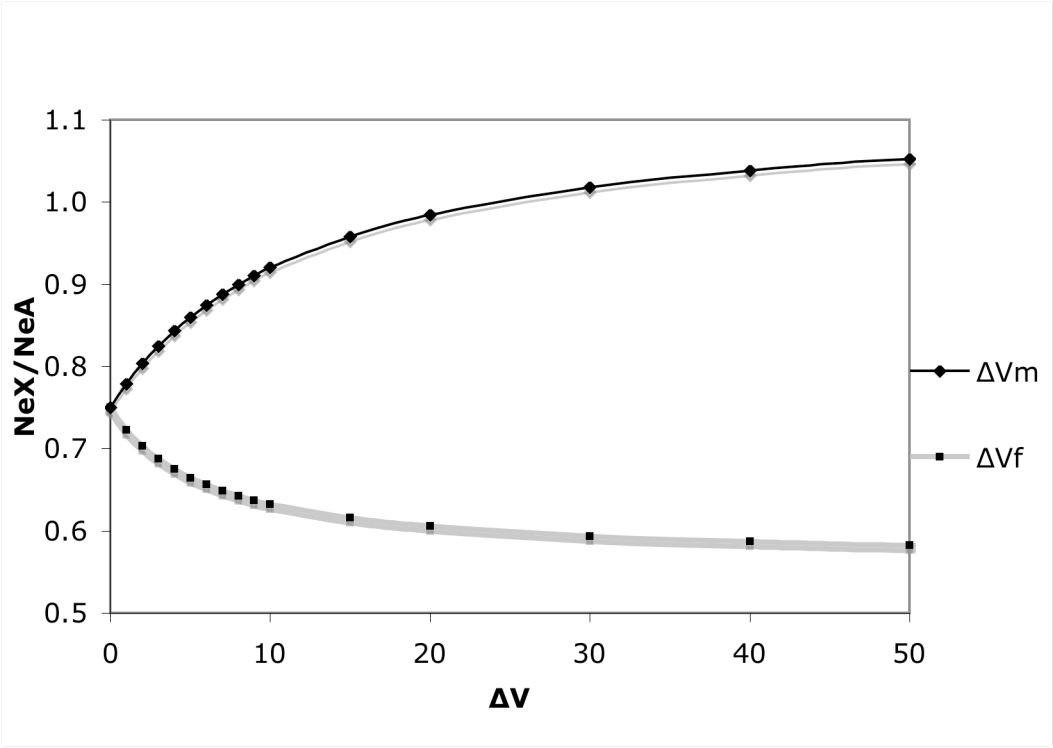


Figure 3.1: The ratio N_{eX}/N_{eA} can be altered by increasing the male (ΔV_m) and female (ΔV_f) deviations from the variance for the Poisson distribution of number of offspring.

3.3 Results

3.3.1 The rate of fixation of beneficial mutations

We can run our program with equal sex ratio (5000 males, 5000 females), equal selection on males and females ($s_f = s_m$, $N_{eA}s = 1$), and no non-random variance in male or female reproductive success (so that $N_{eX} = 3N_{eA}/4$), to check that it yields the same results as those obtained by Charlesworth *et al.* (1987). The results presented in Figure 3.2 for this case are indeed consistent with those obtained with previous models, with the *X* chromosome accumulating more recessive or partially recessive beneficial mutations than the autosomes, but less dominant or partially dominant beneficial mutations.

The ratio N_{eX}/N_{eA} can be increased, by increasing ΔV_m, the male deviation from a Poisson distribution of offspring number, which represents sexual selection on males (Figure 3.1). This also reduces N_e, the overall effective population size, which affects the rates of evolution, as more mutations fall within the zone of near neutrality. In order to focus only on the effect of modifying N_{eX}/N_{eA}, we scaled *s* to maintain a constant $N_{eA}s$ for all the values *i* of ΔV_m studied, by using

$$s_i = s\left(\frac{N_{eAi}}{N_{eA0}}\right)$$

where N_{eAi} is N_{eA} when $\Delta V_m = i$ and N_{eA0} is N_{eA} when $\Delta V_m = 0$.

When we use the same parameters as previously, but changing ΔV_m to 1 ($N_{eX} = 0.86N_{eA}$), there is a faster-*X* effect even when mutations are slightly dominant (Figure 3.2).

Current estimates of neutral polymorphism in African populations of *D. melanogaster* and *D. simulans* suggest that N_{eX} is, in fact, very similar to N_{eA} (if anything, it seems to be slightly higher (Kauer *et al*., 2002; Andolfatto, 2001; Mousset and Derome, 2004)). We examine this case by using ΔV_m=100 ($N_{eX} \approx 1.1 N_{eA}$, Figure 3.2). In this case, there is a faster-*X* effect for all levels of dominance of new mutations.

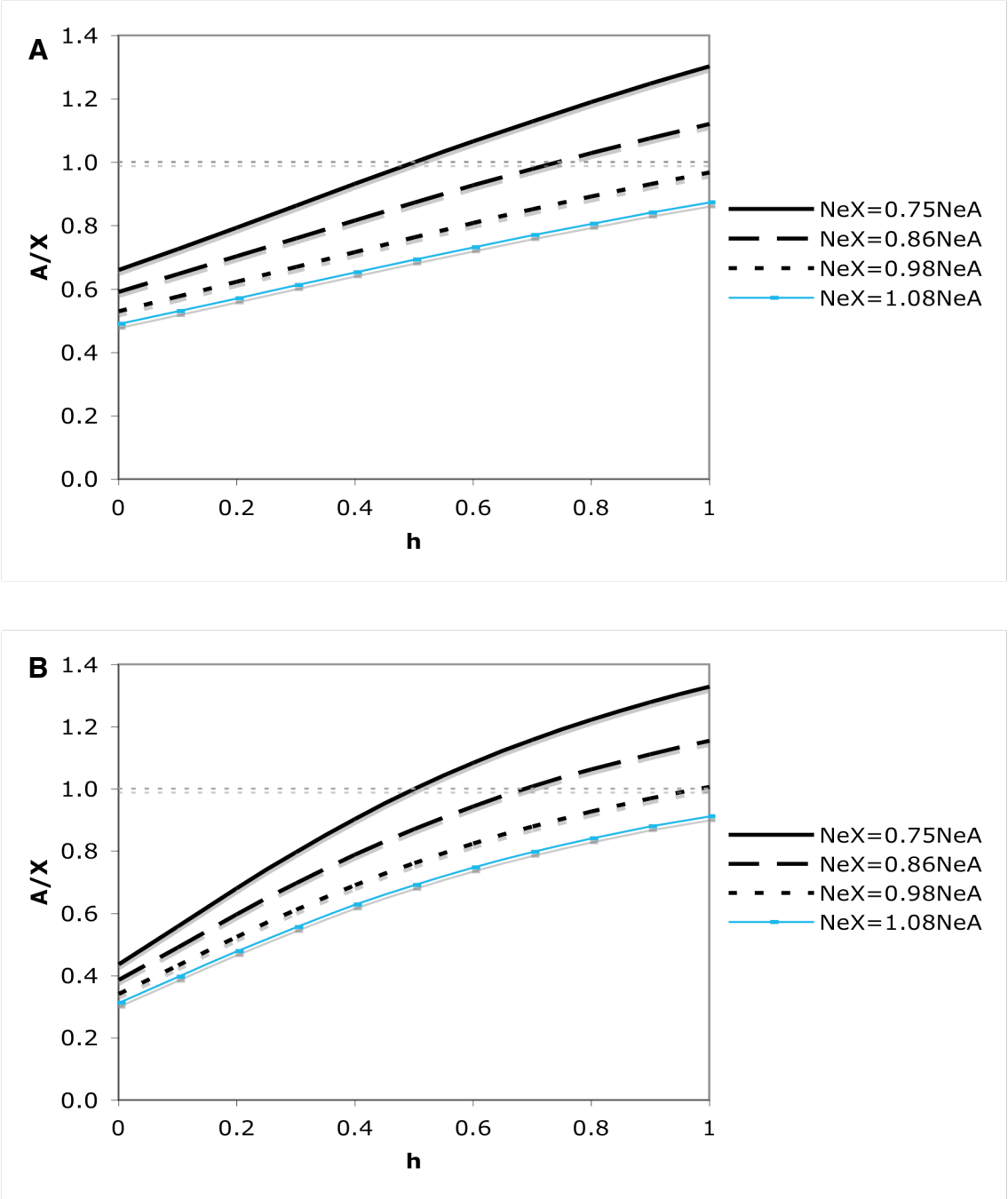


Figure 3.2: The normalized rate of adaptive evolution at *X*-linked and autosomal sites when $N_{eX} > {}^{3}/_{4} N_{eA}$. A/X is the ratio of autosomal to *X*-linked normalized rates of adaptive evolution (N=10000, $s_m = s_f$). A) $N_{eA} s_m = 3$; B) $N_{eA} s_m = 10$)

We also computed the substitution rates for new beneficial mutations at *X*-linked and autosomal sites when N_{eX} is smaller than $3N_{eA}/4$ (Figure 3.3). Unsurprisingly, in this scenario a faster-*X* effect can only be observed for more recessive mutations ($h<0.4$ in the case of $N_{eX}\approx 0.65N_{eA}$). Although this result may be of interest for other organisms, it is probably irrelevant to the discussion of Drosophila.

3.3.2 The rate of fixation of deleterious mutations

We have once again tested our program by reproducing the results of Charlesworth *et al.* (1987) for the fixation of deleterious mutations when $N_{eX}=3N_{eA}/4$ (Figures 3.4 and 3.5). In this case, we focus on mutations that are nearly neutral ($N_e s=-1$) or under weak negative selection ($N_e s=-3$) as mutations under strong negative selection are expected to contribute very little to the overall rates of evolution, as they are effectively removed from the population (Kimura, 1983). The results are consistent with what was previously found: recessive or partially recessive mutations ($h<0.5$) accumulate more slowly on the *X* chromosome, whereas dominant or partially dominant mutations ($h>0.5$) accumulate faster on the *X*.

When $N_{eX}>3N_{eA}/4$ (Figure 3.4), this scenario changes drastically, as there is a slower-*X* effect for most levels of dominance of new deleterious mutations. Furthermore, the amplitude of this phenomenon is much stronger than the faster-*X* effect observed for beneficial mutations, with, for the case of $N_{eX}=1.08N_{eA}$, the ratio of autosomal to *X*-linked rates of deleterious evolution exceeding 12 (when $N_{eA}s=-3$). The opposite effect is observed when $N_{eX}<3N_{eA}/4$ (Figure 3.5).

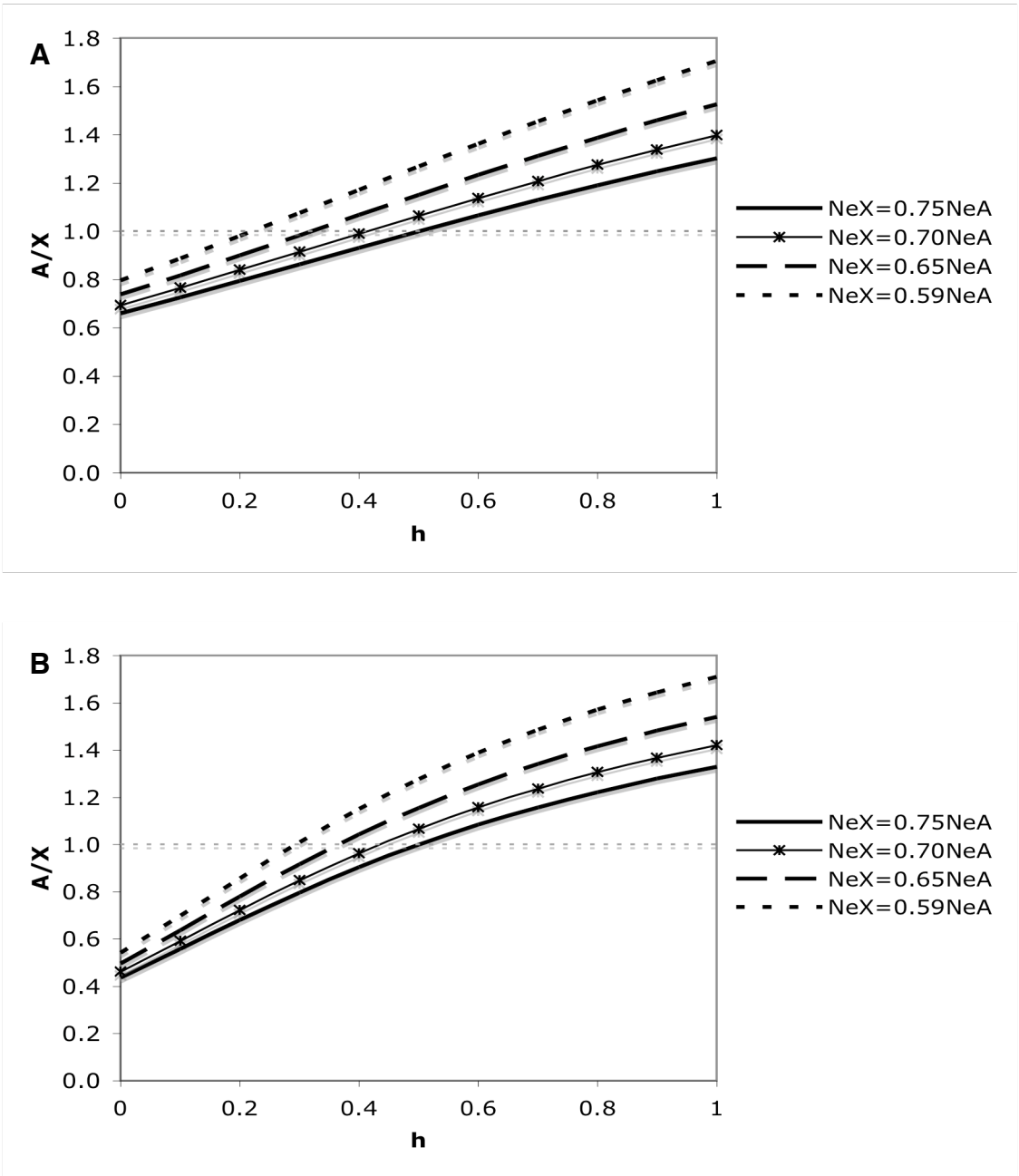
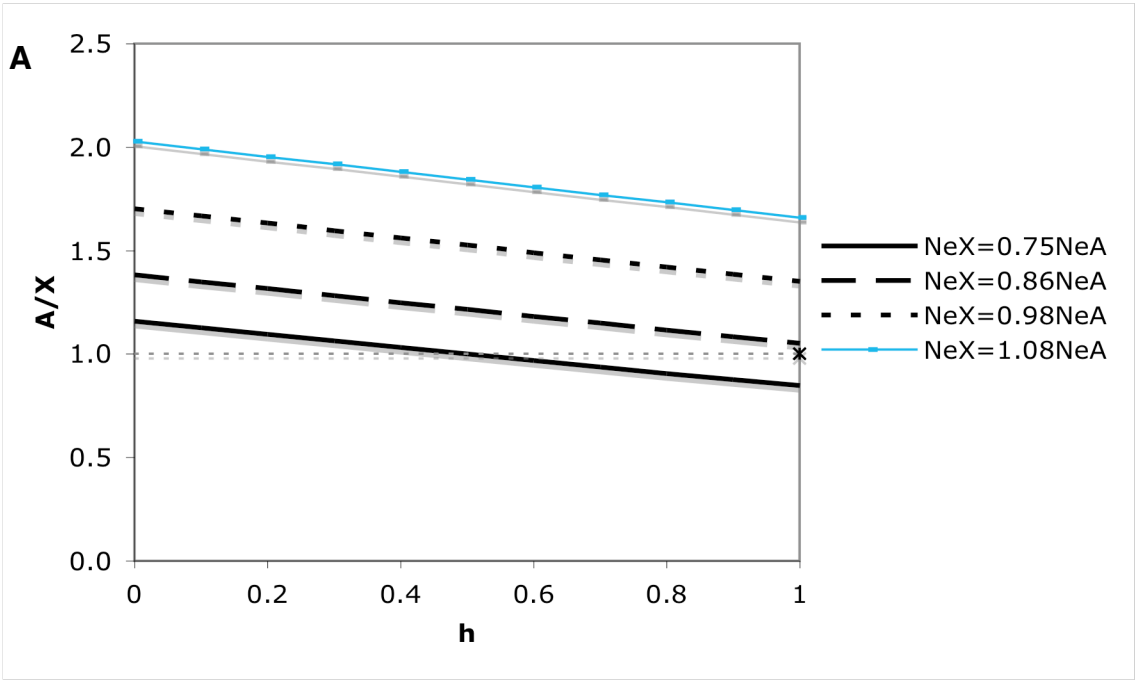


Figure 3.3: The normalized rate of adaptive evolution at *X*-linked and autosomal sites when $N_{eX} < {}^{3}/_{4} N_{eA}$. N=10000, $s_m = s_f$. A) $N_{eA} s_m$=3; B) $N_{eA} s_m$=10.



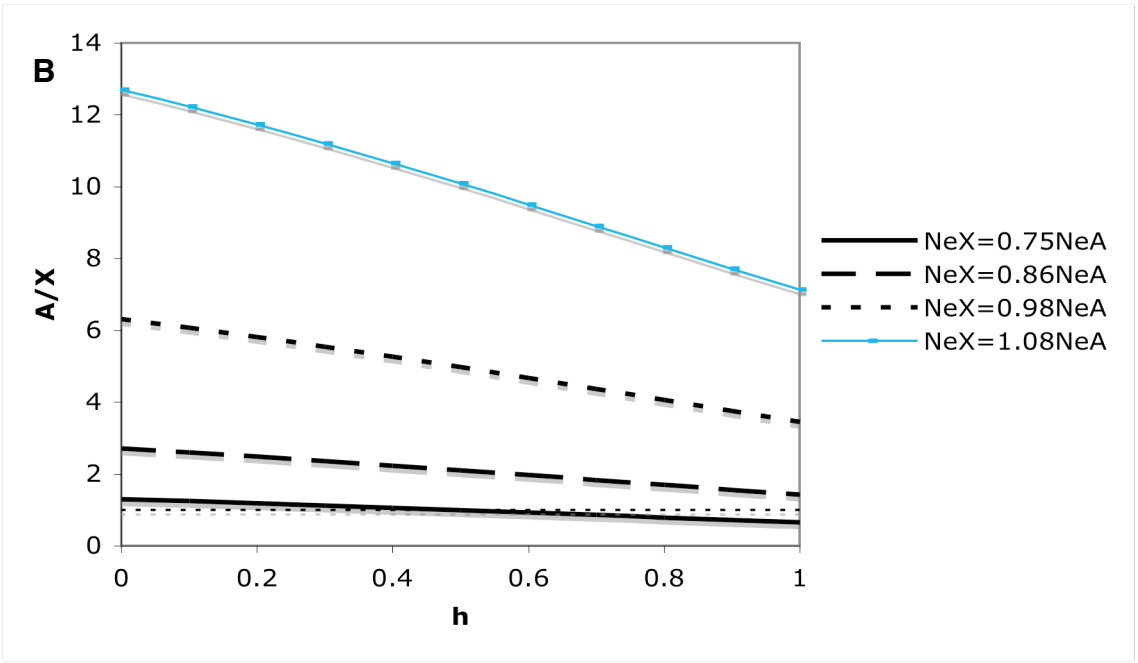
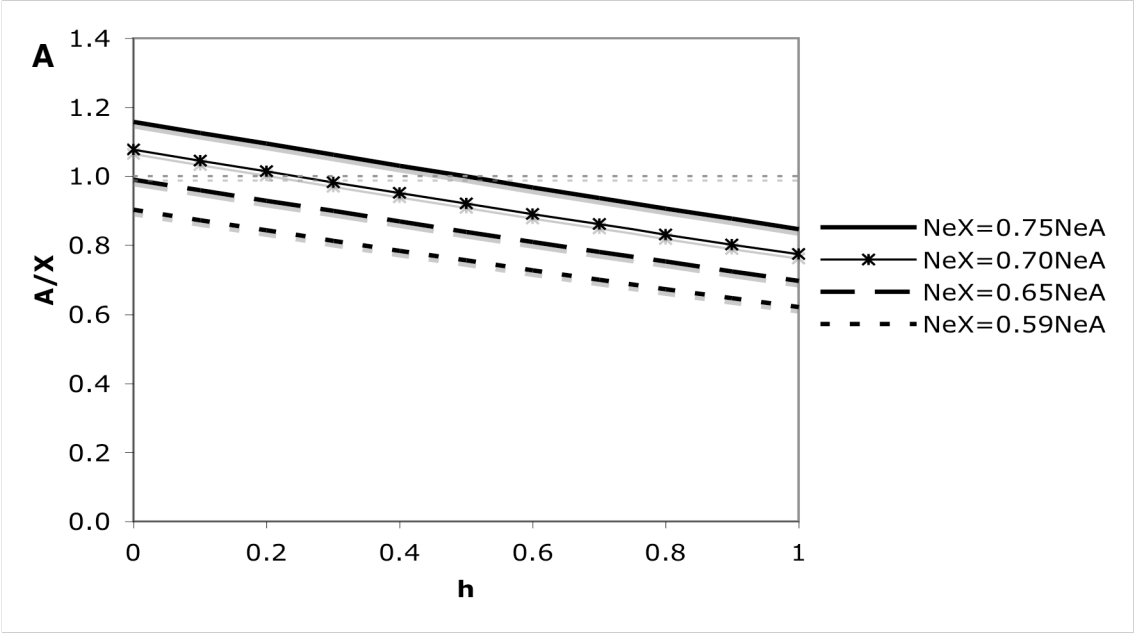


Figure 3.4: The normalized rate of fixation of deleterious mutations at *X*-linked and autosomal sites when $N_{eX} > {}^{3}/_{4} N_{eA}$ ($s_m = s_f$, N=10000). A) $N_{eA} s_m$=-1; B) $N_{eA} s_m$=-3.



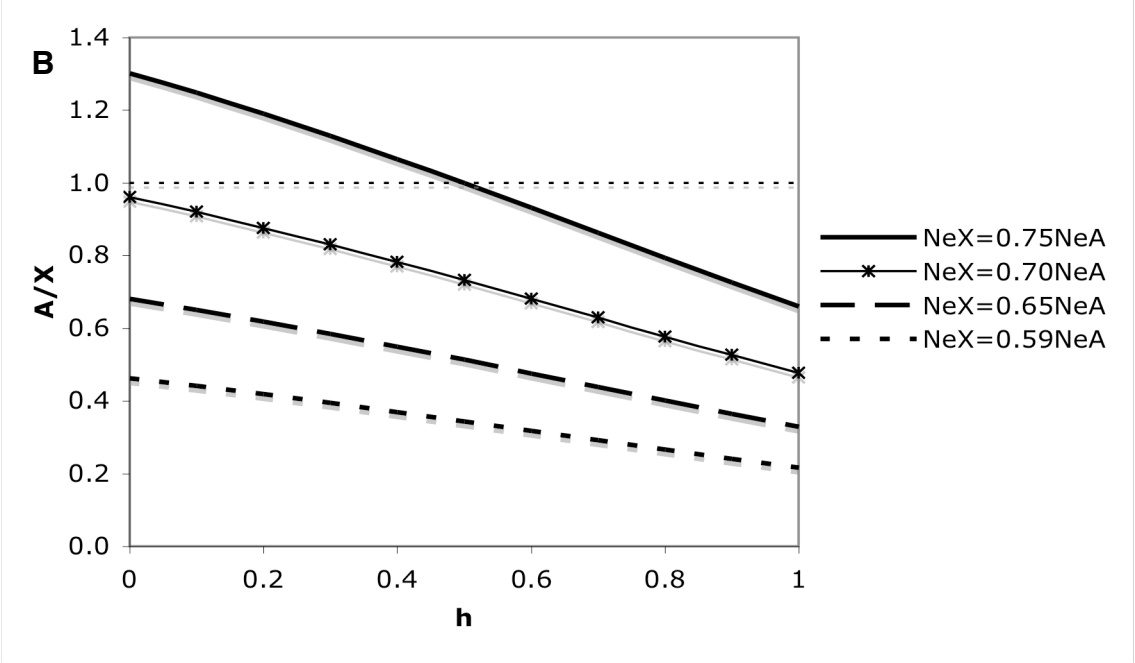


Figure 3.5: The normalized rate of fixation of deleterious mutations at *X*-linked and autosomal sites when $N_{eX} < {}^{3}/_{4} N_{eA}$ ($s_m = s_f$, N=10000). A) $N_{eA} s_m$=-1; B) $N_{eA} s_m$=-3.

3.3.3 Different mutation rates in males and females

In some organisms, males and females have different mutation rates ($\alpha\mu$ and μ, respectively), and this is reflected in the rates of evolution of *X*-linked and autosomal sites, as the time these spend in males and females differs (Miyata *et al*., 1987, Vicoso and Charlesworth, 2006). Kirkpatrick and Hall (2004) argued that a "male-driven" evolution ($\alpha>1$) reduced faster-*X* evolution, whereas the opposite scenario ($\alpha<1$) enhanced the faster-*X* effect to some extent. We have used our program to further investigate how α influences the rates of evolution at *X*-linked and autosomal loci, for both beneficial and deleterious mutations, when N_{eX} differs from $3N_{eA}/4$.

The first, surprising, result, is that both K_{aX}/K_{sX} and K_{aA}/K_{sA} are independent of α. In the case of K_{aA}/K_{sA}, this is easily explained, as, in our model, Equations (15) and (16) imply that:

$$\frac{K_{aA}}{K_{sA}} = \frac{(\mu+\alpha\mu)\left[N_f U_f + N_m U_m\right]}{(\mu+\alpha\mu)/2} = \frac{N_f U_f + N_m U_m}{2} \qquad (3.20)$$

In the case of K_{aX}/K_{sX}, if we write down the formulae for two different values of α (α_1 and α_2), it is easily shown that $K_{aX}/K_{sX}(\alpha_1) = K_{aX}/K_{sX}(\alpha_2)$ when α_1 and α_2 are equal, or when $N_f U_f = N_m U_m$. This last condition is always approximately satisfied in our results, even when N_f is different from N_m, and when we use different values of ΔV_m and ΔV_f (an example is shown in Table 3.2. The small differences observed in the table occur because these are numerical approximations; the demonstration is given in Appendix 3.2).

This suggestion that the normalized rates of evolution are independent of α led us to focus on the effects of varying α on the non-normalized rates of evolution (K_{AA} and

K_{AX}). In agreement with what was previously found, the faster-*X* effect is enhanced in female-driven evolution scenarios ($\alpha < 1$) and somewhat counteracted in male-driven evolution scenarios ($\alpha > 1$, Figure 3.6). A similar pattern occurs for deleterious mutations (Figure 3.7).

Table 3.2: An example of the approximate equality of $N_f U_f$ and $N_m U_m$ for all values of N_m, N_f, ΔV_m and ΔV_f in our computations of the fixation probabilities of new mutations on the X chromosome,

When $N_{eA}s$=10, N_m=4000, N_f=6000, ΔV_m=ΔV_f=0				
h	***U_m***	***U_m*N_m***	***U_f***	***U_f*N_f***
0	1.02E-03	4.09	6.82E-04	4.09
0.1	1.17E-03	4.66	7.77E-04	4.66
0.2	1.31E-03	5.26	8.76E-04	5.26
0.3	1.47E-03	5.87	9.78E-04	5.87
0.4	1.62E-03	6.50	1.08E-03	6.50
0.5	1.78E-03	7.14	1.19E-03	7.14
0.6	1.95E-03	7.79	1.30E-03	7.79
0.7	2.11E-03	8.45	1.41E-03	8.45
0.8	2.28E-03	9.12	1.52E-03	9.12
0.9	2.45E-03	9.79	1.63E-03	9.79
1	2.62E-03	10.47	1.75E-03	10.47
When $N_{eA}s$=10, N_m=4000, N_f=6000, ΔV_m=1, ΔV_f=0				
h	***U_m***	***U_m*N_m***	***U_f***	***U_f*N_f***
0	1.05E-03	4.19	6.98E-04	4.19
0.1	1.19E-03	4.78	7.96E-04	4.78
0.2	1.35E-03	5.39	8.98E-04	5.39
0.3	1.50E-03	6.02	1.00E-03	6.02
0.4	1.67E-03	6.66	1.11E-03	6.66
0.5	1.83E-03	7.32	1.22E-03	7.32
0.6	2.00E-03	7.99	1.33E-03	7.99
0.7	2.17E-03	8.67	1.45E-03	8.67
0.8	2.34E-03	9.35	1.56E-03	9.36
0.9	2.51E-03	10.05	1.68E-03	10.05
1	2.69E-03	10.74	1.79E-03	10.75

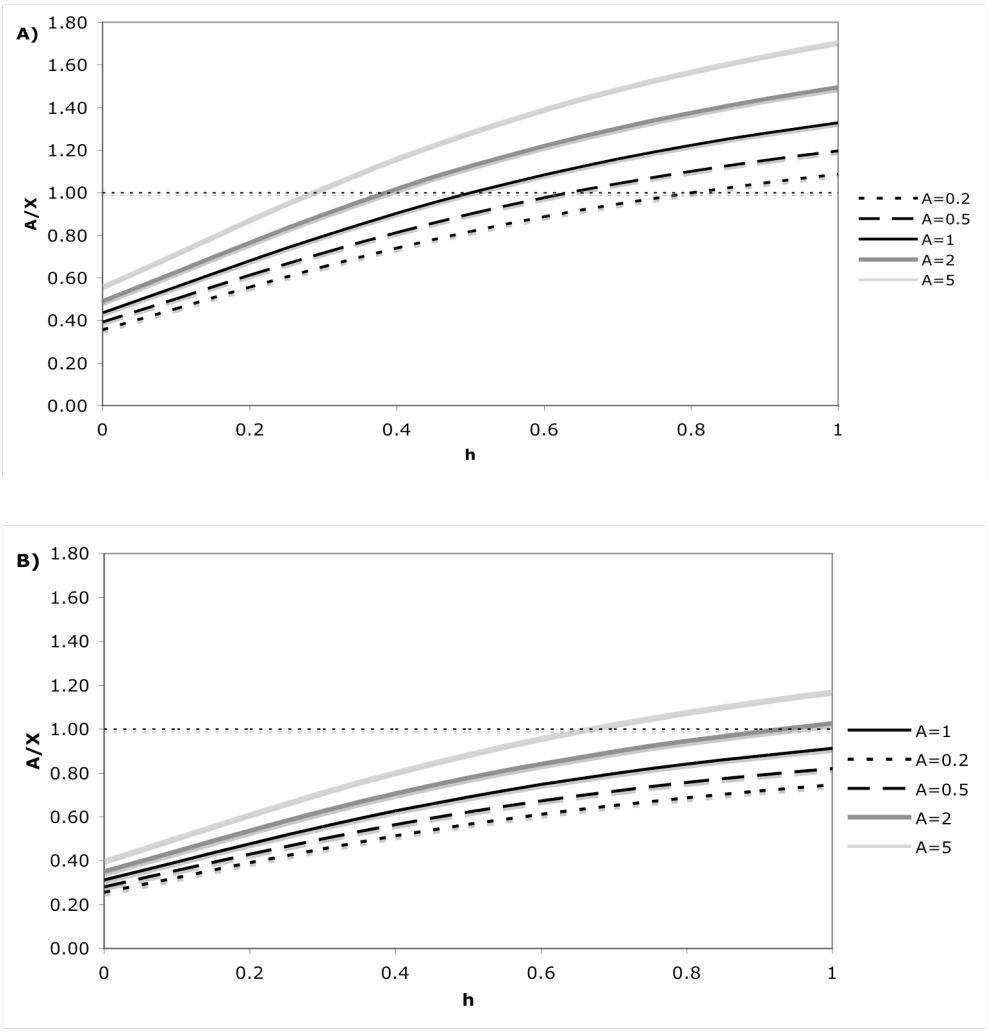
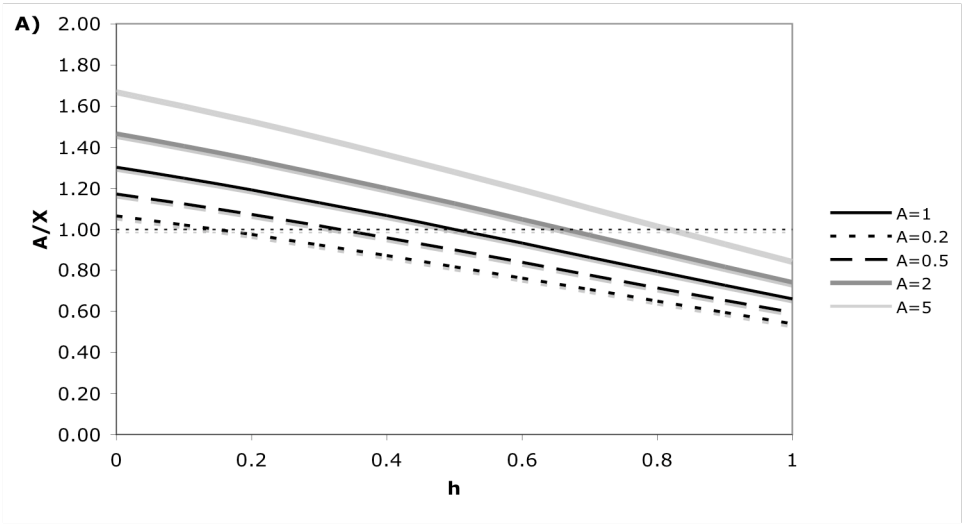


Figure 3.6: The (non-normalized) rate of fixation of beneficial mutations at *X*-linked and autosomal sites ($s_m=s_f$, $N_{eA}s_m=10$) when males and females have different mutation rates (in the figures, A=α). A) $N_{eX}={}^3\!/_4 N_{eA}$; B) $N_{eX}=1.08N_{eA}$.



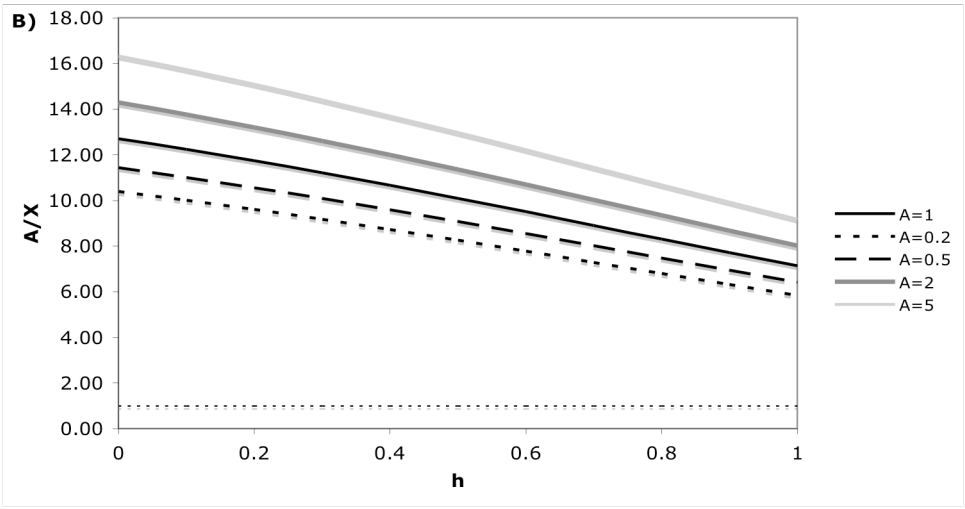


Figure 3.7: The (non-normalized) rate of fixation of deleterious mutations at *X*-linked and autosomal sites ($s_m=s_f$, $N_{eA}s_m=-3$) when males and females have different mutation rates (in the figures, A=α). A) $N_{eX}={}^{3}/_{4}N_{eA}$; B) $N_{eX}=1.08N_{eA}$.

3.3.4 The rate of fixation of sexually antagonistic mutations

We can use our program to compare the rate of accumulation of sexually antagonistic mutations at *X*-linked and autosomal loci, as these are of great interest when studying the evolution of the *X* chromosome (see discussion). Predictions concerning their rates of substitution at *X*-linked and autosomal loci were made first by Rice (1984), but using a complex model, that required the evolution of expression inhibitors in the harmed sex before the mutation could be fixed in the population. Our model is simpler (Table 3.1), as it makes no assumptions about the evolution of expression modifiers, but the results are similar (Figures 3.8 and 3.9): the *X* chromosome accumulates an excess of recessive mutations that are beneficial for males and dominant mutations that are beneficial for females, and autosomes accumulate an excess of dominant mutations favourable to males and recessive mutations favourable to females. This is true when the benefit to the favoured sex is equal to the fitness loss in the harmed sex, when the overall benefit is larger than the loss of fitness, but also when the loss of fitness is larger than the benefit (Figures 3.8 and 3.9).

By using a zero selection coefficient in one of the sexes, we can use our program to see how completely sex-specific genes are expected to evolve on the *X* and the autosomes. In the case of female-specific genes, the haploidy of the *X* chromosome in males is no longer a factor, and these genes are expected to evolve at the same rate as their autosomal counterparts (Figure 3.10). Male-specific genes, on the other hand, are subjected to an enhanced faster-*X* effect, consistent with Charlesworth *et al.* (1987).

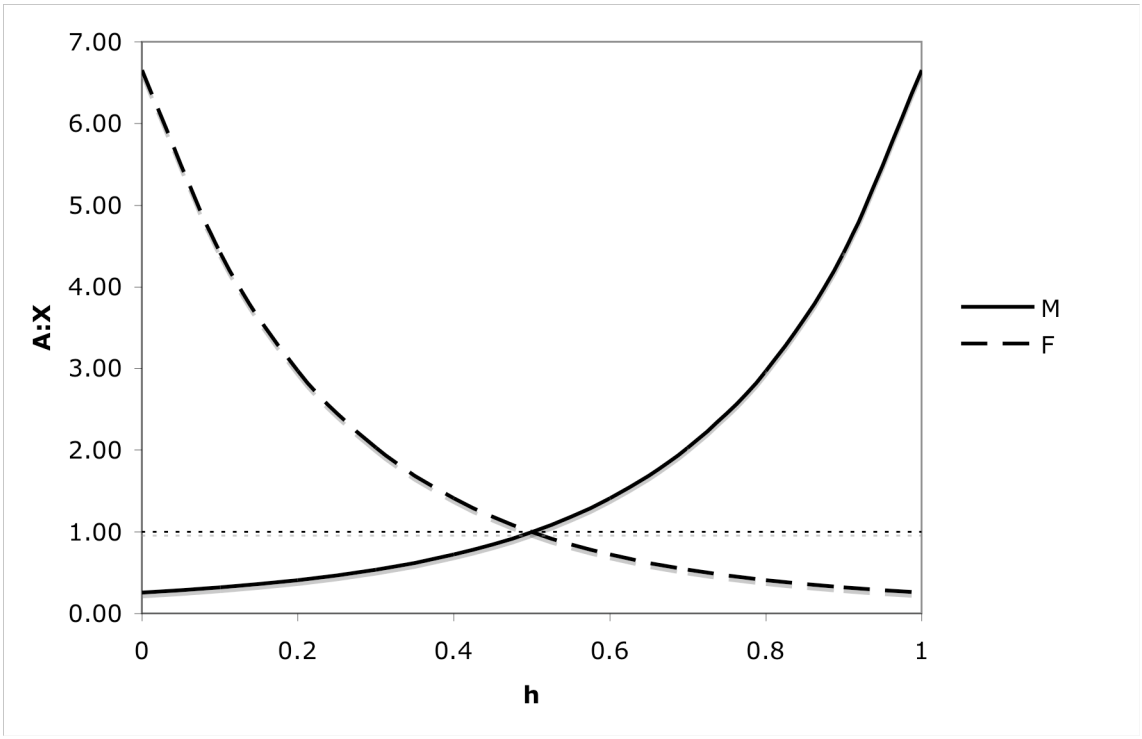
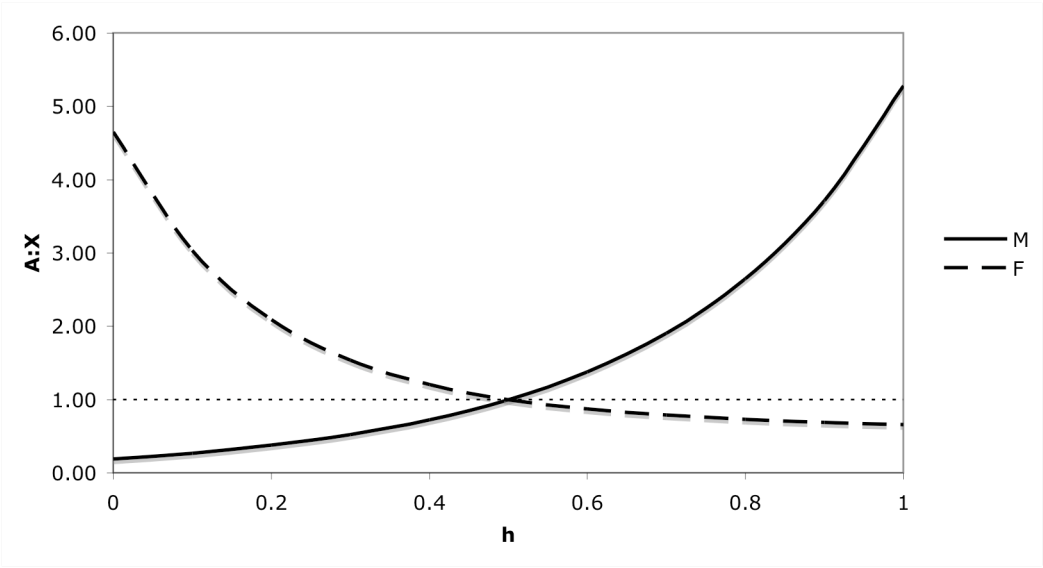


Figure 3.8: The accumulation of sexually antagonistic mutations, when the advantage to the beneficiary sex is equal to the disadvantage to the harmed sex (N_f=N_m=5000, ΔV_m=ΔV_f=0). The dashed line (F) represents the case where a mutation is beneficial to females (s_f=0.001) and detrimental to males (s_m=-0.001). The continuous line (M) shows the opposite scenario (s_f=-0.001, s_m=0.001).



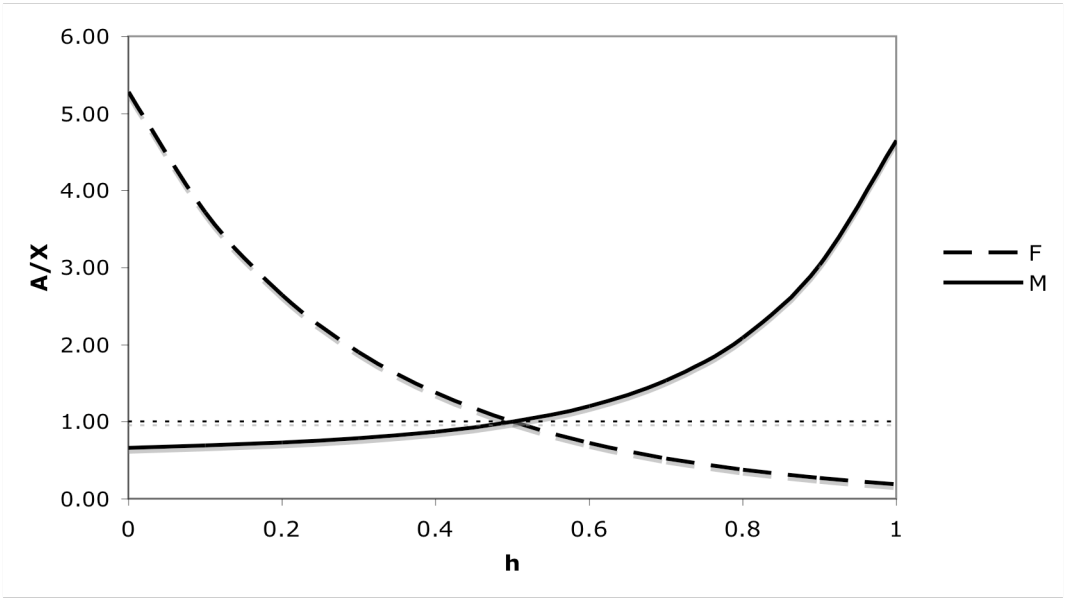


Figure 3.9: The accumulation of sexually antagonistic mutations, when **A)** the advantage to the beneficiary sex is stronger than the disadvantage to the harmed sex (The dashed line (F) represents the case where a mutation is beneficial to females (s_f=0.002) and detrimental to males (s_m=-0.001). The continuous line (M) represents the opposite scenario (s_f=-0.001, s_m=0.002)). **B)** the disadvantage to the harmed sex is stronger than the advantage to the beneficiary sex (F:s_f=0.001, s_m=-0.002; M:s_f=-0.002, s_m=0.001). In both A) and B), N_f=N_m=5000, and ΔV_m=ΔV_f=0.

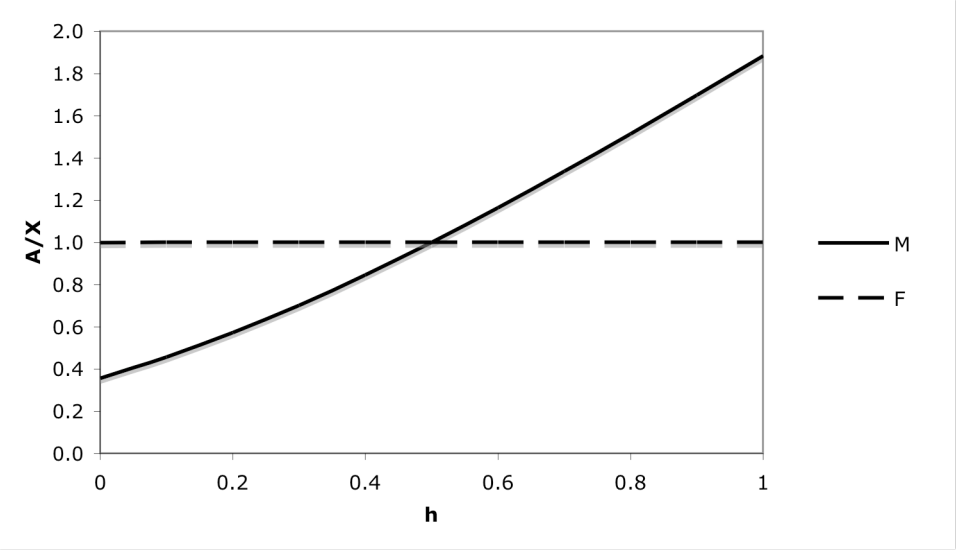


Figure 3.10: The accumulation of sexually antagonistic mutations, when the mutation is advantageous in one of the sexes and neutral in the other (F: s_f=0.001, s_m=0.0; M: s_f=0.0, s_m=0.001), as in the case of sex-specific genes (N_f=N_m=5000, ΔV_m=ΔV_f=0).

3.4 Discussion

3.4.1 The importance of estimating NeX/NeA

The discussion on faster-*X* evolution has crucial implications, as it can provide clues to essential parameters of evolution, such as the mean dominance level of new beneficial and deleterious mutations. However, it has often been reduced to a question of faster-*X*/recessivity of new beneficial mutations versus absence of faster-*X*/dominance of new beneficial mutations. Our results highlight the need for more quantitative analyses, as under different conditions of N_{eA}/N_{eX}, faster-*X* evolution can occur even if new mutations are on average dominant.

In African populations of Drosophila, non-coding polymorphism studies suggest similar population sizes for the *X* and the autosomes (Mousset and Derome, 2004), but this is rarely included in the discussion on faster-*X* evolution. It has been argued, for instance, that the overall dominance of new beneficial mutations could explain an absence of faster-*X* evolution. However, as figure 3.2 shows, this would not be a plausible explanation if Ne_X=Ne_A, and other factors must be involved, as, even when Ne_X=0.98Ne_A, there is a faster-*X* effect even when the mean dominance coefficient of new beneficial mutations is equal to 0.9. Figures 3.2 and 3.4 further show that the ratio N_{eX}/N_{eA} can have a stronger effect on faster-*X* evolution than the dominance coefficient, particularly in the case of the accumulation of deleterious mutations (Figure 3.4B).

Although our results emphasize the extent to which differences in the effective population size of the *X* chromosome can affect the faster-*X* effect, some of the values of ΔV_m that we have used to increase N_{eX}/N_{eA} seem too high to have any biological relevance in Drosophila, the organism that instigated this study. For instance, with equal

sex ratios, the Poisson expectation for variance in total offspring number for both females and males (V_m and V_m, respectively) is 2 (Laporte and Charlesworth, 2002). A ΔV_m value of 100, as we used for N_{eX}=1.1N_{eA}, would therefore require extremely strong sexual selection. It is more likely that several factors are contributing to the high levels of polymorphism at *X*-linked versus autosomal sites in African populations of Drosophila. As discussed in the previous chapter, the Drosophila *X* chromosome is subject to more recombination than the autosomes, since Drosophila males do not recombine, and the *X* chromosome spends less time in males than the autosomes (Connallon, 2007). Higher recombination on the *X* decreases interference amongst sites under selection and therefore increases its effective population size. Hutter *et al.* (2007) also argued that biased sex ratios in African populations of *D. melanogaster* are contributing to this phenomenon. Whilst the exact extent to which each of these factors contribute remains to be determined, this should not affect our results, as we used ΔV_m and ΔV_m as tools to manipulate the effective population sizes, and not just to study the effect of reproductive success per se.

3.4.2 Different mutation rates in males and females

The idea that different mutation rates in males and females could influence the evolutionary rates at *X*-linked and autosomal sites was examined by Miyata *et al.* (1987), who pointed out that male-driven evolution would lead to decreased rates of neutral evolution at *X*-linked sites. Kirkpatrick and Hall (2004) extended this analysis to sites under positive selection and noted that the faster rate of beneficial evolution expected on the *X* chromosome could be somewhat counteracted by this male-driven evolution. We

have used our numerical estimations to confirm this, and have applied the same approach to deleterious mutations, which are also expected to accumulate at lower rates on the *X* when evolution is male-driven than they would otherwise. Our results concerning K_{aA} and K_{aX} are consistent with those expectations. An important point that came out of our analysis, however, is that none of these patterns should affect K_a/K_s analyses, as, once the neutral rate of evolution is taken into account, the A/X ratio is independent of α. This occurs for all the scenarios tested (different numbers of males and females, and different variances of reproductive success). This result is of great interest, as different mutation rates in males and females have been put forward as an alternative to differences in selection efficiency to account for differences in K_a/K_s between the *X* and the autosomes. Differences in male and female mutation rates remain useful for non-synonymous rates of evolution, and to account for biological phenomena such as the large-*X* effect (Dobzhansky, 1936, Tao *et al.*, 2003), but care should be taken when discussing *X*-chromosome molecular evolution, as this is often done in terms of K_a/K_s.

3.4.3 Sex-biased genes and faster-X evolution

Although our analysis has focused on beneficial or deleterious mutations, mutations that have a beneficial effect in one sex but are deleterious for the other sex (antagonistic mutations) are also of interest when considering the evolution of the *X* chromosome. Rice (1984) modelled the accumulation of such mutations on the *X* chromosome and the autosomes, and found that, because recessive or partially recessive mutations are selected when in males, antagonistic mutations that are beneficial for

males are expected to accumulate faster on the *X* than on the autosomes when they are recessive or partially recessive, but faster on the autosomes when they are dominant. The opposite pattern is expected for antagonistic mutations favourable to females (Rice, 1984).

In Rice's model (1984), a modifier of expression, which represses the gene expression in the harmed sex, is required for the mutation to be fixed in the population, leading to the creation of a sex-biased gene (a gene expressed mainly in one sex). Consequently, sex-biased genes are expected to accumulate at different rates on the *X* chromosome (Rice, 1984). This has been consistently observed in Drosophila, mammals and nematode (reviewed in Parsch and Ellegren, 2007). However, the biological soundness of Rice's (1984) model has been brought into question, as sex-biased genes often reflect an increase of expression in the beneficiary sex and not a decrease in the harmed sex (Connallon and Lacey-Knowles, 2005, and see next chapter). It is therefore of interest to investigate whether the same results are expected with our more straightforward model of the accumulation of genes with opposite fitness effects in males and females. Figures 3.8 and 3.9 show that a complex model involving expression modifiers for the fixation of sexually antagonistic mutations is not necessary to explain the different rates of accumulation of such mutations (and consequently of sex-biased genes) at *X*-linked and autosomal sites.

The accumulation of sexually antagonistic mutations and sex-biased genes influences the molecular evolution of the *X* in two ways. If there are enough antagonistic mutations, and they accumulate faster on the *X*, this can enhance a faster-*X* effect. Inversely, if they accumulate more slowly on the *X*, they can counteract the faster-*X*

effect. Which of these actually occurs still remains to be determined, as no information is yet available about the levels of dominance of antagonistic mutations.

Sex-biased genes have also been shown to have different rates of non-synonymous evolution. Male-biased genes, in particular, consistently show increased K_a/K_s compared to unbiased and female-biased genes (Parsch and Ellegren, 2007). Furthermore, as Charlesworth *et al.* (1987) pointed out and as shown in Figure 3.10, male-specific genes also experience an enhanced faster-*X* effect. The proportion of such male-biased and male-specific genes found on the *X* and the autosomes is therefore likely to influence the overall rates of evolution on the *X* and the autosomes. In Drosophila, for instance, the paucity of male-biased genes on the *X* (Parisi *et al*., 2004) could be one factor that makes it harder to detect faster-*X* evolution in this organism (Betancourt *et al.*, 2002). Female-specific genes, on the other hand, do not experience faster-*X* evolution at all (Figure 3.10), and could perhaps provide an interesting control group for future studies on faster-*X* evolution.

3.5 Conclusions

The molecular evolution of the *X* chromosomes and the autosomes has been the subject of much theoretical and empirical research, but the results are often inconsistent. One of the reasons for this is that many factors can influence the rates of evolution of these chromosomes, including their effective population size, their rate of mutation, and the proportion of mutations fixed by positive selection and drift, and these are hard to estimate and analyse together. We have written a program that evaluates the probability of fixation of new mutations, both beneficial and deleterious, as well as the rate of

substitution of such mutations, but allowing for different values of selection, effective population sizes, and mutation rates in males and females. Our results emphasize the need to consider all these parameters, and in particular the ones affecting N_{eX}/N_{eA}, as very different results are expected when there is a large deviation from $N_{eX}/N_{eA}=3/4$.

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Chapter 4: Sex-biased gene expression in Drosophila

Abstract

Sex-biased genes are of great interest, as they are thought to encode most of the differences between sexes in morphology, behaviour and metabolism. The evolution of sex-biased expression was first modelled by Rice (1984), who suggested it resulted from the repression of expression of genes that are carrying sexually antagonistic mutations, in the harmed sex. This should lead sex-biased genes to have, overall, lower levels of expression than unbiased genes.

We have created expression profiles for sex-biased genes, using EST data as a proxy for expression level, in order to compare them with expression profiles of unbiased genes. Our results suggest that the evolution of sex-biased expression occurs primarily through an increase of expression in the beneficiary sex, unlike what was predicted by Rice (1984). Furthermore, the expression profiles of female- and male-biased genes are widely different, and provide us with some clues as to why male- and female-biased genes have very different rates of evolution, a pattern that is consistently observed in Drosophila.

4.1 Introduction

4.1.1 Sex-Biased genes

In species with separate sexes, males and females often have very different morphology, behaviour and physiology. A lot of work has been put into understanding how these between-sexes differences arose, as many characteristics that are advantageous to one of the sexes are disadvantageous to the other (Rice, 1984; Parsch and Ellegren, 2007). Obvious examples include the outrageous tail of the peacock, which makes males an easy target for females but also for predators, or the gigantic horns of the bighorn sheep, which are a lot less effective against predators than the shorter, female version, but more efficient in male head to head fights. Whilst these are extreme cases, it has been suggested that "sexual antagonism" is actually a common feature, and that most metabolic and physical traits have different optimal values for males and females (Glucksman, 1981).

Rice (1984) modelled the evolution of mutations that provide an advantage to one of the sexes but are deleterious to the other. He noted that, for the autosomal case, a sexually antagonistic mutation is predicted to increase in frequency when the advantage it provides to one sex is larger than the harm to the other. As the frequency increases, it becomes beneficial for the harmed sex to develop a modifier that decreases the now deleterious expression of the gene. Once this has occurred, the mutation is expected to become unconditionally advantageous and will be fixed in the population, with the gene predominantly expressed in one of the sexes (it is now a "sex-biased" gene). Predictions for the X chromosome are described in section 4.1.2.

Microarray and EST technologies have made it possible to study the expression level of genes in different organisms or tissues. Several groups have taken advantage of these approaches to determine which genes have a sex-biased expression, by comparing their expression in males and females (these can be whole-body comparisons, or comparisons of specific tissues in both sexes) (Reinke *et al*., 2004; Wang *et al*., 2001; Parisi *et al*., 2003; Lercher *et al.*, 2003; Meiklejohn *et al*., 2003; Zhang *et al*., 2004; Kaiser and Ellegren, 2006). These studies have shown that a large proportion of the genome of several organisms is indeed sex-biased in its expression, and that these sex-biased genes differ from other genes in their rates of evolution and genomic distribution (reviewed in Parsch and Ellegren, 2007). Most of the discussion of these results has been based on Rice's 1984 model. However, the relevance of this model has not been clearly assessed.

4.1.2 Predictions of Rice's model

Rice's model makes two predictions that can be easily tested. First, he showed that, under certain conditions, the *X* chromosome is expected to accumulate more sex-biased genes than the autosomes. Dominant mutations that are beneficial for females, but deleterious for males, accumulate there simply because the *X* spends more time in females (where they are beneficial) than in males (where they are deleterious), whereas autosomes spend the same amount of time in each (Rice, 1984). On the other hand, recessive *X*-linked mutations that are beneficial for males, but deleterious for females, are masked in heterozygous females but have an immediate beneficial effect on males, whereas autosomal mutations only start having a beneficial effect on males when they

appear in homozygous individuals, and therefore have a deleterious effect in females. In Rice's model, in both cases, a modifier that decreases the expression of the gene in the harmed sex is required for the mutation to become fixed in the population. This would, in principle, lead to an accumulation of sex-biased genes on the *X* chromosome. The predictions concerning sex-biased genes are summarized in Table 4.1.

Table 4.1: Rice's (1984) predictions of the distribution of sex-biased genes on the X and the autosomes, depending on the dominance coefficient of the initial antagonistic mutation.

	Dominant favourable mutations	**Recessive favourable mutations**
Male-biased genes	Deficit on the X	Excess on the X
Female-biased genes	Excess on the X	Deficit on the X

Whilst the distribution of sex-biased genes in the genome is not random, and the *X* in particular often shows a deficit or excess of sex-biased genes, the results for different species are often inconsistent. In *Drosophila melanogaster* and *Caenorhabditis elegans*, there seems to be a deficit of male-biased genes on the X, whereas in mammals an excess of male-biased genes is observed (Parisi *et al*., 2003, Lercher *et al*., 2003, Khil *et al.*, 2004, Reinke *et al*., 2004). Most of the discussion of these discrepancies has focused on dominance effects, even though there seems to be no clear reason to expect a systematic difference in dominance coefficients between different species (Kaiser and

Ellegren, 2006). Another possibility is that Rice's model (1984) is in fact an unrealistic representation of the evolution of most sex-biased genes. A better understanding of the actual mechanistic evolution of sex-biased genes is needed to assess how useful this model really is (Vicoso and Charlesworth, 2006).

The second prediction concerns the overall level of gene expression: if sex-biased genes evolve through the appearance of a modifier that inhibits their expression in the harmed sex, they should, on average, have lower levels of expression than non-biased genes. One study has addressed this issue; Connallon and Lacey-Knowles (2005) pointed out that whilst Rice's model has often been quoted and antagonism used as the explanation for the distribution of sex-biased genes, no one had tested it directly. They attempted to do this, using published Drosophila microarray data. One of their findings was that sex-biased genes are, on average, more highly expressed than non-biased genes. This seems to be true even for genes that are sex-biased in *D. melanogaster* but not in *D. simulans*, suggesting that new sex biased genes arise through an increase of expression in the favoured gene, and not, as Rice (1984) predicted, by a decrease in the disfavoured gene. Taken together, these studies suggest that Rice's model (1984) may not be sufficient to explain the overall evolution and distribution of sex-biased genes, and that experimental studies are needed to ensure that the models that are used are relevant to the discussion.

4.1.3 The rates of evolution of male- and female-biased genes

Whilst the differential accumulation of sex-biased genes in the genome can sometimes be accounted for by Rice's model, the unusual rates of evolution observed for

sex-biased genes still need explaining: rates of molecular evolution are particularly high for male-biased genes but particularly low for female-biased genes compared to non-biased genes (Meiklejohn *et al*., 2003; reviewed in Ellegren and Parsch, 2007). The general view is that males are subjected to more positive – often sexual – selection, leading male-biased genes to evolve faster and have high rates of turnover between close species (Zhang *et al*., 2007). Although male- and female-biased genes could be expected to be involved in an arms race, and therefore evolve similarly fast, female-biased genes tend to be more conserved between species and show, overall, lower rates of evolution, even than non-biased genes (Zhang *et al*., 2007). One suggestion has been that female-biased genes are involved in developmental processes, as knocking down female-fertility genes has been shown to often be lethal, which could lead to these genes being strongly conserved (Perrimon *et al.*, 1986; Davis *et al*., 2005).

Although these explanations are intuitively appealing, the evidence supporting them is mostly anecdotal, and more systematic testing is required. Other factors could be influencing these rates of evolution, such as the level, breadth and developmental time of expression, which are all correlated with rates of evolution (Lemos *et al*., 2005). There is some indirect evidence concerning these. Connallon and Lacey-Knowles (2005) found that sex-biased genes had higher levels of expression than non-biased genes, and that this pattern was stronger for male-biased genes. This suggests that differences in expression levels are not the cause of faster evolution of male-biased genes, since high levels of expression are usually accompanied by slow rates of evolution. The breadth of expression of sex-biased genes has been assessed, using microarray comparisons of different male and female tissues, to determine sex-biased gene expression in different

tissues (Parisi *et al.*, 2004). The results suggest that female-biased genes are sex-biased in a wider range of tissues than male-biased genes.

A more straightforward approach to better understanding the relation between the evolution of sex-biased genes and their expression patterns is to select genes that have been classified as sex-biased, and compare their overall expression levels in different tissues with the expression levels of non-biased genes. We have done this, using the mean number of ESTs in an EST database as a proxy for expression levels of genes that have been classified as male-, female- or non-biased in microarray studies of sex-biased genes, to examine tissue-specificity of sex-biased genes and their overall levels of expression. EST datasets provide a simple method to approximate expression levels as, unlike microarray datasets, they do not depend on the number of genes included in the microarray chip, and can therefore be used for any list of genes (see for instance Duret and Mouchiroud, 1999).

4.1.4 Goals of this study

-Creating expression profiles for sex-biased and non-biased genes, to further extend our understanding of what the biological definition of a sex-biased gene is.
-Investigating which steps lead to the creation of sex-biased genes, by comparing the expression profiles of genes that have conserved sex-biases in *D. melanogaster* and its sister species *D. simulans*, and genes that are sex-biased only in *D. melanogaster* (as this last category should be enriched for newly sex-biased genes).

-Examine the differences between male- and female-biased genes in their evolution and current expression patterns, and discuss how this relates to our current knowledge of the evolution of sex-biased genes.

The results reported below suggest that male- and female-biased genes evolve differently and have different expression profiles. Male-biased genes are primarily expressed in the testis, at very high levels, and their expression appears to be reduced in other tissues. The first step in their evolution seems to be a large increase in expression in the testis. The germline appears to have a smaller importance in the evolution of female-biased genes, which are over-expressed in several tissues (although the biggest increase is in the ovary). Furthermore, there seems to be an association between female-biased and embryo-expressed genes.

4.2 Materials and methods

4.2.1 Datasets used

4.2.1.1 Microarray data

A dataset of *Drosophila melanogaster* male-, female- and non-biased genes was downloaded from genes included in the SEx-BIas DAtabase (SEBIDA, http://sebida.de), a repository of microarray data from several studies of sex-biased genes in Drosophila. For this dataset, genes were classified as sex-biased when they had a two-fold excess of expression in one sex. The sex-ratio of expression values for *D. simulans* were also downloaded.

4.2.1.2 EST data

The *D. melanogaster* EST dataset was downloaded from the NCBI UNIGENE database (http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=&db=unigene).

4.2.2 Analysis

4.2.2.1 Cleaning the EST database

The downloaded dataset consists of a list of genes, a list of ESTs associated with each gene and the EST library they come from. A Perl script extracted, for each gene, the gene name, and counted how many ESTs from each tissue library were associated with them. This resulted in a large table containing a list of genes and their expression profile (all Perl scripts used are described in Appendix A4.1).

4.2.2.2 Matching the sex-biased genes and their expression profile

For each gene in the male-, female- and non-biased gene lists, a second Perl script was used to search the EST dataset and extract the expression profile for them. This resulted in 3 groups of expression profiles; male-, female- and non-biased genes, which were then compared.

4.2.2.3 Two-species analysis

The second Perl script was run again, after separating the sex-biased genes into conserved genes (when they were also sex-biased in *D. simulans,* as described on the SEBIDA website) and non-conserved genes (when their sex-bias was specific to *D. melanogaster*), in order to create expression profiles for conserved and non-conserved male-biased and female-biased genes. The non-conserved group is likely to be enriched for newly evolved sex-biased genes, and should therefore give us some information about the early steps of sex-bias evolution.

4.2.3 Statistical analysis

The significance of the differences in expression of male- and female-biased genes compared to the non-biased genes was assessed using two-tailed unpaired t-tests, with *p*-values adjusted for multiple comparisons with the Dunn-Sidak method (Ury, 1976). Since the number of genes in the sample was very large (682 female-biased genes, 636 male-biased genes and 3501 non-biased genes), we used parametric tests.

4.3 Results

4.3.1 The importance of the germline

Parsch and Ellegren (2007) claimed that most of the sex-biased differences could be accounted for by differences in expression in reproductive tissues (Parisi *et al*., 2003, Parsch and Ellegren, 2007). This is hard to reconcile with Parisi *et al*.'s (2004) finding that microarray comparisons using the ovary resulted in a lower fraction of the genome being detected as sex-biased than comparisons using female soma.

Two types of microarray comparisons were used in Parisi *et al*.'s (2003) study on *Drosophila melanogaster* sex-biased genes: ovary versus testis, and whole-female versus whole-male comparisons. By combining these two types of datasets, the importance of the germline for sex-biased genes can be evaluated. Simply comparing the "sex-ratio of expression" (the male to female ratio, in the case of male-biased genes, or female to male ratio, for female-biased genes) for whole-body comparison versus germline comparisons (Figure 4.1) shows a clear difference between female- and male-biased genes: male-biased genes are more sex-biased for germline

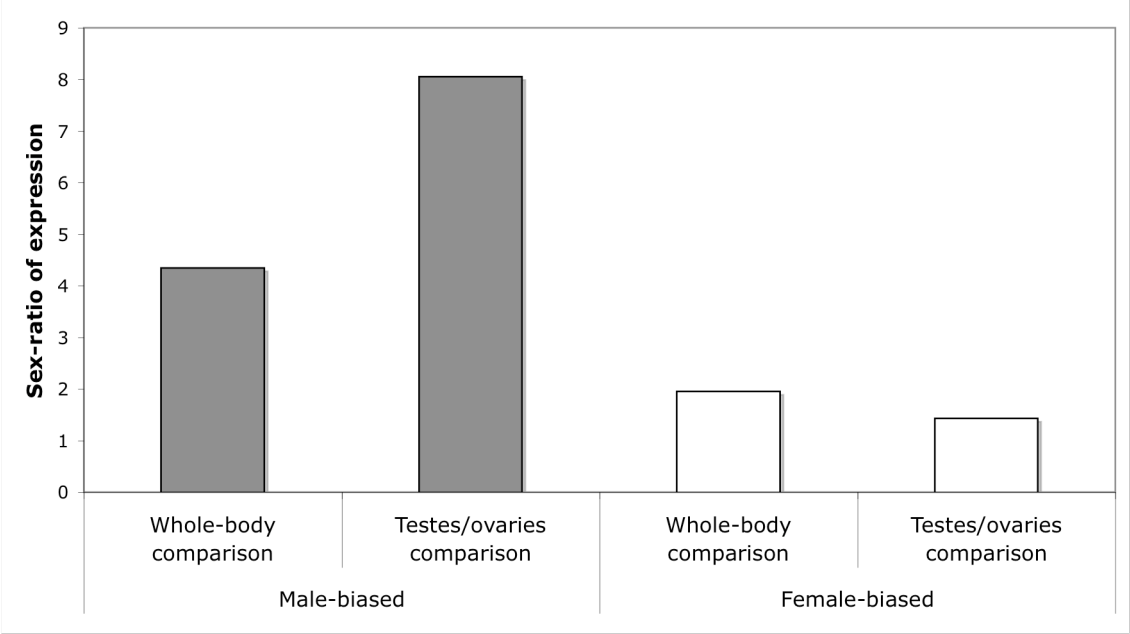

Figure 4.1: The male-bias is stronger in the germline, whereas the female-bias is stronger in whole-body comparisons. The sex-ratio of expression corresponds to the mean male to female expression ratio, for male-biased genes, and mean female to male expression ratio, for female-biased genesin the Parisi et al. (2004) whole-body and ovary-testis comparisons, as given in the Sebida website.

comparisons, whereas such an effect is not observed in female-biased genes (if anything, the bias seems stronger for whole-body comparisons, in agreement with Parisi *et al.* (2004)). This is a first suggestion that a single model to explain the evolution and distribution of all sex-biased genes might not be adequate, as female-biased genes seem to be more systemic whereas male-biased genes are expressed mostly in germline. They therefore may have different sex-specific functions and be under different selective pressures.

4.3.2 Expression profiles of sex-biased genes

Table 4.2 shows the mean number of ESTs from each tissue, for the three classes of genes (seqcount is the total number of sequences found in UNIGENE, and should be representative of overall expression levels). The first thing to note is that different tissues have a different representation in the dataset, and that the results for some tissues are probably not very reliable (whole larva, for instance, is only represented by one small library). However, it is interesting to note that overall, female-biased genes have higher mean expression levels than non-biased genes, but male-biased genes show the opposite pattern. To further analyse in which tissues sex-biased genes are over- and under-expressed, the mean EST counts for female- and male-biased genes, for each tissue, can be divided by the corresponding non-biased value, so that the difference between the sex-biased genes and the non-biased genes is observed independently of the library size. This is represented in Figure 4.2.

The most noticeable pattern is the very large increase in testis expression for male-biased genes. Female-biased genes show a more modest increase of ovary expression, in

Table 4.2: Mean EST count per gene for non-biased and sex-biased genes. Seqcount is the total number of sequences found in Unigene

	Non-biased genes	**(SD)**	**Male-biased genes**	**(SD)**	**Female-biased genes**	**(SD)**
Seqcount	45.20	(60.77)	28.57	(52.51)	61.00	(126.50)
Ovary	0.79	(2.84)	0.15	(0.63)	2.38	(6.54)
Testis	1.57	(5.09)	11.89	(23.50)	1.14	(3.45)
Brain	0.01	(0.08)	0.00	(0.04)	0.00	(0.05)
Head	6.07	(17.13)	4.37	(32.56)	7.82	(47.09)
Whole embryo	9.67	(21.97)	1.76	(6.51)	20.63	(40.75)
Whole larva	0.00	(0.03)	0.00	(0.00)	0.00	(0.00)
Whole adult	0.44	(4.40)	0.42	(2.57)	0.71	(11.36)
Gonad	0.31	(1.86)	0.05	(0.27)	0.70	(4.98)
Fat body	0.52	(3.83)	0.45	(2.38)	0.35	(1.71)
Blood	0.48	(1.89)	0.08	(0.58)	0.61	(2.14)
Salivary gland	0.31	(1.89)	0.11	(0.89)	0.16	(0.82)
Other	21.67	(27.29)	6.61	(13.78)	23.31	(37.95)

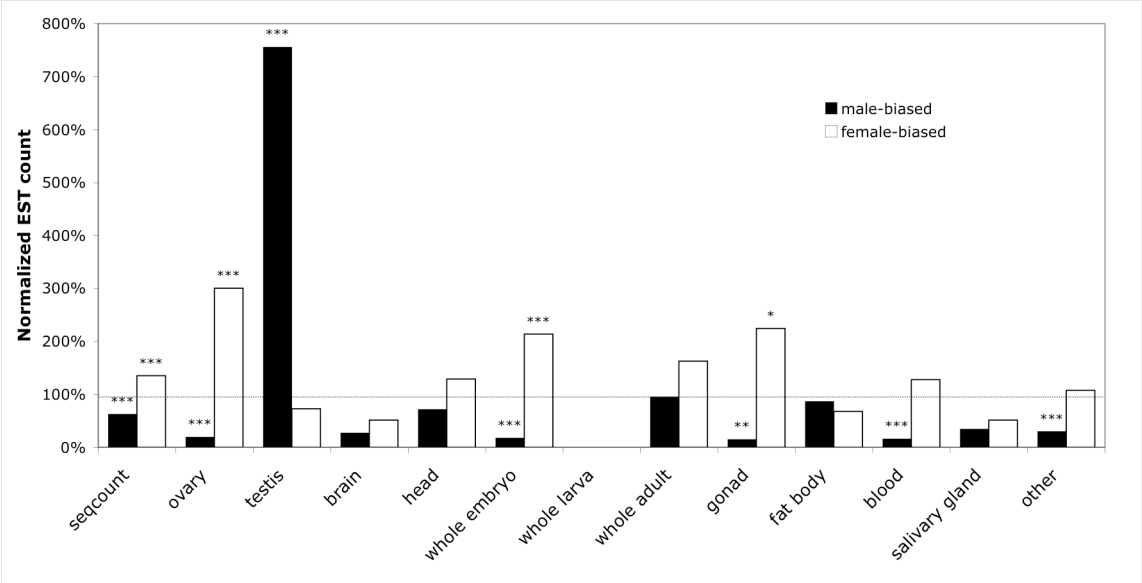

Figure 4.2: Normalized expression levels of sex-biased genes in different tissues. The normalized values were obtained by dividing the mean number of ESTs per sex-biased gene found in each tissue by the corresponding value for non-biased genes, so that we are now observing, for each tissue, the over- (more than 100%) or under- (under 100%) expression of sex-biased genes compared to the non-biased genes. Levels of significance using unpaired two-tailed t-tests, after applying a Dunn-Sidak correction for multiple comparisons, are represented by asterisks (* < 0.05, ** < 0.01, *** < 0.001).

agreement with the microarray data. As far as the other tissues go, male-biased genes are under-expressed in all of them, whereas female-biased genes tend to be over-expressed (they are never significantly under-expressed). This again suggests that male- and female-biased genes are different in their behaviour and may require different models to explain their evolution and distribution. Another interesting result is that the libraries for which female-biased genes show a significant increase in EST count (apart from ovary) are all embryonic. Possible explanations for this pattern are put forward in the discussion.

4.3.3 Conserved versus non-conserved sex-biased genes

These profiles show the current expression of sex-biased genes, whereas Rice's model is concerned with their early evolution. It is possible, for instance, that female-biased genes arise by a decrease of expression in males, later followed by an increase in females. It is therefore of interest to analyse newly evolved sex-biased genes. By analysing the genes that are only sex-biased in *D. melanogaster* but not *D. simulans*, it is possible to obtain a sample that is enriched for genes with newly evolved sex-biased expression.

By considering only the genes that are in all three datasets, the sex-biased genes can be separated into conserved, if they are also sex-biased in *D. simulans*, and non-conserved categories. The analysis can then be repeated. Table 3 shows the mean EST counts for sex-biased and non-biased genes. Since the non-conserved category is likely to be enriched for genes that have recently acquired their sex-bias in *D. melanogaster*, they can give us useful information about the initial steps of evolution of sex-biased

Table 4.3: Mean EST count per gene for conserved and non-conserved non-biased and sex-biased genes. Seqcount is the total number of sequences found in Unigene.

	Non-biased	Conserved male-biased	Non-conserved male-biased	Conserved female-biased	Non-conserved female-biased
Seqcount	45.20	41.93	60.75	64.79	47.21
Ovary	0.79	0.16	0.70	2.81	1.27
Testis	1.57	19.06	11.11	1.11	0.98
Brain	0.01	0.00	0.00	0.00	0.01
Head	6.07	8.17	8.02	8.29	3.75
Whole embryo	9.67	2.29	10.30	22.72	15.76
Whole larva	0.00	0.00	0.00	0.00	0.00
Whole adult	0.44	0.34	2.39	1.21	0.21
Gonad	0.31	0.05	0.09	0.45	0.52
Fat body	0.52	0.50	1.80	0.40	0.22
Blood	0.48	0.09	0.45	0.68	0.40
Salivary gland	0.31	0.09	0.86	0.17	0.14
Other	21.67	8.29	21.16	23.68	20.70

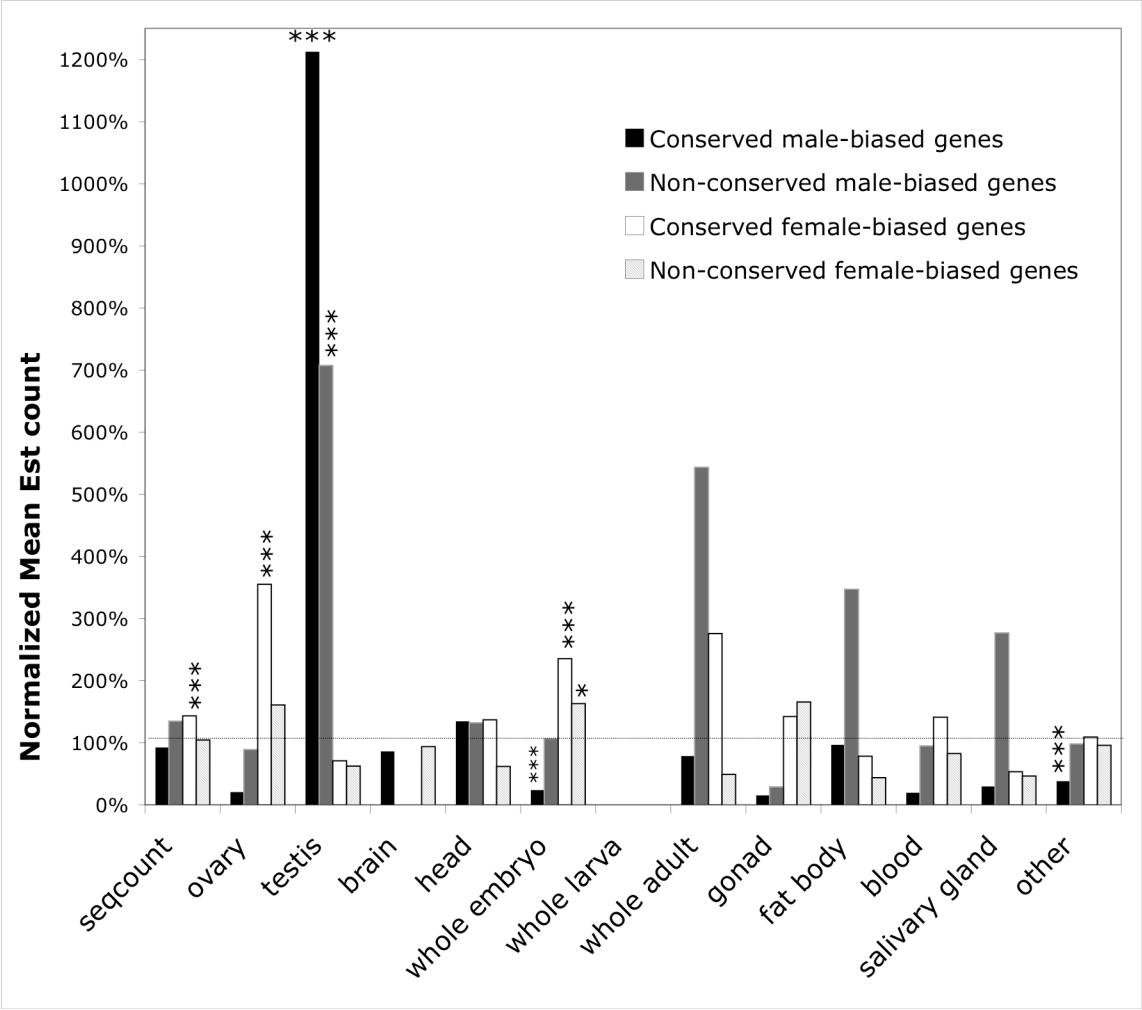

Figure 4.3: Normalized expression levels of conserved and non-conserved sex-biased genes in different tissues. The normalized values were obtained by dividing the mean number of ESTs per sex-biased gene found in each tissue by the corresponding value for non-biased genes. Levels of significance using unpaired two-tailed t-tests, after applying a Dunn-Sidak correction for multiple comparisons, are represented by asterisks (* <0.05, **<0.01, ***<0.001).

genes. The male-biased case is particularly interesting, as non-conserved genes, unlike the conserved genes, have a higher level of expression than non-biased genes. This is in agreement with the findings of Connallon and Lacey-Knowles (2005), that the initial evolution of male-biased genes involves an increase of expression. Female-biased genes always have higher levels of expression than non-biased genes, but the difference is stronger for the conserved genes.

It is again of interest to determine in which tissues these changes in expression occur (Figure 4.3). For the male non-conserved genes, the only significant difference is a strong increase in testis expression. The repression of expression in other tissues is not consistently observed (even expression in the ovary is still at 88% of the expression level of non-biased genes in the ovary), which may suggest that this only occurs at a later stage of male-biased gene evolution, and would explain why their overall level of expression is higher than that of the non-biased genes. For female-biased genes, the non-conserved group shows an increased level of expression in the ovary and in embryonic tissues, but the increase in expression levels is not as large as for the conserved female-biased genes. The consistency of the association between female-biased genes and embryonic tissues suggests this is not a random event (but a bias in the libraries cannot be excluded).

4.4 Discussion

4.4.1 The expression of sex-biased genes

Connallon and Lacey-Knowles (2005) had hinted at the inadequacy of Rice's model to fully account for the evolution and distribution of sex-biased genes. The results

reported here differed from theirs, as male-biased genes had, overall, a lower level of expression than non-biased genes. Their expression was, in fact, repressed in all tissues except the testis, where it was greatly increased. Female-biased genes, on the other hand, have a higher level of expression in most tissues analysed, and this is reflected in the higher mean expression level of female-biased genes.

It is interesting to note that in *D. melanogaster* the rates of non-synonymous divergence are negatively correlated with the level and the breadth of expression (Lemos *et al*., 2005). Since the results show that female-biased genes are expressed at higher levels in several tissues than non-biased genes (resulting in a higher overall expression level), but male-biased genes are expressed at lower levels in most tissues, low rates of divergence for female-biased genes and high rates in male-biased genes are expected. Low rates of evolution have indeed been consistently detected in *Drosophila melanogaster* for female-biased genes, contrasting with the high rates detected for male-biased genes (Zhang *et al*., 2004, Proeschel *et al.*, 2006).

4.4.2 The evolution of sex-biased genes

Although male-biased genes have, overall, a lower level of expression than non-biased genes (represented by seqcount in Figure 4.2 and 4.3), the opposite is true when only non-conserved male-biased genes are considered. In fact, unlike what has mostly been previously assumed, our results suggest that the initial step in the evolution of both male- and female-biased genes appears to be an increase of expression, possibly in the beneficiary sex, and not a decrease in the disfavoured sex, in agreement with Connallon and Lacey-Knowles (2005). Our results further suggest that in the case of male-biased

genes, this increase in expression is mostly limited to the testis, and later followed by the repression of expression in most other tissues. Female-biased genes, on the other hand, arise by a consistent increase of expression not only in the ovary, but in embryonic tissues as well.

How can this be reconciled with the genomic distribution results, which often are in agreement with Rice's (1984) model, at least if sexually antagonistic mutations are assumed to be dominant? It has been pointed out that similar predictions to those in Table 3.1 can be made, without invoking a modifier of expression, for the accumulation of antagonistic mutations (Vicoso and Charlesworth, 2006; Chapter 3). Once a sexually antagonistic allele is fixed, it becomes advantageous for the beneficial sex to increase its expression and/or for the harmed sex to decrease it, so that this is likely to be followed by the accumulation of mutations in the regulatory region of the gene, which lead to the sex-biased patterns of expression. Although the reasoning is similar to that of Rice's (1984) model, in this case the fixation of the antagonistic mutation precedes the evolution of sex-biased expression, and does not require inhibition of the expression in the harmed sex to cause a differential accumulation of sex-biased genes on the *X* chromosome.

4.4.3 An association between female- and embryo-expressed genes

In this dataset, the tissues where the biggest increase in expression of female-biased genes is observed (excluding the ovary) are the embryonic tissues. An experimental bias could account for this, if the embryonic libraries were made from a

pool of flies containing more females than males. It is, however, unclear why this should be the case for all the embryonic libraries.

A more likely alternative is that a large proportion of the female-biased genes are in fact "maternal genes", that is, genes whose mRNAs are expressed in the egg and used in the development of the embryo. It has been suggested that genes required in early development tend to be strongly conserved, and such an effect has been observed in *D. melanogaster* (Davis *et al*., 2005). In the same study, the authors postulate that maternal genes should be under similar selective pressures as embryonic genes, and evolve at similarly low rates, and they did indeed find maternal genes to have slow rates of evolution (Davis *et al*., 2005). This would further explain why a large proportion of *D. melanogaster* female-biased genes have been found to be under stronger negative selection and evolve slower than not only male-biased genes, but also non-biased genes (Zhang *et al*., 2004, Pröschel *et al*., 2006).

4.5 Conclusions

The analysis of EST and microarray data can further improve our knowledge of the evolution of sex-biased genes in *D. melanogaster*. The results presented here suggest that the evolution of a non-biased gene into a sex-biased gene occurs primarily through an increase of expression in the beneficiary sex. This goes against the predictions made by Rice (1984), and the relevance of this model to the overall evolution of sex-biased genes needs to be assessed carefully.

The expression pattern of female- and male-biased genes is widely different. Male-biased genes are strongly over-expressed in the testis, but under-expressed in all

other tissues. Female-biased genes, on the other hand, are over-expressed in a wide array of tissues. Although this effect is stronger in the ovary, embryonic tissues also show an enrichment of female-biased genes, possibly because many female-biased genes are maternal genes. These differences in the expression profiles of male- and female-biased genes also shed some light into the different rates of evolution and different selective pressures that have been detected for female-and male-biased genes in *D. melanogaster*.

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Chapter 5: Male-biased genes and the hyperactivated *X*

Abstract

In Drosophila, there is a consistent deficit of male-biased genes on the *X* chromosome. The results presented in Chapter 4 suggest that male-biased genes arise through an initial large increase of expression levels in the testis. If transcription rates are limited, a large increase of expression in the testis may be harder to achieve for single-copy *X*-linked genes than for autosomal genes, as the latter are present in two copies in males. Furthermore, dosage compensation mechanisms that duplicate the expression on the single *X* in Drosophila males imply that, in males, *X*-linked genes are more likely to be close to this upper limit of transcription than autosomal genes.

This hypothesis predicts that the larger the increase of expression required to make a male-biased gene, the smaller the chance of it being located on the *X*. Consequently, highly expressed male-biased genes should be located on the *X* chromosome less often than lowly expressed male-biased genes.

This pattern is consistently observed in our data, whether microarray data or EST data are used to detect male-biased genes in *D. melanogaster* and to measure their expression levels, consistent with the idea that limitations in transcription rates may prevent male-biased genes from accumulating on the *X* chromosome.

5.1 Introduction

5.1.1 The evolution of dosage compensation

5.1.1.1 The evolution of sex chromosomes

Sex chromosomes have evolved independently from pairs of autosomes in many clades, suggesting similar evolutionary forces and overall similar steps in their evolution (reviewed in Charlesworth *et al*., 2005). Once a male-determining gene has arisen on an autosome, this new autosome will only be transmitted from fathers to sons. Unlike the autosomes, which spend half of their time in females, this new sex chromosome will only be under selection in males: this will lead to the accumulation of mutations that are favourable to males. It then becomes highly advantageous to keep the sex-determining gene and the alleles that are advantageous to males linked (that is, to avoid recombination on the new sex chromosome), so that they are only transmitted to males. This can, for instance, occur through successive inversions around the sex-determining gene region, as these abolish the recombination within the inverted fragment.

This reduced recombination on the *Y* (we will refer to the chromosome carrying the sex-determining region as *Y,* but the same applies to the *W* chromosome, in the case of *ZW* sex determination systems, such as birds and butterflies) also has consequences for the rest of the *Y*-linked genes, as follows (Charlesworth and Charlesworth, 2000):
-Selective sweeps: when a new mutation arises on the non-recombining *Y,* which is beneficial for males, it can sweep to fixation. If there is no recombination, it will carry with it any other mutations present on the chromosome, including deleterious ones.
-Muller's ratchet: when there is no recombination, the chromosome with the fewest deleterious mutations is the fittest in the population. Soon after the repression of

recombination, this is likely to be a chromosome free from strongly deleterious mutations. However, after some time, all chromosomes accumulate some mutations, so that even the fittest *Y* chromosome will carry at least one deleterious mutation: the ratchet has clicked. This process then starts again, eventually leading to the accumulation of deleterious mutations on the *Y* chromosome.

-A lower recombination rate causes a reduction in the efficacy of selection, by decreasing the effective population size. This means that more deleterious mutations will behave neutrally, and therefore may become fixed by drift. The removal of transposable elements is expected to be less efficient for the same reasons.

Taken together, these theories predict that after recombination is repressed, the *Y* chromosome is expected to quickly accumulate deleterious mutations and transposable elements, and this has been amply documented. In species that have had sex chromosomes for a long time, such as mammals, the *Y* chromosome has lost most of its gene content (Lahn *et al.*, 2001). More recently, genomic data from clades that have only recently acquired their sex chromosomes has shown that the *Y* chromosome shows higher rates of substitution (possibly due to the accumulation of deleterious mutations), accumulation of transposable elements and disrupted patterns of gene expression (Guttman and Charlesworth, 1998; Liu *et al*., 2004; Charlesworth, 2004; Bachtrog, 2005; Bartolomé and Chalesworth, 2006).

5.1.1.2 Dosage compensation

As the *Y* chromosome degenerates, males are left with only one copy of *X*-linked genes, and an absent or defective *Y*-linked copy. This creates a dosage problem, since

the expression of functional *X*-*Y*-linked genes is halved in males. Dosage compensation mechanisms, that redress this, have been found and studied extensively in several species (Lyon, 1961; Gupta *et al*., 2006). Discussion of dosage imbalances initially focused on the difference between *XX* females and *XY* males and the means to readjust this difference. This view has however been abandoned, since, as Charlesworth (1978) and Gupta *et al*. (2006) pointed out, selection acts on the individual and is not affected by the difference between females and males. It has therefore been hypothesized that dosage compensation mechanisms arise in two steps (reviewed in Straub and Becker, 2007):

1) Expression levels on the *X* chromosome are increased to compensate for the absence of *Y*-linked copies. This readjusts the expression in males. If the increase of expression is limited to males, as in Drosophila, no further mechanisms are necessary. However, if the X chromosome is over-expressed in both sexes, females suffer from an excess of *X*-linked expression, leading to step 2;

2) Expression of the female *X* is inhibited, so that the two sexes now have levels of *X*-linked expression similar to the initial ones, and also similar between females and males.

Several studies have analysed the expression of *X*-linked genes in male and female soma and germline in *Drosophila melanogaster*, *Caenorhabditis elegans* and *Mus musculus* to determine precisely how the dosage compensation mechanisms regulate expression in these organisms (Gupta *et al.*, 2006; Nguyen and Disteche, 2006; Lin *et al.,* 2007; reviewed in Straub and Becker, 2007), and their findings are summarized below.

Drosophila melanogaster

The male *X* chromosome is hypertranscribed in the male germline and soma, as *X*-individuals produce as much *X*-linked mRNA as *XX* individuals. In somatic tissues, this hyperactivation seems to be male-specific, and *XX* females produce *X*-linked and autosomal RNA at similar rates (slightly higher on the *X*, actually). This is consistent with the previous observation that when dosage compensation mechanisms are repressed, female *X*-linked expression remains unchanged, whilst male *X*-linked expression is halved (Mukherjee and Beermann, 1965).

C. elegans

X-linked somatic transcription is similar in *XX*- and *X*- individuals, with females reducing transcription on the *X* (Straub and Becker, 2007). However, even in females, transcription on the *X* is higher than on the autosomes (Gupta *et al.*, 2006). This means that the female *X* might be slightly hyperactive, and the male *X* is very hyperactive (as it achieves these high levels of transcription from one chromosome only). No information is available for the germline.

Mammals

One of the *X* chromosomes is inactivated in each somatic female cell, so that *XX* and *X*- individuals produce similar levels of X-linked RNA (Lyon, 1969; Straub and Becker, 2007). Studies of expression levels in mouse and human detected no difference between *X*-linked and autosomal gene expression (Gupta *et al.*, 2006; Nguyen and

Disteche, 2006; Lin *et al.,* 2007), suggesting that there was hyperactivation of the *X* in both males and females.

5.1.2 The distribution of sex-biased genes: theory and practice

Rice (1984) showed that, under certain conditions, the *X* chromosome is expected to accumulate more sex-biased genes than the autosomes (see previous chapter). Dominant mutations that are beneficial for females but deleterious for males accumulate there because the *X* spends more time in females than in males, whereas autosomes spend the same amount of time in each sex (Rice, 1984). Recessive *X*-linked mutations that are beneficial for males, but deleterious for females, are masked in heterozygous females but have a beneficial effect in males instantly, whereas autosomal mutations only start having a beneficial effect in males when they appear in homozygous individuals, and therefore have a deleterious effect in females. In both cases, a modifier that decreases the expression of the gene in the harmed sex is required for the mutation to become fixed in the population. This would in principle lead to an accumulation of sex-biased genes on the *X*.

Microarray and EST datasets comparing female and male expression have made the identification of male- and female-biased genes possible in several organisms. As predicted, their distribution in the genome is not random, and the *X* chromosome always differs from the autosomes in its content of sex-biased genes (Parisi *et al*., 2003, Khil *et al*., 2004, Lercher *et al*., 2003, Reinke *et al*., 2003, Kaiser and Ellegren, 2006). However, the patterns are highly inconsistent between species (see Table 1.2 in Chapter 1). Since Rice's (1984) theory predicts different results for different levels of dominance

of the new mutations, most of the discussion on these patterns has relied on differences in dominance to explain the discrepancies. Why there should be such systematic differences in dominance between organisms remains unclear.

5.1.3 New insights into the evolution of sex-biased genes

Whilst the *X* chromosome differs from the autosomes in its transmission mode and ploidy state, as modelled by Rice (1984), it also has different biological properties that could affect the distribution of sex-biased genes. Meiotic *X* inactivation, for instance, implies that genes required for certain stages of spermatogenesis cannot be located on the *X* chromosomes in organisms where this mechanism is present, such as mammals (Khil *et al*., 2004), and possibly Drosophila (Hense *et al.*, 2007). In mouse, it has been shown that genes required for late spermatogenesis are indeed rare on the *X*, whereas genes required for early spermatogenesis (before *X*-inactivation) are located on the *X* more often than expected with a random distribution (Khil *et al*., 2004). Meiotic *X*-inactivation cannot, however, explain other peculiarities of the sex-gene distribution, as many are not limited to testis-expressed genes (Sturgill *et al.*, 2007). The potential effect of dosage compensation on the distribution of sex-biased genes has so far not been studied. This phenomenon, however, might be of particular interest because, whilst it is present in all the organisms analyzed (apart from birds, Ellegren *et al.*, 2007; Itho *et al*., 2007), it evolved independently and has different properties in each of them. This could lead to different predictions for the sex-biased gene distribution in these species, which we attempt to test using published microarray and EST data.

5.1.4 Can dosage compensation affect the distribution of sex-biased genes?

In Rice's model (1984), genes become sex-biased when expression in the harmed sex is decreased or abolished. Therefore, on average, sex-biased genes should have lower levels of expression than unbiased ones. Connallon and Lacey-Knowles (2005) tested this by comparing microarray expression data for male-biased, female-biased and unbiased genes. Surprisingly, they found that sex-biased genes are on average transcribed at higher rates than unbiased genes. Our analysis of EST data for sex-biased and unbiased genes (see previous chapter) also suggests that the first step in the evolution of male-biased genes is an increase of expression in the testis, whereas for females it is an increase in both ovary and embryonic tissue expression. If that is the case, and if there is a limit to how much mRNA can be produced from a single gene, then this increase might be harder to achieve in genes that are already being heavily transcribed. The *X* chromosome is often hyperactivated to achieve dosage compensation, so that most of its genes may be close to this limit and therefore an increase to make a gene sex-biased may be rare on these hyperactive chromosomes. This yields two predictions:

1) hyperactive *X* chromosomes should accumulate less sex-biased (with respect to the sex where the hyperactivity occurs) genes than the autosomes;

2) this deficit should be expression-dependent, as lowly expressed sex-biased genes either arose from genes that were very lowly expressed to start with, or by a decrease of expression levels in the harmed sex, as predicted by Rice's model (1984). The evolution of their sex-bias should therefore not have been affected by limited transcription rates.

These predictions are further complicated by the fact that most of what is known about dosage compensation and *X* chromosome hyperactivation concerns the soma (Gupta *et al.*, 2006; Nguyen and Disteche, 2006), whereas a large proportion of sex-biased genes are primarily expressed in the germline (Parsch and Ellegren, 2007). In Drosophila, however, predictions are made particularly simple by the fact that *X* chromosome hyperactivation has been detected both in the germline and in the soma (Gupta *et al.*, 2006).

5.1.5 Aims of this chapter

In Drosophila, we expect to observe (see previous section):

1) A deficit of male-biased genes on the X-chromosome (which has been consistently observed);
2) A small deficit of male-biased genes on the *X* chromosome when only low-expression genes are considered, and a marked deficit of highly expressed male-biased genes on the *X*-chromosome.

We test the second prediction by checking if the deficit of male-biased genes observed on the *D. melanogaster X* chromosome is stronger for highly expressed genes than for lowly expressed genes, using microarray and EST data to measure levels of expression of male-biased genes.

We have focused on genes expressed in the testis and the ovary, as there are only very small numbers of somatic male-biased genes on the *X*, making the sample size too small to perform meaningful analyses.

5.2 Materials and methods

5.2.1 EST analysis

The UNIGENE database (http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=&db=unigene) is a collection of EST and cDNA libraries organized by sequence similarity, so that, for each gene, it returns the ESTs that have been detected in all the libraries in the dataset. The results can be filtered to return all the genes found in one particular species, tissue and/or chromosome. We have taken advantage of this to select *D. melanogaster* autosomal and *X*-linked genes that are expressed in the testis and ovary, and scored the number of genes found in each case. We used this to compare the fraction of *X*-linked and autosomal genes expressed in the testis, separating them according to their expression level (measured by the total number of ESTs detected for each gene). We followed the same procedure for autosomal and X-linked genes expressed in the testis (ovary) but not ovary (testis) and finally, to avoid most of all ubiquitously expressed genes, testis (ovary) but not ovary (testis) or head, as the head library is the largest library from a characterized somatic tissue.

5.2.2 Microarray

We downloaded four microarray datasets (Ovaries versus Testes 5a, dataset ID: GSM2464; Ovaries versus Testes 5b, ID: GSM2465; Testes versus Ovaries 6a, ID: GSM2466; and Testes versus Ovaries 6b, ID: GSM2467) that compared expression levels in *D. melanogaster* testes and ovaries (Parisi *et al.*, 2004) from the NCBI GEO website, a repository of microarray datasets (http://www.ncbi.nlm.nih.gov/geo/). The genes were ordered according to the natural log-transformed ratio of the corrected ratio

of ovary to testis signals, as this should be representative of their sex-bias. Genes with scores higher than 1, or smaller than -1, were considered to be sex-biased.

Once the genes were classified into male-, female- or unbiased genes, we organized them according to their overall expression in males (for the case of male-biased genes), females (for female-biased genes), and the average of males and females (for unbiased genes). We used the overall probe signal, after normalization for background signaling, as the measure of expression levels (this corresponds to P1S/B and P2S/B in the datasets).

5.3 Results

5.3.1 Microarray data

Using two datasets from Parisi *et al.* (2004), which compared *D. melanogaster* ovary and testis expression (Ovaries versus Testes 5a and Ovaries versus Testes 5b), we can test if the distribution of male-, female- and unbiased genes is dependent on the expression level (Figure 5.1). To maximise our capacity to detect the deficit of male-biased genes for all levels of expression, we divided the sample of male-biased genes into three groups of equal size (low-expression, medium-expression and high expression). The boundaries of these groups were used to classify the unbiased and the female-biased genes according to their expression level, so that, for each expression class, we could test if there was a deficit of male-biased genes.

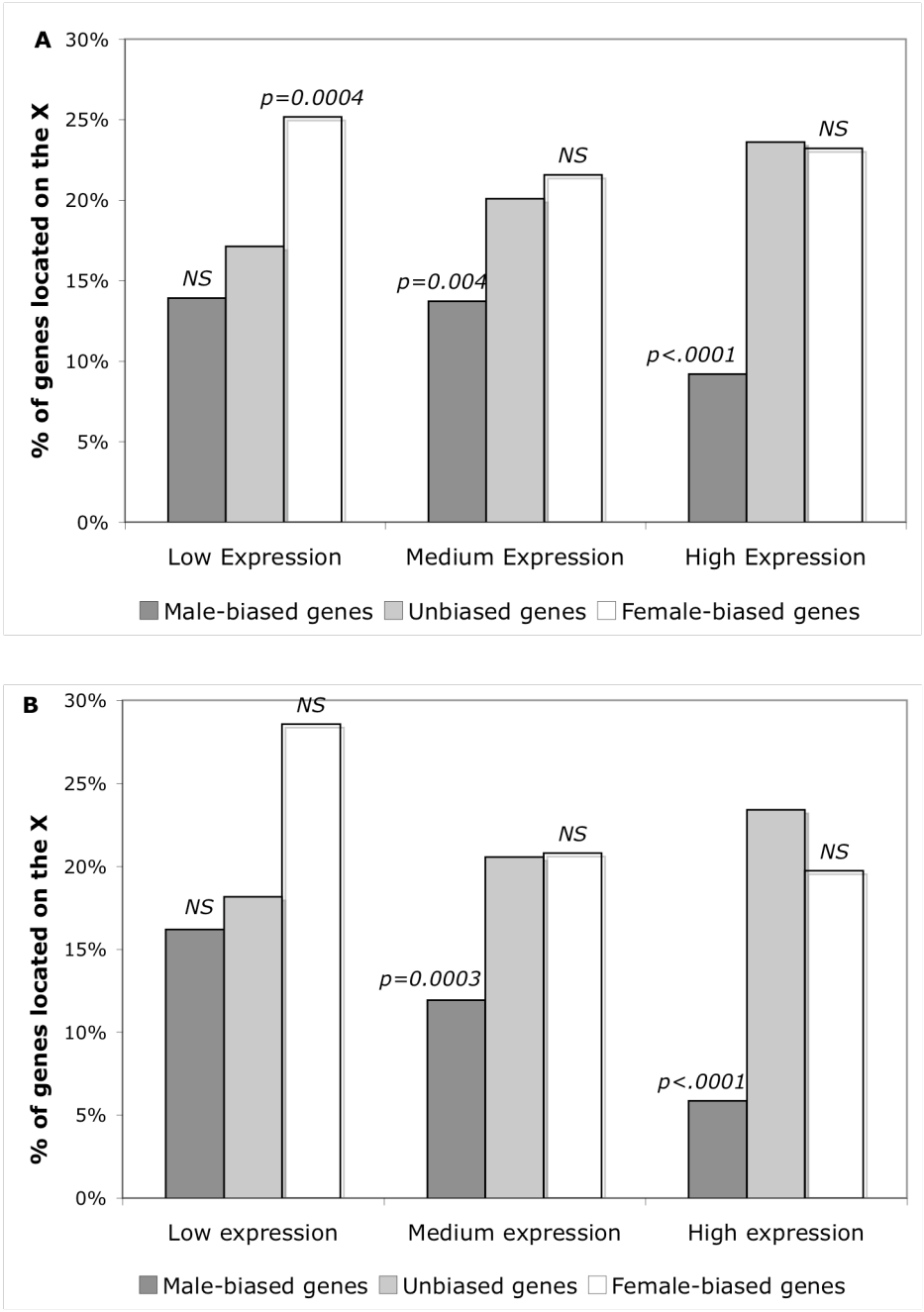


Figure 5.1 (Legend on the next page)

Figure 5.1: The percentage of male-, female- and unbiased genes located on the X for three classes of expression levels (low, medium and high expression), using two comparisons of testis and ovary expression levels (A: Ovaries versus testes 5a, B: Ovaries versus testes 5b). The male-biased genes were organized according to the probe signal for testis RNA, and divided into three groups of equal size (low, medium and high expression genes). The probe signal range of these classes was used to classify female-biased and unbiased genes into low, medium and high expression classes. The female-biased genes were separated according to the probe signal for ovary RNA, and the unbiased genes according to the average of the ovary and the testis signals. The *p*-values denote significant deficits or excesses of low-, medium- and high-expression sex-biased genes on the *X* compared with the number of unbiased genes for that class, and were obtained with Chi-square tests (*NS* denotes non-significant differences).

In both datasets (Figure 5.1A and 5.1B), the deficit of male-biased genes on the *X* (compared with the number of unbiased genes that are located on the *X*) is non-significant for the low expression genes, using a Chi-square test. The high expression genes have, in both cases, the most highly significant deficit of male-biased genes, suggesting that the deficit of male-biased genes on the *X* chromosome is indeed stronger for highly expressed genes. The patterns for female-biased genes are mostly non-significant, apart from a significant excess of lowly expressed female-biased genes on the *X* in one of the datasets (Figure 5.1A).

In order to see if the pattern observed for male-biased genes was consistent, we used two more datasets (Testes versus Ovaries 6a and 6b) and repeated the analysis for male-biased genes (Figure 5.2A). In all four datasets, the percentage of male-biased genes located on the *X* was lowest for highly expressed genes, and highest for lowly expressed genes, and this difference was significant in three of the cases (using a three by two Chi-square test). This contrasts with unbiased genes (Figure 5.2C), which show the opposite pattern: in three of the four datasets, a larger proportion of highly expressed than lowly expressed unbiased genes is located on the *X* chromosome (although this difference is only significant for two of the datasets). No significant differences were detected for female-biased genes (Figure 5.2B).

Overall, these results agree with the prediction that the deficit of male-biased genes on the *X* chromosome should be stronger for highly-expressed genes, and weaker for lowly-expressed genes. However, this analysis suffers from two drawbacks:

- truly lowly expressed genes may not to be identified as sex-biased amongst the generally high levels of background noise;

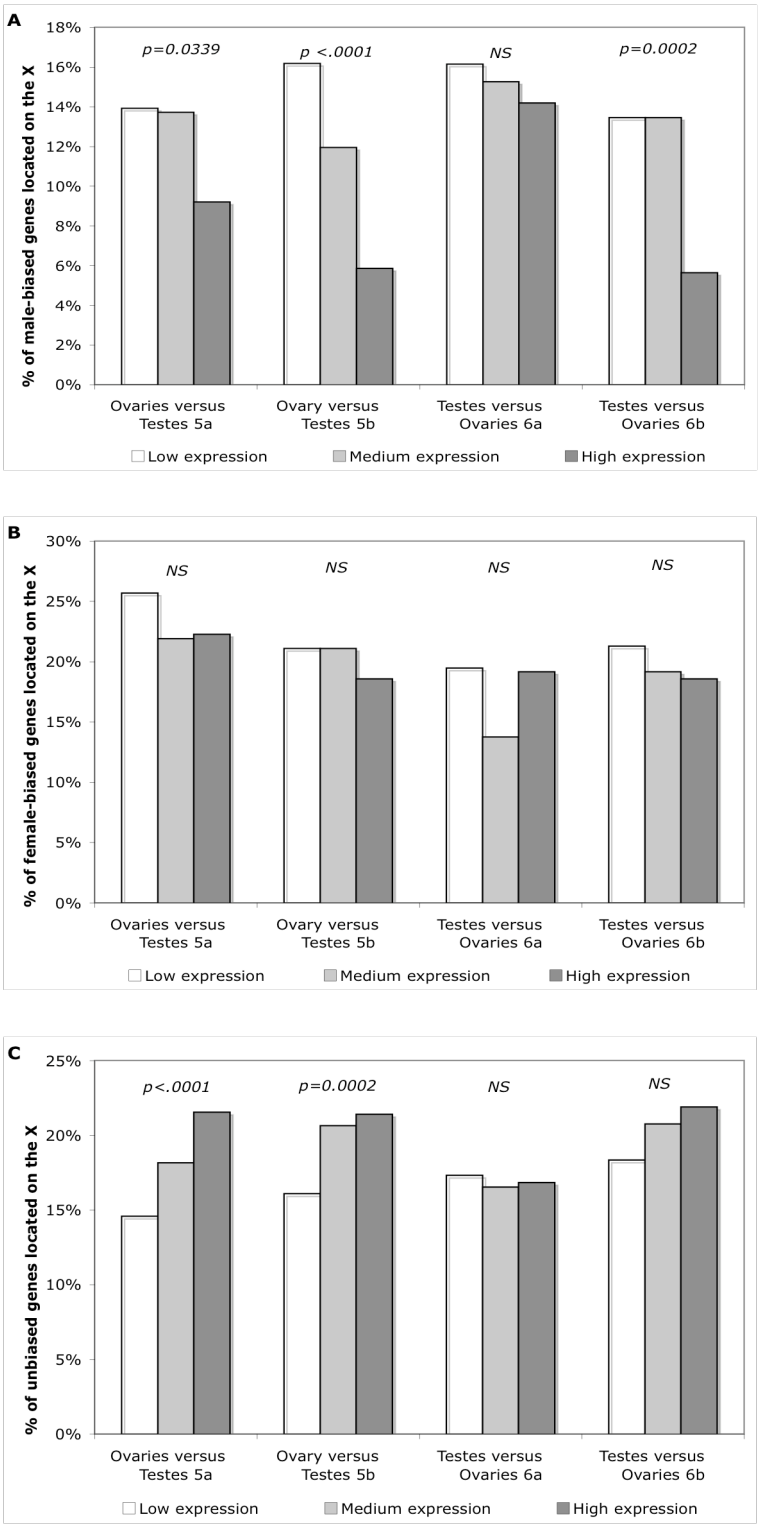


Figure 5.2 (Legend on the next page)

Figure 5.2: The percentage of A) male-biased B) female-biased and C) unbiased genes with low, medium and high expression that are located on the *X* in the four datasets studied (Ovaries versus Testes 5a and 5b, and Testes versus Ovaries 6a and 6b). In this case, the three classes of genes (male-biased, female-biased and unbiased) were organized according to the probe signal (of the testis RNA for male-biased genes, ovary RNA for female-biased genes and the average of the two for unbiased genes), and divided into three groups of equal size. The *p*-values are for the comparison between low, medium and high expression for each class of genes, and they were obtained using 3x2 Chi Square tests (*NS* denotes non-significant differences).

- this type of microarray is meant to be used in a comparative analysis (male versus female in this case), and is not ideal for estimating absolute expression levels.

It therefore seemed useful to see if these results held, when using EST data as a proxy for expression level.

5.3.2 EST data

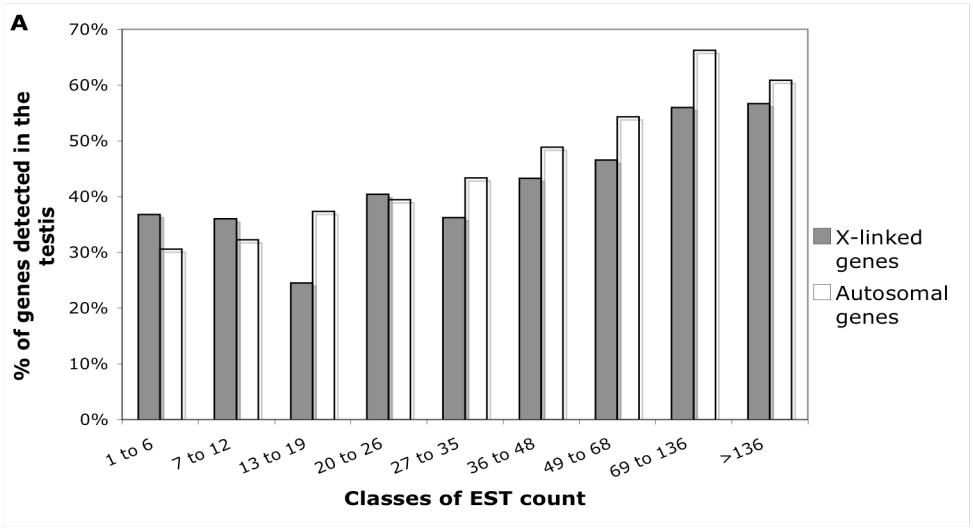
EST data from several cDNA libraries can be easily queried on the NCBI Unigene database. Whilst it is difficult to determine which genes are male-, female-biased, or unbiased from EST datasets, we can select them according to the tissues they have been detected in. In this case, we selected genes that are expressed in the testis, testis but not ovary and finally, to avoid most of all ubiquitously expressed genes, testis but not ovary or head, as the head library is the largest library from a characterized somatic tissue. To examine genes with female-biased functions, we chose genes detected in the ovary, in the ovary but not in the testis and, finally, in the ovary but not in the testis or the head. The results are shown in Figures 5.3 and 5.4.

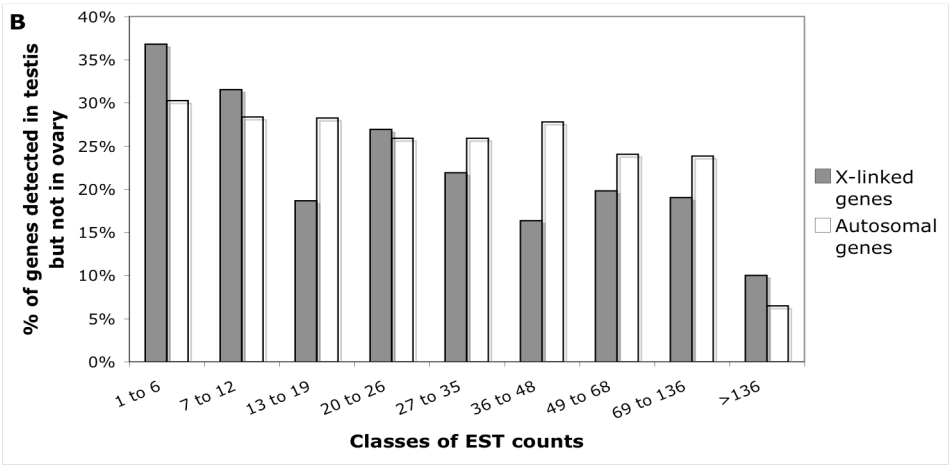
Male-biased gene distribution (Figure 5.3), is heavily expression dependent, with an excess of low-expression *X*-linked genes being detected in the testis compared to autosomal genes, but a deficit of highly expressed genes, yielding an overall deficit of testis-expressed genes on the *X*, as described previously (41% of *X*-linked genes are detected in the testis versus 45% of the autosomal genes, for all testis-expressed genes, 23% versus 25% for genes detected in testis but not in ovary, 10% versus 11% for genes

detected in testis but not ovary or head). On the other hand, consistent with the microarray data, the ovary-expressed gene distribution does not appear restricted to any class of gene expression, with an excess of genes on the *X* chromosome being expressed in the ovary for most EST count classes (Figure 5.4).

To test the significance of these patterns, we calculated, for each case, the difference between the percentage of *X*-linked genes and autosomal genes expressed in the testis (or ovary), and plotted it as a function of the median of their class of EST count (Figures 5.5 and 5.6). We can test if these two variables are correlated by means of a Kendall Rank correlation (Figures 5.5 and 5.6). In the case of testis-expressed genes, there is a significant negative correlation for the two first cases (all the genes expressed in the testis, shown in Figure 5.5A, and genes expressed in the testis but not in the ovary, in Figure 5.5B), suggesting that the deficit of *X*-linked genes expressed in the testis is stronger for highly expressed genes, in agreement with the microarray data. No significant trend is observed for the last case (genes detected in testis but not in ovary or head), but this may be due to the fact that the number of genes analysed is in this case greatly reduced.

Ovary-expressed genes show the opposite trend: the excess of X-linked genes that are expressed in the ovary seems to be stronger for highly expressed genes (Figures 5.6A and 5.6B, though this is only significant for the case of genes expressed in the ovary but not in the testis). Once again, the analysis excluding genes expressed in the head yields no observable trend (Figure 5.6C).





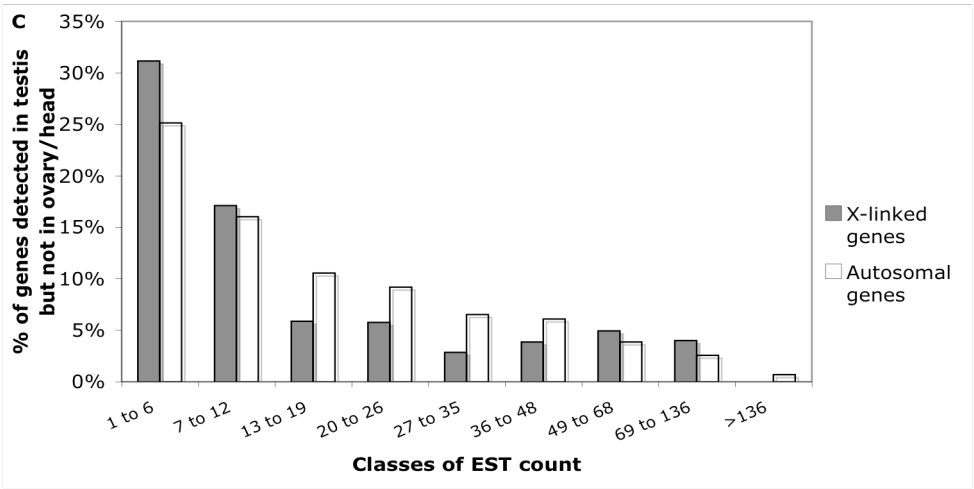


Figure 5.3 (Legend on the next page)

Figure 5.3: The percentage of *X*-linked and autosomal genes expressed in the *Drosophila melanogaster* testis (A), testis but not ovary (B), and testis but not ovary or head (C), for different classes of expression level. Genes were separated into different classes of expression according to their EST count in the Unigene database. For each expression class, we determined the percentage of genes that had ESTs from testis libraries (A), from testis libraries but not from ovary libraries (B), and from testis libraries but not from ovary or head libraries (C).

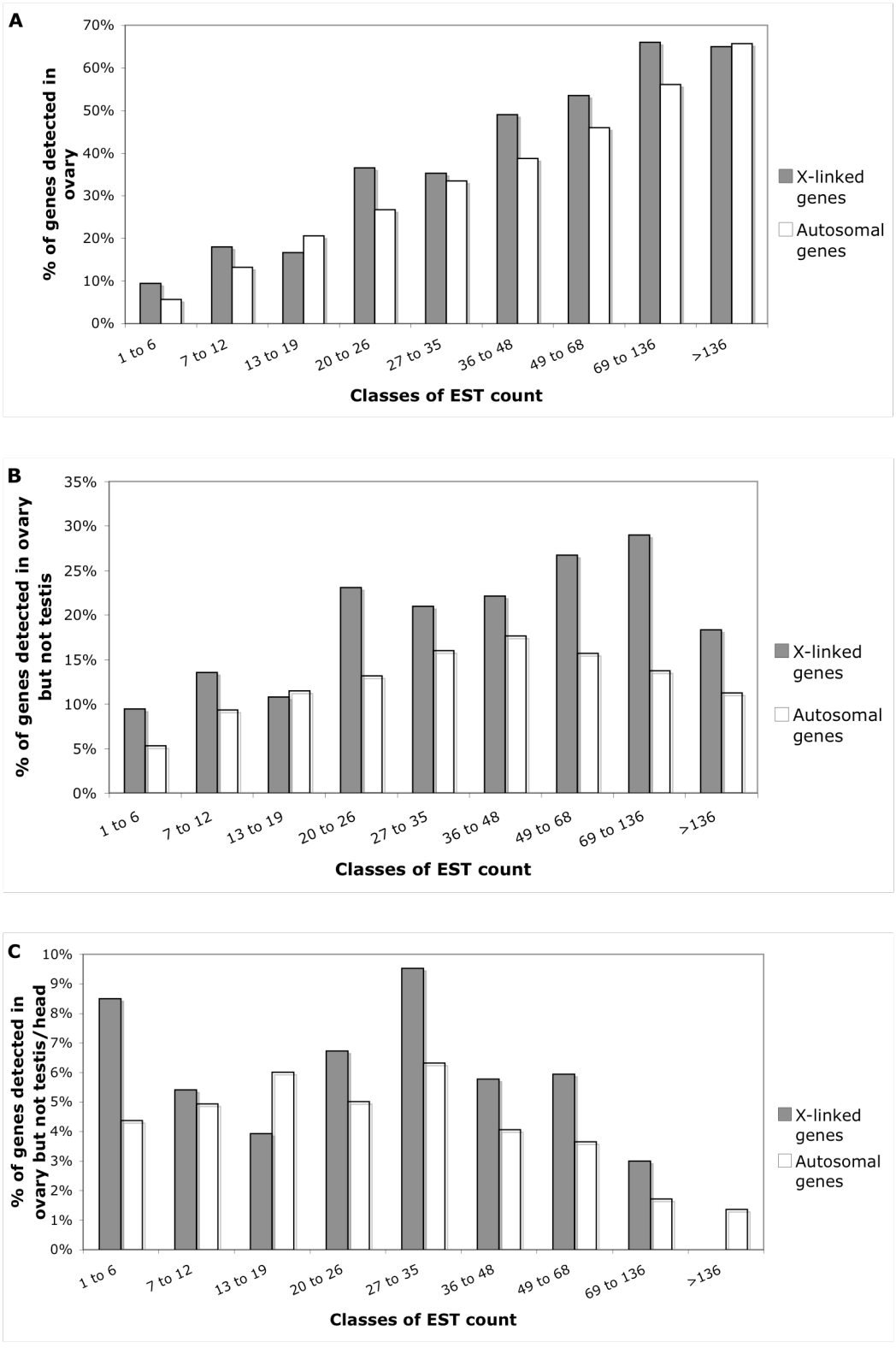
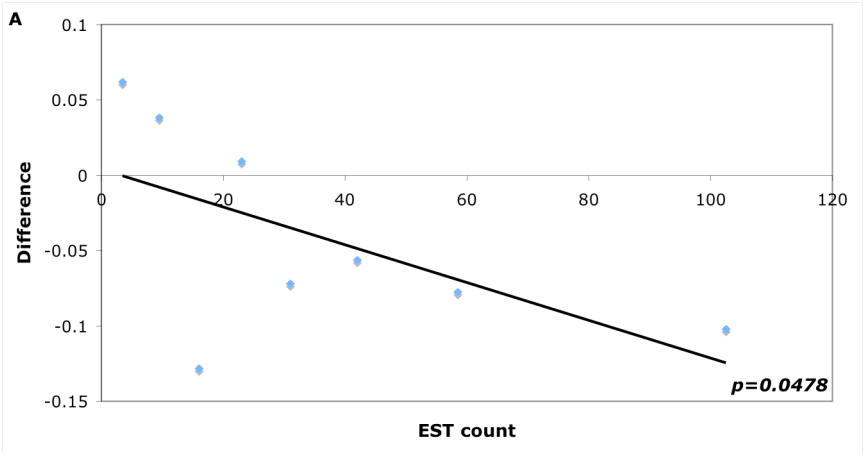
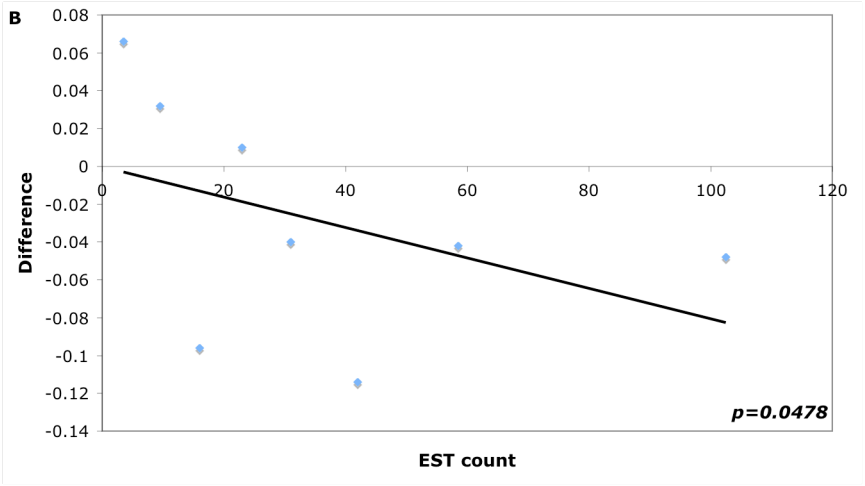


Figure 5.4 (Legend on the next page)

Figure 5.4: The percentage of *X*-linked and autosomal genes expressed in the *Drosophila melanogaster* ovary (A), ovary but not testis (B), and ovary but not testis or head (C), for different classes of expression level. Genes were separated into different classes of expression according to their EST count in the Unigene database. For each expression class, we determined the percentage of genes that had ESTs from ovary libraries (A), from ovary libraries but not from testis libraries (B), and from ovary libraries but not from testis or head libraries (C).





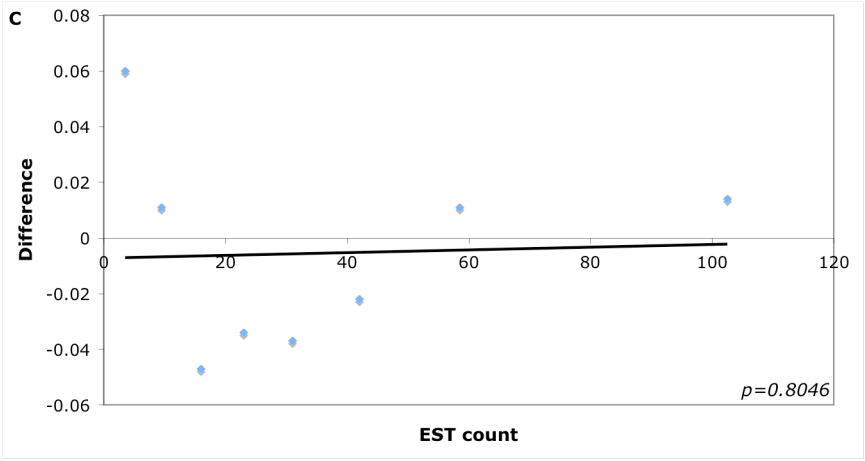
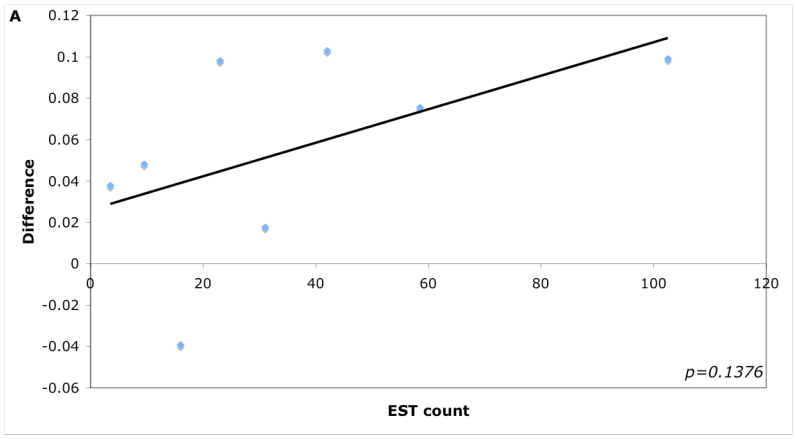
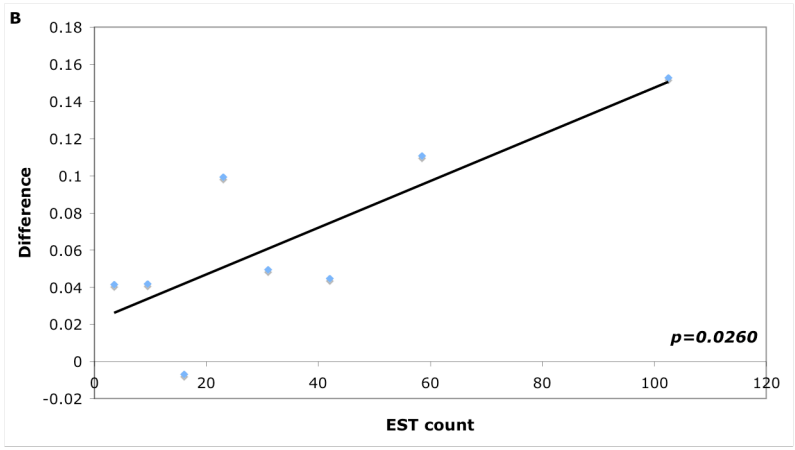


Figure 5.5 (Legend on the next page)

Figure 5.5: The correlation between the difference in frequency of *X*-linked genes and autosomal genes that are expressed in the testis (A), in the testis but not the ovary (B), and in the testis but not in the ovary or head (C) and their level of expression (difference=fraction of X-linked genes expressed in the testis minus fraction of autosomal genes expressed in the testis, EST count is the central value of each EST count class). The *p*-values give the significance of the Kendall rank correlation.





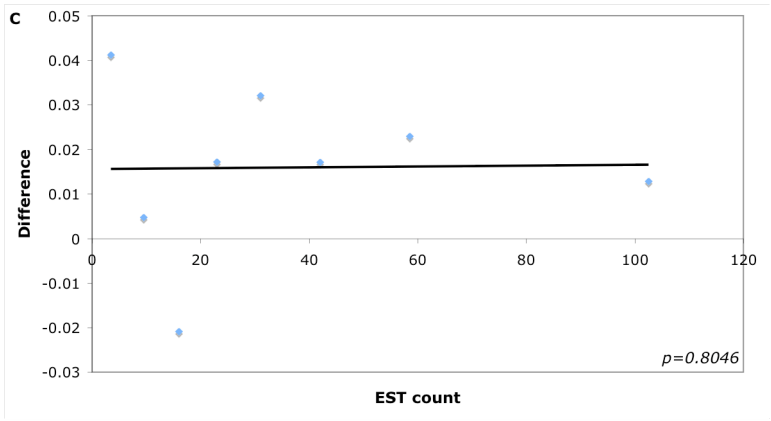


Figure 5.6 (Legend on the next page)

Figure 5.6: The correlation between the difference in frequency of *X*-linked genes and autosomal genes that are expressed in the ovary (A), in the ovary but not the testis (B), and in the ovary but not in the testis or head (C) and their level of expression (difference=fraction of X-linked genes expressed in the testis minus fraction of autosomal genes expressed in the testis; EST count is the central value of each EST count class). The *p*-values give the significance of the Kendall rank correlation.

5.4 Discussion

Since microarray data became widely available, a lot of work has focused on testing Rice's (1984) predictions for the genomic distribution of sex-biased genes. As predicted, the *X* chromosome shows peculiar patterns of accumulation of sex-biased genes, but these are highly inconsistent between the different species analyzed, and sometimes between studies in the same groups (Parsch and Ellegren, 2007). After a promising first paper that detected an excess of male-biased genes in mice (Wang *et al*., 2001), most follow-up studies found that, in fact, there was a deficit of male-biased genes on mammalian *X* chromosomes. This apparent discrepancy was resolved by Khil *et al*. (2004), when they analysed separately genes that are expressed in early and late spermatogenesis. Their reasoning was that, since the *X* is inactivated in late spermatogenesis, genes that are required for this process cannot be located on the *X*. As expected, there is an excess of early spermatogenesis genes on the *X*. This was the first study that highlighted the need to complement Rice's evolutionary model (1984) with other biological mechanisms that might affect the distribution of genes on the *X*.

In this chapter, we have suggested a possible effect of dosage compensation mechanisms on the distribution of male-biased genes. Our main result is that, in *Drosophila melanogaster*, the deficit of male-biased genes on the *X* chromosome is dependent on their expression level, with a small excess of low-expression testis genes being detected on the *X* in the EST dataset. This is consistent with the idea that, if dosage compensation mechanisms lead the *X* to become hyperactivated in males, any increase of expression required to make a new male-biased gene could be harder to achieve than it would on an autosome.

Other hypotheses could lead to an expression-dependent deficit of male-biased genes on the *X* chromosome. For instance, genes that have low-expression in the testis could be genes that avoid meiotic X-inactivation. This would, however, not explain why somatic male-biased genes are also rare on the *D. melanogaster X* chromosome (Sturgill *et al*., 2007). Another possibility is that these lowly-expressed testis genes are not really male-biased genes, but are so rarely detected in EST screens that by chance they were only found in the testis. It is unclear why there would be an excess of these genes on the *X*, but this needs further examination.

Whilst the data is not sufficient to prove that dosage compensation is the cause of the male-biased gene deficit observed in *D. melanogaster*, it is still interesting to note that it follows the predictions made by the theory and provides a another possible line of explanation for the differences between mammals, flies and worms, that have otherwise not convincingly been accounted for. In mammals, the ratio of *X* to autosomal expression in the testis is lower than 1, but this could to be due to the presence of spermatocytes containing an inactivated *X* chromosome, as in spermatogonia the ratio of *X* to autosomal transcript levels is about 1 (Nguyen and Disteche, 2006). In *C. elegans*, no information is yet available. Once more precise information on the transcriptional state of the *X* chromosome in the germline and soma of mammals and nematodes becomes available, more precise predictions can be made and tested in all three clades.

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Chapter 6: Discussion

As discussed in the Introduction to this thesis (Chapter 1), the *X* chromosome is home to various peculiar patterns of evolution. The initial focus of this thesis was faster-*X* evolution, the idea that male haploidy of the *X* chromosome leads to recessive mutations being selected for and against more effectively on this chromosome, therefore implying higher rates of adaptive evolution on the *X* than on the autosomes (Chapters 2 and 3). The second theme of this thesis is the evolution and differential accumulation of sex-biased genes on the *X* chromosome and the autosomes (Chapters 4 and 5).

6.1 Faster-*X* evolution: is selection more efficient on the X?

We have used an empirical approach to assess faster-*X* evolution in Drosophila (Chapter 2) and a theoretical approach to identify evolutionary scenarios, which could lead to this effect (Chapter 3). Although the mean dominance of new mutations plays an important role in determining whether the *X* evolves faster or more slowly than the autosomes, a much stronger effect was shown to derive from differences in the effective population size of the *X* compared to the autosomal effective population size. Different mutation rates in males and females, on the other hand, were shown not to have an effect on K_a/K_s, the normalized rate of non-synonymous evolution.

Our empirical work involved estimating K_a, K_s and K_a/K_s for a set of genes that are *X*-linked in some species of Drosophila but autosomal in others. This was intended to detect changes in evolutionary rates that are caused solely by being located on the *X*, and not by other factors that could differ between chromosomes (such as systematic differences in selection or dominance coefficients). Although there was some indication of increased K_a for fast evolving genes, when these are

located on the *X* chromosome, we detected no significant overall difference in K_a between the *X* chromosome and the autosomes. Since the species we used were nearly ideal for estimating K_a, it is likely that this truly reflects similar rates of non-synonymous evolution at *X*-linked and autosomal sites in Drosophila, and not an experimental difficulty to estimate rates of evolution. More exciting results came from the rate of synonymous evolution of genes when they were located on the autosomes and on the *X*, as the relocation to the *X* chromosome caused a strong reduction in K_s. Since this is accompanied by an increase in codon usage bias, it is likely to be caused by more efficient selection on the *X* chromosome impeding unpreferred codons from accumulating there.

6.2 How is this expected to affect the results for the different organisms studied?

Faster-*X* evolution has typically been studied in mammals and Drosophila. These species differ widely not only in the ratio of N_{eX} to N_{eA}, but also in the effective population size itself. Although the quantitative results are similar for small and large effective population sizes, the amplitude of the faster-*X* effect decreases with the size of the population, as more mutations fall within near-neutrality ($N_e s<1$). This suggests that any faster-*X* effect, if existent at all, should be easier to detect in Drosophila than in mammals.

This effect is further enhanced by the fact that, in Drosophila, N_{eX}/N_{eA} is higher than ¾, as shown by polymorphism data in African populations of *D. melanogaster* and *D. simulans* (these are thought to be more representative of the ancestral population sizes than the European and American populations, which have only recently expanded from African populations). In mammals, lower polymorphism

levels have consistently been observed on the X chromosome compared to the autosomes (Schaffner, 2004; Vallender, 2005), suggesting that N_{eX}/N_{eA} does not exceed the expected value of 3/4.

It is therefore puzzling that, in fact, K_a/K_s is much higher for *X*-linked than autosomal sites in mammals, whereas this effect is either small or non-detectable in Drosophila (J. Mank and S. Berlin, unpublished). We are combining our theoretical results with empirical data compiled by J. Mank and S. Berlin to investigate what is causing this pattern.

6.3 The dominance of new mutations

One surprising result of our theoretical analysis (Chapter 3) was that, compared to the effect of increasing N_{eX}/N_{eA}, the mean dominance coefficient is not as crucial a factor as previously assumed. In Drosophila, where the ancestral N_{eX} is likely to have been as large or larger than the ancestral N_{eA}, faster-*X* evolution should have occurred if mutations had on average a smaller dominance coefficient than 0.9. This makes it rather unlikely that overall dominance of new mutations is the reason for lack of faster-*X* evolution in Drosophila, and suggests that the proportion of mutations fixed by positive selection may not be as high as current estimates suggest.

6.4 The proportion of sites fixed by positive selection

The non-synonymous rate of evolution of the *X* chromosome relative to the autosomes is heavily dependent on the proportion of sites that are fixed by positive versus negative selection (Chapters 1 to 3). If most sites are fixed by drift affecting slightly deleterious mutations, then we expect a slower-*X* evolution. This is particularly true because the effect on slightly deleterious mutations is often stronger

than the faster-*X* effect for beneficial mutations.

Can this also help us understand why mammals show a stronger faster-*X* effect than Drosophila? The ratio of *X*-linked to autosomal rates of evolution is much more sensitive to $|N_e s|$ values when considering the rate of fixation of deleterious mutations than it is for the rate of fixation of beneficial mutations (Chapter 3, Figures 3.2 and 3.4). An increase in N_e will therefore exacerbate to a much greater extent the "slower-*X* effect", resulting from more efficient removal of *X*-linked deleterious mutations, than the faster-*X* effect caused by higher rates of adaptive evolution on the *X* chromosome. Depending on the fitness distribution of new mutations, this can have the puzzling result of masking an existing faster-*X* effect in species with large population sizes, even though these are the ones with higher rates of adaptive evolution on the *X* compared to the autosomes (Figure 3.2).

6.5 The accumulation of sex-biased genes

When Rice (1984) modeled the accumulation of sex-biased genes, he assumed that these would result from the accumulation of sexually antagonistic mutations. This idea is so appealing that further studies on sex-biased genes have often accepted it without further testing. The antagonistic mutations themselves remain somewhat elusive. Rice (1984) mentions that most metabolic features have different optimal values in males and females, so that, technically, we might expect any gene to have accumulated antagonistic mutations. Studies on sex-biased genes, on the other hand, tend to focus on gonadal expression, as this is where most of the differences can be found. For this reason, it seemed of interest to see where the genes that are classified as sex-biased are expressed, as this can give us some clues to their function (Chapter 4). The results were interesting, although not particularly surprising: male-biased

genes correspond to genes that are highly over-expressed in the testis and under-expressed elsewhere, and female-biased genes correspond to genes over-expressed in the ovary but also in embryonic tissues.

The purpose of that analysis was also to investigate the physical steps that lead to sex-biased expression, in the hope of understanding why the distribution of sex-biased genes differs so drastically between Drosophila and mammals, a pattern for which there is so far no clear explanation (Chapters 1, 4 and 5). In both cases, the initial step in the acquisition of a sex-biased expression was an increase in the level of expression in the testis (in the case of male-biased genes) or ovary/embryonic tissues (female-biased genes). One possibility considered here is that the high levels of testis expression observed for male-biased genes in Drosophila might be harder to obtain from the single male *X* chromosome, if there is an upper limit to the amount of mRNA that can be produced from one copy of each gene, and that this could limit the number of male-biased genes evolving on the *X* chromosome (Chapter 5). Consistent with this hypothesis, the deficit of male-biased genes was stronger for genes with higher expression levels.

6.6 Why the differences between *D. melanogaster*, *C. elegans*, and mammals?

If there are no systematic differences in dominance coefficients of antagonistic mutations between *D. melanogaster*, *C. elegans* and mammals, Rice's (1984) model is not sufficient to explain why the mammalian *X* chromosome is enriched for male-biased genes, whereas the *X* chromosome of *D. melanogaster* and *C. elegans* has a consistent deficit of germline-expressed and somatic male-biased genes. In order to test if limitations in transcription efficiency on the single copy of the male *X* chromosome are limiting the number of male-biased genes appearing on this

chromosome in these organisms, we should compare the overall levels of *X*-linked expression per cell in the testis of *D. melanogaster*, *C. elegans* and mammals. Should this value be smaller for mammals, it could provide a new line of investigation for the opposite distribution of *X*-linked sex-biased genes in mammals and *D. melanogaster* and *C. elegans*.

6.7 References

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Appendix A2.1: List of primers used

3L-XR		
CG10415	F1	TCACCGAGGTCCCCAGCAGTCTTA
	F2	TGGCCCACTTTAACGAGCAACTGC
	R1	GCAGGGCATTCGGTTTCGTGATAC
	R2	TGGCGTTATCAATGTCCTTCTCGTC
CG10575	F1	GAAGACTCTGCCCGATCTGA
	F2	GTACGAGCCAGGTCAGGTGT
	R1	ACCTGACCTGGCTCGTACAC
	R2	CTGTGTTGCGAGTCCAGTTC
CG10809	F1	GGCCACCAACAGTGACTCCT
	F2	CCGACGAGTACAATCGGAGT
	R1	CGGACTCCGATTGTACTCGT
	R2	GCTGGCCAGAAGCTCGTC
CG11010	F1	GACATACGCGAGGGATTTGT
	F2	TGTCTTCACCATCGCCTTG
	R1	GAGACCGGCACATACGGATA
	R2	CACCAGCCATCTCGTCACT
CG11274	F1	ATGAAGTTCGGCGACTGTCT
	F2	TCAACGTGATCGATCTCCAA
	R1	CTTGGTACCGAGCCATTGTT
	R2	TCCTCCTCCACCAAACTGAC
CG11349	F1	ACCCAGATCAACGTCCAGTC
	F2	ATGACCCCAATGCACAATTT
	R1	AAATTGTGCATTGGGGTCAT
	R2	GTGGGATCGGTATCGTCATC
CG11350	F1	CTGTTGTTGATCTGCGCATT
	F2	TGAGTACGAGACCGCTGCTT
	R1	GGCAGGTACTCAGAGGTTGG
	R2	CAGCAGGGGCAGAGTAAGTC
CG12034	F1	TCTGCCCTATTCCCATTACTTCCACA
	F2	CGGCATTCGCATAGACCACATA
	R1	AGCTAAACTTTTGACCAGGCACACG
	R2	GCACGCCATTCCGCTCCATA
CG12182	F1	ACTCTCTGCGGGAGTTCGTA
	F2	CCTGGATCACGATGTTCACTT
	R1	CGGGCATCAGAACTCAAAGT
	R2	GCTGAACAGCCCAATCATCT
CG12362	F1	CAGCGGGCGCATCTCGTCAAT
	F2	TGCGGCAGTGGCTCAAGAAGTG
	R1	AGTCGTAGCGGCAGTTGGGGTTCTT
	R2	CGCCGCCGCTCGCAGTAGT

CG1291	F1	GAGCGCGGTCACGAGGTCAGC
	F2	CGTTCTCTACCCCTCCATTCACAC
	R1	AGCGTGGTTTTTCTTTCTTTCGTA
	R2	CTTCAGGCGCAGCGGTTCGTC
CG13287	F1	CTTTGACGTGGCATTTCTGA
	F2	AATCCCTTGCCACCCAAT
	R1	GGTACTCTGGCCGAAAGTTG
	R2	GTCACTGGCCTTGTCCTGAT
CG13810	F1	GCTCGTGGGGCGCTTTGTTGT
	F2	GGTGTAGCGCATCAGCAGCAGAGC
	R2	CGTCGTGTACCTGGCGGGCTTTAG
	R1	CTGCTCTTCTCCCCCGCCTACCTG
CG13924	F1	CAACGGCAAGGTGACCAAAGTGA
	F2	GAACGATTCCTCCACGGTTTC
	R1	AGAGATTCTTGTAGGCTTGAC
	R2	CCGGTGTAGCGAGGCAGTTTCTC
CG14110	F1	GAGATCCATTAAGATACACCCTTCC
	F2	ATGCGGAAGTGGATGATGACTATGAA
	R1	GCTGTAGTACTGCGGCGTTTGTGG
	R2	TCTGGAGTTCTATGTTTCGCAGTAA
CG14160	F1	CCGGCGTTCTGAATCTGGCACTGC
	F2	CCGCGATTGGGAGCAGTTTGTGGAG
	R1	CCAGGAAAGCCCGGTTGAAGGACAGG
	R2	CTGAGGGCGCCGATGGTGAAGAACA
CG14165	F1	CAGGCGCAACAACAGCAGCACAA
	F2	GCCGCCCACCTCGCATCCAG
	R1	GCCAGGTAGGCCTTGGGTTGG
	R2	CGTTAGGGGGCGGCAGATTCA
CG14834	F1	GATGAAACATCGCAGCCTCT
	F2	ACGGTCAATGCTCTGGAAAC
	R1	CGAGGATGTTTCCAGAGCAT
	R2	GCTAGGGTCACAGCTTCTGG
CG15812	F1	GGCGGTGGAGGCAGCTAAATCAAT
	F2	GATGCCGATTTGGACGACGATGAA
	R1	GCGTTCGCTTCATAATCTTCGTTCG
	R2	GAAGGTGCCGCCAGAGTGTGCT
CG17152	F1	GTCCTGCCCGATGATAACGAGTCCT
	F2	TGGACGGACTTTCTCGCCTCAAT
	R1	CGTAGTGGCGCTCCGACAGTGTG
	R2	ATAGAAAGCCCCCTGCAATACATCCA
CG17173	F1	GAAACGGGAGCGCGGCGAGTC
	F2	CTAATAGCCCGGCAGATCGAGACCAG
	R1	CCATCAGGTAGGCAAAGTCCAGGTTC
	R2	GGGGGTTGTGGGTGTGAGCATAGT
CG18676	F1	TCAGCAGGATGGAGCAGGAGACGA

	F2	AGATAGACCTTGTCCCCGCTGAGC
	R1	CCCGACCAGGAACAAGAACG
	R2	TGCCGCCACAGGACACCAGGAT
CG18808	F1	CAGCAGGGCGTTGTGATAGG
	F2	GCCCAGAGGTGCGGAATGTATT
	R1	CAATGGGGCTCCGATAATGGTG
	R2	TAATCTGGGGGACTACTCGCTGGTGC
CG1934	F1	AAGGAGGCCATCGATATACCCGTGAC
	F2	AAGACCCTGCCCGCCGAAGACACC
	R1	CCTGCCGCAGATGGGGCTGTCC
	R2	GTTGCCGCCCAGGAAATTGATGATGC
CG2107	F1	CAGGAGCCTCTCCTTCCTCT
	F2	AGCCGGAGGTGTACCACA
	R1	GGCTGTACTGGCTCATGTCC
	R2	CCTTTCCCGCCAGTAGGT
CG32026	F1	ATTGGCCCGGAGATCTCTAT
	F2	ATGAAGCGGAAGCAGATCAC
	R1	CTCGTACTTCTTGGCCGTGT
	R2	GGGTGTACTCCGAGCACTTG
CG32053	F1	TTACGGTCATTGGAGGAACTGT
	F2	GACTGTACGCCATTAGCTCCA
	R1	ATAGACAATGGTGCTTGTGCAG
	R2	GGAACTTTATCTGCGCCTGTAG
CG32100	F1	AACTGATACGCAAGGCAAAGAT
	F2	AGAAGCAGTCGAAGAAGAATGC
	R1	CGTGAGTGTCTACCTCCTGAAA
	R2	TATTGTTGATCTGGCTGTGCTC
CG32121	F1	CAAGACAGCGAAGAGCGGACCCTTAT
	F2	TTCGGGGCCAAGCGACTGAAG
	R1	CGGCAGTTGGGGCGAGAAGTG
	R2	GTGCACGCGCAGATTGTATTGGTTG
CG32236	F1	GCGGAGTTTCGGCGTCTGATGA
	F2	GCCTGGCGATGGTAAAGTCAA
	R1	GCAGCGTATGGGGATCGATTTGACC
	R2	ACTCTTGGGCTTGCCGTTTTTGGTG
CG32238	F1	GGGCATCGGCACTCGGTACTTTGTTC
	F2	GTACGCCTTGAGGGGACGGAATGA
	R1	GCGAGGCCAAGGGACCGTTTCATC
	R2	CGGCGATGATGATTGGCACTTCTACT
CG32242	F1	AACGGGCGCGACACCAACGAACT
	F2	GAGGAGTCCGGCTTCGCTTACCAC
	R1	TCGGGCTGGTAGGCTTGTGGTCTGTG
	R2	GCTGCTCTGCCCTCCTGCTGCTCTT
CG32281	F1	ATCCACCCGTTTACGTCACTACTTTC
	F2	CTGCCGCACGGTAGTCAATAAAG

	R1	GTCTCCACTTGGTACTCGGCTTCTCC
	R2	TCTGTTCCACCTGCGCCCTCACTAAT
CG32353	F1	GCCGAGAAGAAGCACTTTGT
	F2	GGCAACTCCAAAATGGATCT
	R1	CCAGGAGCGTTCGGTATATT
	R2	GGATGTTCACGATGTTGTGC
CG32395	F1	CGCGTACATCAACGCTAAAG
	F2	CATCTACTGCTGCTCGTGCT
	R1	AGAACGGCAGGTATGAGCAG
	R2	AAAGCTGGGCTGATAGTTGG
CG32414	F1	ATCGTGGAAAATACCCAGCA
	F2	ATACTGAAGCCACCCTCCAG
	R1	ACTGGAGGGTGGCTTCAGTA
	R2	GATGGGCTGTGAGAAGGTGT
CG32415	F1	ATCCCATGGACAAGGACAGT
	F2	CCAATCTGGCGGATAATGTC
	R1	ATGCTGTTCTCCTCCTCCAG
	R2	ATAGAGGCGAGCCACTTTGA
CG3434	F1	GGCCGGCTGCACATAAAGGATGGA
	F2	GAGCGCCAAGAAACGCGTTCAGAAA
	R1	AATGGGGGCCAGCAGGGTAGAGTCC
	R2	GGGCCCAGCAGCTCGTTGGTCTT
CG3715	F1	GCCGGAAAGCGACGTCTGTGTTC
	F2	GCCCCGCGTGTGGCTCAAC
	R1	CTAAGGCGATCGCGTGGCTTCTTC
	R2	CTACGGCGACCGCTGCCTGAT
CG3891	F1	CAGTACCCGCACTACAGCAA
	F2	AATTACTCAAGCGGCGACTG
	R1	GCTTGGGGAAGCATGATGA
	R2	CCCGACTGAGGGTTACACTG
CG4167	F1	ACCAGCACCGCATCATCGTCATCACC
	F2	AAACCGGTTCCGACAGAGGCACAT
	R1	GCGGCCACGCTCTTCGGCTCTG
	R2	CTCGGGCTCCTTCTCCTGCTGCTTCT
CG5150	F1	CGGGCCAAGAACATCATACT
	F2	TCCCGAGTTCTACGACAAGG
	R1	CGAATTCCTCCACCAGATTG
	R2	GGGGCAGGTAGGGCTTATTA
CG5645	F1	AAGGAGACGCGTTTCTTCAA
	F2	AAGGGATACGCCAACACAGA
	R1	CTGCTTCTCCAGTTCCTGCT
	R2	TTCGACGTTGATGGATCGTA
CG5653	F1	GGATTGGCGGTCGCATACACA
	F2	CAGCCCGATGACCTTGGAGTG
	R1	TCCATCCGAGCACCGAACATT

	R2	AAATCGGTTAGGTTGGGGCACATC
CG5690	F1	GATACGGACGACACCGATCT
	F2	CATCATCAGCACCACAGTGA
	R1	ATGTCATCCGAATGCTAGGC
	R2	TCGTGCCTCTCTTGGAACTT
CG5714	F1	GGCGACGAATGGTTTATTGT
	F2	GCACAAATGTCCGATTCACC
	R1	TAACGCCCAGAATCTGTTCC
	R2	ATGCCCTCAAAGTTCGACTG
CG5883	F1	GCCCTGCTGCTGGCTCTCGTA
	F2	GCGCCGCCAAATTTGAGTGGT
	R1	TCCCTCATCGCAGGTGTTGTTGACTA
	R2	ACACCGTAGCTTTTAATGGCTGTAGT
CG5897	F1	CACAATGGAGGAGCGGTGCCAGTA
	F2	CAAACAGCTGCCGGTCCTACCTGAG
	R1	GCATTGCCCAGTCTGACGGTTGAAAT
	R2	GCAGACCCTAAAGTTCGGGAAGTGCT
CG6053	F1	CACGCGTACAGTTCGAGGACAAGGAT
	F2	GATGGCGGCATTAAGATGGCTGTCAG
	R1	AGCCATCTTAATGCCGCCATCAGGAG
	R2	TTGCACGAGAACAACATGCCCATTTC
CG6140	F1	CTTGTCCAGGCCATGGGGATACTG
	F2	CTGCGCGATTCCCTGAACAACAA
	R1	GGCCGTTGGTTAGCTGTTGGGTGTA
	R2	CAGAAGTTTGATGGATCGCAGCACAT
CG6404	F1	ATGCACTTGGCCAGCCACAGGATTT
	F2	AACGGAACTCGGCTAAGATGAACAAC
	R1	TAGCGGGCAGACTCAATGGCGTTACC
	R2	TTCGCGAACTGAGGGAATACG
CG6602	F1	TGAAGTGCTCTTCCACATCG
	F2	CCTCACTCAGTTGGCTCTGG
	R1	TTCGGAGATTATCTTGCATGG
	R2	GATGGGCTGCAAATTCAGAT
CG6749	F1	CTTTTCGTGGTCTGCATGGTCTGCT
	F2	CCTACATCCGCTGGCCTTCTCCTC
	R1	GAGGAGAAGGCCAGCGGATGTAG
	R2	TACTGCTGCACTGGCATCGTCTTCAT
CG7083	F1	CGCAGGTGGGGATCATCAAAGGTGTC
	F2	AATCGGCCACAGTTCTTCGCACTTCC
	R1	GGTGTGGCCATAGGAAGTGCGAAGAA
	R2	AAAGGGATTCGCCGTGTTCGTTGAGC
CG7252	F1	CTGTCGAGTGTTTGCCTCTATG
	F2	GACTACCAACGTCAATCTGCAA
	R1	AGGGACAGCTCATCTCATTGAC
	R2	AAGATCAAGTTGTTTGGGCACT

CG7303	F1	GCGAGAATGAGGAGAACTTCC
	F2	GCGTCCTGATGCTGGTCTAC
	R1	CTTGCAGAACACCTCGATCA
	R2	AAGAGCGTCGAGTTGTCGAT
CG7386	F1	CAACTCAATATGCCGCCTTT
	F2	TATCATCCCCTGCACCATTT
	R1	TTCATGTGCTCGTCGAATTT
	R2	AACTGCTTTTCCTGGGTGTG
CG7991	f1	GTAGTGCCGAGAGCCAAAAG
	F2	CCTCCACCAAAGAGTTGCTC
	R1	GGATCTCGTTCCGCTTCC
	R2	TCTGCACGTACAGCCAGTTC
CG8019	f1	AGCAAGGCGATCACTCAGTT
	F2	TCAGTGGAGCAATGGAAACA
	R1	AAGCGGCAGATCATGCTATC
	R2	TTTATCGCCTCTTTGCTCGT
CG8281	F1	GCCCCACACCTTCAAAGTAA
	F2	CCGAAATGTTTCACAAATCGT
	R1	CCGAACGATTTGTGAAACATT
	R2	TAATCTCCACCGTGGCTCCT
CG8308	F1	AATTCCTGCTGGGAGCTGTA
	F2	GCCTCTCAACCGACTACAGC
	R1	GAGGGGTAAACCGCAAAGTC
	R2	GGGACACCAGTCGACAAACT
CG8602	F1	CTGATCGATCGTGTGTTTGG
	F2	CCATTCTGGATGGTCTCGAT
	R1	AGGTACACCAGCGAGTCCAC
	R2	TTTCCCTGCTGCTTCTTGTT
CG8616	F1	CGGGATACCAAGATTGAGGA
	F2	GGAGCAAACAAAACCAAGGA
	R1	GAGCCAGGCAAAGTGTGAAT
	R2	CCGTTCACATGTTTGTCCAG
CG9004	F1	GGGTCTGCTGAATCGTTTGT
	F2	CAGTCGAATGTCTGTTGCTGA
	R1	TTGTTCTTCACGGCATTCAG
	R2	ATGCGCATAGTAGGGATTCG
CG9965	F1	GCCAGTGCCGTGCCCGTCATTT
	F2	GCTCGACGGGCTTCATTGTGG
	R1	AGCTGCTGATGCCCTGCGGATGGT
	R2	CGTACCTTTGGAGCCCGCATTCTG

X-XL

CG10932	F1	CCCGCACACCGATCGGCAGTTT
	F2	GGCCAATGCCTTTGCCGACGAGAT
	R1	CGATCTCGTCGGCAAAGGCATTGG

	R2	CTGTGCGCGAGATGGGTGACCAGA
CG11436	F1	AGCTGGACTCGCCCTTTCACAT
	F2	ACTGACGCGACGGTTTGCTGTTG
	R1	GCCCCGCATTCGCCAGTAGTA
	R2	ACTGCAGTATTGGCCCCGACGAT
CG15324	F1	AGGCGACGCCAACATTACATTCGTGT
	F2	CACGCCGCGACGTAACTTTTCACG
	R1	AAGCGGATTTTTGAGCCGAGCAGTGA
	R2	CGATTGGCCACATCCAGGACTCTGC
CG15465	F1	CTACCGCCGCGAACTCGACGAACAGC
	F2	CACCCTCCCCGCTGCTCCTG
	R1	GGTGTGGGCGTGCGACTGGTGACTGA
	R2	CGCTTTACGCTCACGCACTTG
CG15776	F1	CGCCCTGCCCGGAGCTGAATCTC
	F2	ACTTTGCGGAGTTGGATGAGGACAGC
	R1	CGGCGTGTCCCTCCTCCCTGTCC
	R2	TCTCCGCCAGCTTGGGTGGACTGCT
CG15784	F1	CAGCCAGGCGATCAAGAAGGTAACGA
	F2	GGGTGCCGGATGGCCCTATGG
	R1	GACCCCATGTCCATGACGACGAT
	R2	ATGACGGCTCCGGCGACACGA
CG17758	F1	GAATCCGGACACCAAGTGCCACAATG
	F2	CGCAGCATCTCGCGACCACAGAGTA
	R1	GGGCGTTGCGGCGGATTACTCTG
	R2	GATCTCCCGGCCGGGCTTTTTG
CG18262	F1	GCCATTTGGCGATTTTCCGCAACACT
	F2	GGTCTACACATGCCCCGAGGAGGAGT
	R1	CGCTGACGGTGGCGCATCATA
	R2	GCCGCCGGACTGGGCAAATGTCTTG
CG2116	F1	CAGACGAGCCCGAGCCAGAGC
	F2	TGTGCGGAAACGTATCGTGATTGTAA
	R1	GGCATGACCGGCCAGGTATTTACAAT
	R2	GGCCCATTGCGTCCTCATCGTT
CG2260	F1	TGGGCGGCAGGCGAGGACAT
	F2	GGGCGGCAGTCGTGGCGTAGTCT
	R1	CAGGCCAGCGGTGACCAGATGC
	R2	GCGGGCGGCTTGAGGTGGAATAG
CG2263	F1	CCATGGCCCACGGCTGGATACTG
	F2	CATCCGGCCAGGAGCCACAAGTTC
	R1	TCCTGGCCGGATGGTTGATGAAGAAG
	R2	CGACAGGCCCCAGGCAATCACAT
CG3032	F1	GACGGCCCAGCAGCACTGTCTCAC
	F2	CGGGGCGTGAGAAACAGCCTGAAG
	R1	AGAGGCCGCCCATGAAGCTGTAACAC
	R2	GGCCGCACACCTGGCACTCGT

CG3184	F1	AACATTGCACAGCGTGGATACAAACT
	F2	CTACACCCCGCAGGGAAAACTGTT
	R1	TGTCCAGCGGTCAAAAACACAAGTC
	R2	AGGTGGCCATGTGCATTAGTCCAT
CG3319	F1	TACAAGGCTCGGGACACGGTGACC
	F2	ATTTCGGCCTGGCCAAGACATACG
	R1	AGATCCGAGTCGCCGGGAATAAAAG
	R2	CGCTTCTTCTGTGGCAGGCTGTTG
CG3342	F1	AGCCCGGCGTTTGGACCACTG
	F2	GGTCCCGTCTTGGTCCGAATGTAAG
	R1	GCGCGGCACCGGTGTCATTA
	R2	CTGTAGCGCCGCCACCTGACG
CG3546	F1	CCCACCAGCCGCCCAAAGTCAAT
	F2	CCAACACGAGGCCCAACAATAAG
	R1	GCTGCTCCTCCTTCCGCTTCATCTG
	R2	GCTGGCGCTTCCGGGTCTGCT
CG3599	F1	AACGCCCTGCTATGCCACGGACTACG
	F2	GCTGGGCCTTTGGCAACGATGTCAA
	R1	TCCAGAACGACCCGCATAGAT
	R2	TGTTTGTTGCCCACGCGAATGCTCTC
CG6978	F1	CGTGGCGATTGTGGCGATGGTCA
	F2	CCCGCAGGCGAGCAAGAGGAT
	R1	GGCGTACGTCCGGTGTCCTC
	R2	GGCGAAGCGGGGCGAGAGGTC
CG7952	F1	TCTCTACACCGCCTACGCCTATCAGC
	F2	CCGCCCGCAGCCCAGACCAC
	R1	GGCGATTGTGGGGTCTCCAAAACATC
	R2	CGGCCCGGATGGCAATCTCGT

All the gene names come from *D. melanogaster*. We used two pairs of primers for each gene, F1-R1 and F2-R2. The gene was first amplified using the external primers (the F1/R2 pair). The resulting fragments were about 1000 base pairs long, and the internal primers (R1 and F2) were required for the subsequent sequencing reactions.

Appendix A2.2: Individual K_a, K_s and K_a/K_s values for 3L-XR, X-XL and autosomal genes.

3L-XR loci

	D. pseudoobscura/D. affinis			*D. melanogaster/D. yakuba*		
n=69	ka	ks	ka/ks	ka	ks	ka/ks
CG10415	0.001	0.200	0.007	0.011	0.319	0.036
CG10575	0.034	0.243	0.141	0.038	0.306	0.124
CG10809	0.007	0.140	0.053	0.004	0.197	0.020
CG11010	0.011	0.218	0.049	0.003	0.270	0.013
CG11274	0.007	0.261	0.026	0.006	0.238	0.024
CG11349	0.039	0.271	0.145	0.046	0.386	0.119
CG11350	0.048	0.350	0.137	0.017	0.118	0.141
CG11495	0.024	0.209	0.115	0.024	0.257	0.094
CG12034	0.027	0.280	0.095	0.019	0.338	0.055
CG12182	0.078	0.287	0.273	0.075	0.331	0.226
CG12362	0.061	0.225	0.270	0.018	0.323	0.057
CG1291	0.014	0.209	0.069	0.008	0.327	0.024
CG13287	0.004	0.169	0.022	0.014	0.392	0.036
CG13810	0.048	0.261	0.186	0.014	0.231	0.059
CG13924	0.041	0.265	0.153	0.030	0.279	0.108
CG14110	0.050	0.256	0.196	0.062	0.345	0.181
CG14160	0.015	0.228	0.066	0.023	0.431	0.054
CG14165	0.011	0.291	0.037	0.016	0.300	0.055
CG14834	0.046	0.271	0.170	0.062	0.326	0.189
CG15812	0.049	0.323	0.151	0.042	0.489	0.085
CG17152	0.034	0.292	0.118	0.046	0.357	0.129
CG17173	0.005	0.286	0.018	0.062	0.335	0.187
CG18676	0.001	0.180	0.007	0.002	0.265	0.009
CG18808	0.118	0.322	0.367	0.044	0.373	0.118
CG1934	0.048	0.322	0.149	0.126	0.606	0.208
CG2107	0.049	0.385	0.128	0.011	0.351	0.031
CG32026	0.047	0.303	0.154	0.018	0.423	0.042
CG32053	0.039	0.163	0.242	0.042	0.301	0.139
CG32100	0.033	0.268	0.125	0.019	0.296	0.065
CG32121	0.018	0.237	0.075	0.018	0.326	0.055
CG32236	0.122	0.336	0.364	0.043	0.384	0.113
CG32238	0.003	0.136	0.019	0.003	0.447	0.006
CG32242	0.037	0.109	0.336	0.035	0.086	0.410

CG32281	0.028	0.309	0.090	0.039	0.362	0.108
CG32353	0.018	0.257	0.070	0.018	0.276	0.065
CG32395	0.140	0.473	0.295	0.109	0.509	0.215
CG32414	0.004	0.310	0.012	0.007	0.382	0.017
CG32415	0.025	0.244	0.101	0.029	0.399	0.073
CG3434	0.033	0.274	0.121	0.031	0.283	0.111
CG3715	0.015	0.149	0.101	0.003	0.244	0.011
CG3891	0.018	0.216	0.083	0.025	0.278	0.089
CG4167	0.018	0.129	0.144	0.025	0.125	0.204
CG5150	0.032	0.229	0.139	0.016	0.358	0.044
CG5645	0.014	0.283	0.050	0.016	0.405	0.041
CG5653	0.050	0.285	0.175	0.050	0.357	0.140
CG5690	0.031	0.330	0.093	0.035	0.424	0.082
CG5714	0.041	0.238	0.172	0.021	0.231	0.089
CG5883	0.157	0.250	0.629	0.088	0.484	0.182
CG5897	0.154	0.258	0.597	0.095	0.308	0.308
CG6053	0.001	0.251	0.005	0.004	0.258	0.016
CG6140	0.017	0.294	0.056	0.008	0.390	0.021
CG6404	0.021	0.234	0.091	0.015	0.311	0.049
CG6602	0.034	0.260	0.132	0.039	0.364	0.108
CG6749	0.035	0.177	0.196	0.012	0.379	0.032
CG7083	0.003	0.196	0.014	0.016	0.338	0.047
CG7252	0.108	0.240	0.450	0.048	0.282	0.171
CG7303	0.062	0.209	0.296	0.049	0.330	0.149
CG7386	0.030	0.252	0.118	0.094	0.401	0.234
CG7991	0.011	0.220	0.050	0.013	0.217	0.062
CG8019	0.005	0.274	0.020	0.005	0.317	0.017
CG8281	0.019	0.341	0.055	0.021	0.298	0.070
CG8308	0.010	0.173	0.058	0.004	0.271	0.016
CG8602	0.027	0.162	0.165	0.026	0.292	0.089
CG8616	0.008	0.311	0.026	0.007	0.417	0.016
CG9004	0.046	0.317	0.144	0.020	0.290	0.069
CG9965	0.040	0.354	0.113	0.012	0.285	0.041
Est5B	0.044	0.316	0.140	0.091	0.285	0.320
hsp83	0.001	0.198	0.006	0.007	0.153	0.043
sod	0.012	0.166	0.075	0.014	0.229	0.063
Average	0.036	0.253	0.142	0.031	0.323	0.095

X-XL loci

	D. pseudoobscura/D. affinis			*D. melanogaster/D. yakuba*		
n=27	ka	ks	ka/ks	ka	ks	ka/ks

Cg2116	0.056	0.418	0.133	0.048	0.296	0.162
CG3032	0.037	0.339	0.109	0.022	0.259	0.085
CG3319	0.000	0.227	0.000	0.009	0.396	0.023
CG3342	0.082	0.378	0.218	0.104	0.326	0.319
CG3546	0.117	0.379	0.308	0.088	0.392	0.224
Cg3599	0.021	0.218	0.098	0.063	0.413	0.154
CG7952	0.015	0.292	0.053	0.014	0.249	0.055
CG10932	0.010	0.211	0.048	0.005	0.339	0.015
CG11122	0.092	0.391	0.236	0.024	0.282	0.086
CG11436	0.006	0.216	0.025	0.011	0.340	0.032
CG15324	0.061	0.198	0.307	0.112	0.586	0.192
CG15465	0.023	0.198	0.117	0.024	0.363	0.067
CG15776	0.030	0.293	0.104	0.021	0.392	0.053
CG17758	0.004	0.231	0.018	0.000	0.265	0.000
CG18262	0.026	0.189	0.137	0.067	0.343	0.196
AnnX	0.002	0.262	0.008	0.011	0.111	0.098
Cyp1	0.003	0.069	0.040	0.003	0.121	0.023
gapdh2	0.003	0.170	0.020	0.000	0.107	0.000
scute	0.021	0.257	0.083	0.019	0.216	0.086
sesB	0.009	0.075	0.116	0.002	0.020	0.080
sisA	0.083	0.292	0.284	0.019	0.319	0.060
swallow	0.074	0.392	0.189	0.124	0.346	0.357
Cg6978	0.052	0.225	0.232	0.041	0.481	0.085
CG15784	0.075	0.372	0.203	0.130	0.326	0.397
CG3184	0.073	0.300	0.244	0.057	0.302	0.188
CG2260	0.010	0.274	0.035	0.012	0.221	0.056
CG2263	0.006	0.242	0.026	0.004	0.224	0.020
Average	0.037	0.263	0.140	0.038	0.298	0.129

Autosomal loci

	D. pseudoobscura/D. affinis			*D. melanogaster/D. yakuba*		
n=39	ka	ks	ka/ks	ka	ks	ka/ks
Ade3	0.006	0.257	0.023	0.021	0.311	0.067
Adh	0.021	0.194	0.110	0.012	0.153	0.081
Adhr	0.028	0.352	0.081	0.012	0.368	0.031
Alk	0.005	0.328	0.014	0.004	0.358	0.010
Amd	0.014	0.234	0.062	0.013	0.321	0.041
Asx	0.033	0.198	0.167	0.016	0.178	0.089
Bcd	0.009	0.237	0.039	0.019	0.249	0.075
Bruce	0.014	0.350	0.040	0.019	0.398	0.049
CG11136	0.007	0.284	0.025	0.006	0.271	0.022
Cnk	0.009	0.225	0.038	0.001	0.373	0.004
Cos	0.015	0.326	0.046	0.022	0.350	0.063

Ddc	0.009	0.311	0.029	0.008	0.273	0.028
Dpp	0.023	0.128	0.181	0.027	0.063	0.438
Eno	0.010	0.125	0.084	0.010	0.138	0.071
Exu1	0.081	0.280	0.288	0.056	0.262	0.213
ftz	0.041	0.216	0.192	0.029	0.190	0.155
gld	0.005	0.192	0.023	0.025	0.259	0.096
gpdh	0.000	0.139	0.000	0.000	0.122	0.000
grau	0.012	0.305	0.038	0.021	0.370	0.056
hb	0.011	0.242	0.044	0.006	0.196	0.031
hyd	0.009	0.211	0.041	0.009	0.238	0.036
Lam	0.042	0.262	0.161	0.031	0.210	0.149
NinaE	0.007	0.243	0.031	0.001	0.123	0.010
NompA	0.016	0.273	0.058	0.012	0.285	0.043
Nop56	0.006	0.180	0.034	0.005	0.215	0.024
Plc	0.033	0.317	0.104	0.034	0.257	0.133
Rpl32	0.000	0.176	0.000	0.000	0.088	0.000
sax	0.003	0.349	0.008	0.008	0.260	0.030
smo	0.004	0.142	0.029	0.004	0.333	0.011
srya	0.073	0.288	0.255	0.028	0.334	0.082
stan	0.002	0.322	0.005	0.002	0.303	0.008
t1	0.060	0.257	0.235	0.082	0.367	0.224
toll7	0.002	0.211	0.011	0.002	0.404	0.006
tud	0.074	0.326	0.229	0.038	0.363	0.106
uba1	0.014	0.239	0.058	0.004	0.277	0.014
updo	0.012	0.242	0.051	0.003	0.410	0.008
uro	0.014	0.258	0.053	0.025	0.308	0.082
vlc	0.043	0.253	0.169	0.058	0.220	0.266
xdh	0.022	0.310	0.072	0.011	0.307	0.036
Average	0.020	0.251	0.081	0.018	0.269	0.065

The K_a/K_s average is the ratio of the K_a and K_s averages.

Appendix A.2.3: Perl scripts used to process the data from Shapiro *et al.*, 2007

1) The dataset was downloaded from NCBI by searching for "shapiro AND adaptive"

2) The Perl script “fastacleaner.pl” was used to discard all the sequences from cosmopolitan lines or other species, and create, for each gene, a file containing the african sequences.

3) The protein sequence for each gene was downloaded from the flybase batch download. The corresponding cDNA was extracted from the african sequences using the genewise software (http://www.ebi.ac.uk/Wise2/doc_wise2.html).

4) The Perl script “wisecleaner.pl” extracted the cDNA sequences from the genewise output, but only if the score of the alignment was larger than 100 (this should remove non-specific
alignments).

5) If the initial and the final number of sequences for a gene were the same (1 sequence ➔ 1 cDNA to be analyzed), the sequences were aligned with Clustalw (376 genes). The other 36 genes were "reprocessed" by hand.

6) The π_a and π_s values were evaluated from the ClustalW alignments using DNAsp.

7)The alignments were checked manually (with Se-Al) and the dodgy bits removed (these came mostly from intronic sequence being extracted as coding for some sequences). The π_a and π_s values were re-evaluated for the 33 alignments that were changed.

Perl script 1: fastacleaner.pl

```
#!/usr/local/bin/perl

open (FASTA, "../sequences.fasta") or die "can't open the fasta file: $!\n";
open (AFRICAN, ">>african") ;
open (COSMOPOLITAN, ">>cosmopolitan") ;
open (PROBLEMS, ">>problems") ;

$/ = ">";
while ($line = <FASTA>) {
                ($definition, $sequence) = split("\n", $line);
                ($gi, $genus, $species, $strain, $strainname, $gene) = split (" ",
$definition);
                if ($species eq "melanogaster") {
                        if ($strainname eq "LA20") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "LA66") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "OK17") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "OK91") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "ZH12") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "ZH13") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "ZH27") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "ZH40") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "ZS8") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "ZH18") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "ZH21") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "ZS6") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "Z56") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "ZS11") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "ZS30") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "Z30") { print AFRICAN $line ;
                        }
                        elsif ($strainname eq "ZS56") { print AFRICAN $line;
                        }
                        elsif ($strainname eq "Fr") { print COSMOPOLITAN $line;
                        }
                        elsif ($strainname eq "Can") { print COSMOPOLITAN $line;
                        }
                        elsif ($strainname eq "Hg") { print COSMOPOLITAN $line;
                        }
                        elsif ($strainname eq "Id") { print COSMOPOLITAN $line;
                        }
                        elsif ($strainname eq "TWN") { print COSMOPOLITAN $line;
                        }
                        elsif ($strainname eq "rucuca") { print COSMOPOLITAN $line;
                        }
                        else { print PROBLEMS $line;
                        }
                }
                else {print PROBLEMS $species;}
}
close AFRICAN;
open (AFRICAN, "african") ;
```

```
$/ = ">";

while ($mine = <AFRICAN>) {
                ($definition, $sequence) = split("\n", $mine);
                ($one, $two) = split ( '\(' , $definition);
                ($genename, $other) = split ( '\)' , $two);
                        if ($genename gt 0) {
                        open (GENENAME, ">>$genename") ;
                        chop $mine ;
                        print GENENAME ">$mine" ;
                        close GENENAME ;
                }
                else {
                ($gi, $genus, $species, $strain, $strainname, $genename, $other) = split
( " ", $definition) ;
                        if ($genename gt 0) {
                        open (GENENAME, ">>$genename") ;
                        chop $mine;
                        print GENENAME ">$mine" ;
                        close GENENAME ;
                        }
                        else {
                        print PROBLEMS "can find gene name: $mine" ;
                        }
                }
}
```

Perlscript 2: wisecleaner.pl

```
#!/usr/local/bin/perl

open (LISTGENES, "../genelist.txt") or die "can't open list of genes: $!\n";
open (WISERRORS, ">>wiserrors.txt");

while ($genename = <LISTGENES>) {
        chop $genename ;
        push (@genenames, $genename);
}

$/ = "Score ";
foreach $genename (@genenames)  {
                        open (GENE, "$genename.wise") or die "can't open $genename!\n";
                        open (GENEOUT, ">>$genename.out");
                        while ($line = <GENE>) {
                                ($definition, $cdna) = split ("\n\/\/", $line);
                                ($score, $other) = split (" ", $definition) ;
                                if ($score > 100) {
                                print GENEOUT $cdna;
                                }
                                elsif ($score eq "Wise2") {
                                print "";
                                }
                                else {
                                print WISERRORS "$genename has a bad alignment!";
                                }

                        }
}
```

Appendix A2.4: Fast, medium and slow evolving genes (determined by their Ka/Ks values in the melanogaster group). The number of genes that have higher rates of evolution (*Ka*, *Ks* or *Ka/Ks*) in *D. pseudoobscura/D. affinis* (second column) and *D. melanogaster/D. yakuba* (third column) are shown in the table.

Slow-evolving genes (Ka/Ks < 0.05 in melanogaster-yakuba)			
Ka	pseudo.-aff.	mel.-yak.	Total
non-3L-XR	20	4	27
3L-XR	14	9	23
Fisher test:			
p, 1-tailed	0.08		
p, 2-tailed	0.11		
Ks	pseudo.-aff.	mel.-yak.	Total
non-3L-XR	11	16	27
3L-XR	4	19	23
Fisher test:			
p, 1-tailed	0.07		
p, 2-tailed	0.12		
Ka/Ks	pseudo.-aff.	mel.-yak.	Total
non-3L-XR	21	4	27
3L-XR	16	9	23
Fisher test:			
p, 1-tailed	0.1		
p, 2-tailed	0.2		

Medium-evolving (0.05 < Ka/ks < 0.1 in melanogaster-yakuba)			
Ka	pseudo.-aff.	mel.-yak.	Total
non-3L-XR	13	10	23
3L-XR	11	12	23
Fisher test:			
p, 1-tailed	0.38		
p, 2-tailed	0.77		
Ks	pseudo.-aff.	mel.-yak.	Total
non-3L-XR	9	14	23
3L-XR	5	18	23
Fisher test:			
p, 1-tailed	0.17		
p, 2-tailed	0.34		
Ka/Ks	pseudo.-aff.	mel.-yak.	total
non-3L-XR	12	11	23
3L-XR	18	5	23
Fisher test:			
p, 1-tailed	0.06		
p, 2-tailed	0.12		

Fast-evolving genes (Ka/Ks > 0.1 in melanogaster-yakuba)

Ka	**pseudo.-aff.**	**mel.-yak.**	**Total**
non-3L-XR	6	10	16
3L-XR	12	11	23
Fisher test			
p, 1-tailed	0.28		
p, 2-tailed	0.52		
Ks	**pseudo.-aff.**	**mel.-yak.**	**Total**
non-3L-XR	10	6	16
3L-XR	4	19	23
Fisher test			
p, 1-tailed	0.005		
p, 2-tailed	0.006		
Ka/Ks	**pseudo.-aff.**	**mel.-yak.**	**Total**
non-3L-XR	7	9	16
3L-XR	14	9	23
Fisher test			
p, 1-tailed	0.23		
p, 2-tailed	0.34		

Appendix A3.1: ***The INTEG subroutine***

We are trying to estimate the probablility of fixation of a mutation with initial frequency p ($U(p)$), as given by Equation (3.1):

$$U(p) = \frac{\int_0^p G(x)dx}{\int_0^1 G(x)dx} \tag{3.1}$$

The subroutine INTEG evaluates $\int_a^b G(x)dx$ and can be applied to any a and b. Let's focus for now on the case of $\int_0^1 G(x)dx$, the lower part of the equation.

First, we arbitrarily set n so that we can use a numerical approximation to write:

$$\int_0^1 G(x)dx \approx \sum_{i=x1}^{xn} G(x_i)\Delta x \tag{A1}$$

Where $\Delta x = \frac{(1-0)}{n}$ and

$$x_1 = 0 + \frac{\Delta x}{2},$$

$$x_2 = x_1 + \Delta x,$$

$$\ldots,$$

$$x_n = x_{n-1} + \Delta x$$

In order to obtain a numerical estimation of Equation (A1), all we have to do is, for each x_i, estimate $G(x_i)$.

As we noted before (Equation 3.2):

$$G(x) = \exp\left(-2\int_0^x \frac{M\delta y}{V\delta y}dy\right) \tag{3.2}$$

So that $G(x_i)$ is simply:

$$G(x_i) = \exp\left(-2\int_0^{xi} \frac{M\delta y}{V\delta y} dy\right) \tag{A2}$$

We can once again use the numerical approximation to determine $\int_0^{xi} \frac{M\delta y}{V\delta y} dy$.

$$\int_0^{xi} \frac{M\delta y}{V\delta y} dy \approx \sum_{n=y1}^{y_n} \frac{M(y_i)}{V(y_i)} \Delta y \tag{A3}$$

Where $\Delta y = \frac{x_i}{n}$ and:

$$y_1 = 0 + \frac{\Delta y}{2},$$

…,

$$y_n = y_{(n-1)} + \Delta y$$

Since $M(y)$ and $V(y)$ are simple functions, they can be resolved for each y_i, and we obtain a numerical estimate of $G(x_i)$ that is then used to estimate numerically Equation (A1). The precision of this estimate increases as n increases, but, when n is large enough, this increase becomes insubstantial. Therefore the integration is repeated, increasing n, until the difference between rounds of integration is smaller than 0.01% of the integral.

The same principle can be used to estimate $\int_0^p G(x)dx$, the upper part of Equation (3.1), by using $\Delta x = \frac{p}{N}$.

Appendix 3.2 K_a/K_s for the *X* chromosome with a sex difference in the mutation rate

For small q, $U(q)$ in equation (1a) can be approximated using a Taylor's series by:

$$U(q) \approx U(0) + q\left(\frac{\mathrm{d}U(q)}{\mathrm{d}q}\right)_{q=0} = q\left(\frac{\mathrm{d}U(q)}{\mathrm{d}q}\right)_{q=0} \qquad \text{(A1)}$$

This provides an excellent approximation for the fixation probability of a new mutation, except in very small populations. From equation (1a), we have:

$$\frac{\mathrm{d}U(q)}{\mathrm{d}q} = \frac{G(q)}{\int_0^1 G(x)\,\mathrm{d}x} \qquad \text{(A2)}$$

where $G(q)$ is given by equation (1b).

But $G(0) = 1$, so that substituting from equation (A2) into (A1), we have:

$$U(q) \approx \frac{q}{\int_0^1 G(x)\,\mathrm{d}x} \qquad \text{(A3)}$$

$U(q)$ is thus proportional to q. For the case of *X*-linkage, U_f and U_m in equation (6b) are therefore proportional to $1/(3N_f)$ and $1/(3N_m)$, respectively, since the same integral appears in each of their denominators. The terms in N_f and N_m therefore cancel out, leaving the final expression:

$$K_{aX} \approx \frac{(2 + \alpha)\mu}{3\int_0^1 G(x)\,\mathrm{d}x}$$

Since $K_{sX} = (2 + \alpha)\mu/3$, it follows that K_{aX}/K_{sX} is independent of α.

Appendix A4.1: Perl scripts used

A4.1.1 Transforming the EST dataset into a table of expression profiles

The input for this section is the *D. melanogaster* EST dataset downloaded from the Unigene website. This consists, for each gene, of the gene identifier followed by its chromosomal location and the list of ESTs and the libraries they come from (other unnecessary information was removed beforehand). We have edited it by hand, so that the name of each EST library has been replaced by the tissue it was made from. Our script counts the number of ESTs from each tissue and outputs them to a table.

Input:

```
Genename1
Chromosome
Sequence count
EST1 EST2 EST3 EST4 … ESTn //
Genename2
Chromosome
Sequence count
EST1 EST2 EST3 EST4 … ESTn//
[…]
GenenameN
Chromosome
Sequence count
EST1 EST2 EST3 EST4 … ESTn //
```

Script:

```
#!/usr/local/bin/perl

open (GENES, "dm") or die "can't open dm: $!\n";
open (RESULT, ">>results") ;
print RESULT  "genename, chromosome, seqcount, ovary, testis, brain, head,
embryo, whole_body, salivary_gland, blood, gonad, other\n";
$/ = "\/\/\n";
while ($line = <GENES>) {
       $ovary = 0 ;
       $testis = 0 ;
       $brain = 0;
       $head = 0 ;
       $embryo = 0;
       $whole_body = 0;
       $salivary_gland = 0;
       $blood = 0;
       $gonad = 0;
       $other = 0;
       ($genename, $chromosome, $seqcount, $ests) = split("\n", $line);
       @ests = split(" ", $ests);
       if ($seqcount > 0) {
              foreach $est (@ests)  {
                     if ($est eq "ovary") { $ovary++ ;
```

```
                    }
                    elsif ($est eq "testis") { $testis++ ;
                    }
                    elsif ($est eq "brain") { $brain++ ;
                        }
                    elsif ($est eq "head") { $head++ ;
                        }
                    elsif ($est eq "embryo") { $embryo++ ;
                        }
                    elsif ($est eq "whole_body") { $whole_body++ ;
                        }
                    elsif ($est eq "salivary_gland") { $salivary_gland++ ;
                        }
                    elsif ($est eq "blood") { $blood++ ;
                        }
                    elsif ($est eq "gonad") { $gonad++ ;
                        }
                                                                        else
{ $other++ ;
                    }
             }
             print RESULT  "$genename, $chromosome, $seqcount, $ovary,
$testis, $brain, $head, $embryo, $whole_body, $salivary_gland, $blood, $gonad,
$other \n";
       }
       else { print STDOUT "$genename\n" ;
       }
}
```

Output: “results”

```
genename, chromosome, seqcount, ovary, testis, brain, head, embryo, whole_body,
salivary_gland, blood, gonad, other
fs(1)M3, X, 7, 2, 0, 0, 0, 0, 2, 0, 0, 0, 1
CG5966, X, 31, 0, 0, 0, 7, 0, 13, 0, 0, 0, 10
sqh, X, 121, 0, 1, 0, 25, 3, 25, 0, 3, 5, 58
Rpt4, X, 69, 1, 0, 0, 18, 3, 25, 0, 0, 1, 19
CG15892, X, 40, 0, 14, 0, 9, 2, 1, 0, 0, 0, 10
CG12219, X, 21, 1, 0, 0, 0, 0, 10, 0, 0, 0, 9
CG15893, X, 71, 4, 1, 0, 0, 0, 49, 0, 0, 0, 16
l(1)G0030, X, 135, 9, 7, 0, 21, 4, 20, 0, 3, 0, 68

[…]

fzy, 2L, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 1
```

A4.1.2 Matching sex-biased genes to their expression profile

We now have two files: the first is the list of male-, female- and unbiased genes that we downloaded from the SEBIDA website. The other is the table with the EST counts for each gene. A second script was used to, for each list of genes, find the matching gene in the table.

Input:

```
Input 1: list of male genes, "males.txt"
CG10014
CG10026
CG10029
CG10053
CG10064
CG10091
CG10124
CG10126
PpD5

[…]

CG9970

Input 2: the table "results", as described in previous section (A1).
```

Script:

```
#!/usr/local/bin/perl

open (LISTMALES, "males.txt") or die "can't open list of males: $!\n";
open (RESULTS, "results") or die "can't open list of males: $!\n";
open (MALEEXPRESSION, ">>maleexpression") ;
while ($genename = <LISTMALES>) {
       chop $genename ;
       push (@genenames, $genename);
}
while ($line = <RESULTS>) {
              ($gene, $others) = split(/, /, $line);
              foreach $genename (@genenames)  {
                     if ($genename eq  $gene) {
                     print MALEEXPRESSION $line;
                     }
                     else {
                     print MALEEXPRESSION "";
                     }
              }
}
```

Output: "maleexpression"

The output is a table similar to "results", but containing only male-biased genes. Similar scripts were used for female-biased and unbiased genes, and later for conserved and non-conserved genes.