

Trade-off Between Selection for Dosage Compensation and Masculinization on the Avian Z Chromosome

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ABSTRACT Following the suppression of recombination, gene expression levels decline on the sex-limited chromosome, and this can lead to selection for dosage compensation in the heterogametic sex to rebalance average expression from the X or Z chromosome with average autosomal expression. At the same time, due to their unequal pattern of inheritance in males and females, the sex chromosomes are subject to unbalanced sex-specific selection, which contributes to a nonrandom distribution of sex-biased genes compared to the remainder of the genome. These two forces act against each other, and the relative importance of each is currently unclear. The *Gallus gallus* Z chromosome provides a useful opportunity to study the importance and trade-offs between sex-specific selection and dosage compensation in shaping the evolution of the genome as it shows incomplete dosage compensation and is also present twice as often in males than females, and therefore predicted to be enriched for male-biased genes. Here, we refine our understanding of the evolution of the avian Z chromosome, and show that multiple strata formed across the chromosome over ~130 million years. We then use this evolutionary history to examine the relative strength of selection for sex chromosome dosage compensation vs. the cumulative effects of masculinizing selection on gene expression. We find that male-biased expression increases over time, indicating that selection for dosage compensation is relatively less important than masculinizing selection in shaping Z chromosome gene expression.

MALES and females of the same species are subject to distinct selective forces, often resulting in contradictory sex-specific selection pressures acting on a given locus. These selective forces have been shown to affect large portions of the genome (Connallon *et al.* 2010) and can place a significant genetic and evolutionary burden on a species (Chippindale *et al.* 2001; Arnqvist and Rowe 2005; Morrow *et al.* 2008). At the genetic level, sexually antagonistic selection pressures are thought to contribute to gene expression differences observed between the sexes in many species (Rice 1984; Connallon and Knowles 2005; Ellegren and Parsch 2007).

The effect of sex-specific selection is particularly evident on the sex chromosomes, where the unbalanced pattern of

inheritance creates uneven sex-specific selection pressures (Rice 1984; Charlesworth *et al.* 1987; Connallon and Clark 2010). Consequentially, the sex chromosomes are a hotspot of intralocus sexual conflict within the genome (Mank 2009; Innocenti and Morrow 2010), and unequal sex-specific selection is predicted to have contributed to the complex pattern of sex-biased gene distribution observed across the sex chromosomes of many animals (Parisi *et al.* 2003; Khil *et al.* 2004; Vicoso and Charlesworth 2006; Sturgill *et al.* 2007; Zhang *et al.* 2010a,b; Chen *et al.* 2011). Indeed, the avian Z chromosome exhibits a pervasive pattern of male-biased gene expression, which could be interpreted in the light of masculinizing selection for dominant male-benefit genes (Rice 1984; Ellegren *et al.* 2007; Itoh *et al.* 2007).

At the same time, sex chromosomes experience changes in gene dose. Due to lack of recombination with their homologs, Y and W chromosome gene activity slowly degenerates by neutral processes (Charlesworth 1996) and the buildup of nonsynonymous and nonsense mutations as well as small indels (Zhou and Bachtrog 2012), where the rate of decay declines with the number of functionally

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constrained loci (Bachtrog 2008). Gene expression loss shows a range of dominance (Agrawal and Whitlock 2011), and for some genes, loss-of-function mutations on the W or Y chromosome will cause negative fitness effects (Charlesworth 1978).

The resulting loss of gene activity in the heterogametic sex and thus unbalanced gene dose with the autosomes is predicted to be costly, especially for X- or Z-linked genes that interact with autosomal genes in large protein complexes (Pessia *et al.* 2012), thereby selecting for the evolution of dosage compensation mechanisms (Ohno 1967; Charlesworth 1978). Selection for dosage compensation can be strong, leading in some organisms to complex regulatory machinery to equalize gene dose across the entirety of the chromosome (Muyle *et al.* 2012; Pessia *et al.* 2012); however, more often selection acts on dosage sensitive genes rather than entire chromosomes (Mank and Ellegren 2009a; Vicoso and Bachtrog 2011; Pessia *et al.* 2012). In *Drosophila*, males hypertranscribe the single X chromosome (Lucchesi 1973), whereas in mammals a subset of X-linked genes appears to be up-regulated in both sexes and then down-regulated in females via X chromosome inactivation to restore balanced gene dose with the autosomes (Deng *et al.* 2011; Julien *et al.* 2012; Lin *et al.* 2012; Pessia *et al.* 2012). A by-product of the evolution of dosage compensation is that gene expression levels are also balanced between the heterogametic and homogametic sexes (Bachtrog *et al.* 2011; Mank *et al.* 2011).

Birds lack complete Z chromosome dosage compensation, and the majority of Z-linked genes are expressed more in males than females due to the fact that males have two copies of Z-linked loci and females only one (Ellegren *et al.* 2007; Itoh *et al.* 2007). For dosage-sensitive genes, the predicted cost of unbalanced gene dose with the autosomes selects for the evolution of dosage compensation mechanisms, such as hypertranscription of Z-linked dosage-sensitive genes in females, to restore balanced expression levels, and consequentially equalize expression between the sexes (Ohno 1967; Pessia *et al.* 2012). Therefore, selection for dosage compensation theoretically reduces male-biased expression on the Z chromosome, and this predicted outcome is opposite to what would be expected under a model of masculinizing selection for gene expression, where sex-specific differences in Z-linked expression are exacerbated.

The evolutionary history of the Z chromosome itself can be used to distinguish signatures of masculinizing selection from selection for dosage compensation. The avian Z chromosome resulted from at least two (Handley *et al.* 2004; Suh *et al.* 2011) recombination cessation events over the last 130 million years, resulting in strata of different ages. These strata are subject to unbalanced sex-specific selection and selection for dosage compensation, for different lengths of time (Charlesworth *et al.* 2005).

Consequently, patterns of sex-biased expression between the different strata will reflect the selective regime to which the Z chromosome has been subject. Under selection for

dosage compensation with no masculinization, Z-linked expression in the youngest stratum is predicted to reflect the ratio of expression from two copies in males to only one in females. Changes in gene dose show a range of expression level effects (Torres *et al.* 2008), and twofold change in human gene copy number produces an average fold change in expression of 1.5 (Pollack *et al.* 2002), consistent with the sex-biased expression ratio observed under lack of dosage compensation (Deng *et al.* 2011; Pessia *et al.* 2012), potentially due to the robust nature of gene networks (Oliver 2007). Therefore, dose effects without masculinization are expected to have, on average, 1.5-fold effects on expression. Z-linked expression is predicted to become increasingly less male biased in older regions where effective compensation mechanisms have evolved. However, the opposite pattern is expected under masculinizing selection. In this scenario, the extent of male-biased expression should increase with the age of the stratum and thus length of exposure to cumulative masculinizing selection.

Here we expand and refine our understanding of the topology of the chicken Z chromosome through the addition of several newly discovered Z-W orthologs (gametologs), and find evidence for up to four evolutionary strata. We then use this evolutionary history to test these two predictions for gene expression evolution.

Materials and Methods

Identification of misassembled W-linked genes

To expand the known coding content of the chicken W chromosome, we used RNA-seq data obtained from fertilized Red Jungle Fowl eggs (T. Pizzari, Oxford University), kept under standard incubator conditions. After collecting the left gonad at embryonic day (ed) 19, we stored the tissue in RNAlater until preparation. Previous work has shown this stage is just prior to meiotic sex chromosome inactivation (MSCI) in chickens, which affects both the W and Z chromosomes (Schoenmakers *et al.* 2009). Therefore, we used ed19 gonads to maximize the number of active W genes and minimize the risk that our Z expression estimates are confounded by MSCI. Detailed methods for the discovery of new W-linked genes are described elsewhere (Moghadam *et al.* 2012). Briefly, we extracted RNA from four samples of each sex using standard purification methods, which was then used for Illumina RNA-seq at the Wellcome Trust Centre for Human Genetics facility at Oxford University, resulting in 16 million 50-bp paired-end mappable reads on average per sample. We mapped sequences to the chicken reference genome (Washington University Genome Sequencing Center, WUGSC 2.1/galGal3) using Bowtie (v.0.12.7) (Langmead *et al.* 2009) and Tophat (v.1.1.1). To estimate transcript abundances for the Ensembl annotated genes we used Cufflinks (v.0.9.3) (Trapnell *et al.* 2010) and identified putative W-linked genes through *in silico* analysis of the gene expression profiles between

males and females. We validated that these genes had been misassembled in the current Ensembl annotation (WUGSC 2.1/galGal3) and were indeed W-linked by PCR genotyping of genomic DNA (Moghadam *et al.* 2012).

Identification and divergence of Z-W orthologs

In total, we had sequence data on 14 known and 12 newly identified W-linked genes from Ensembl, some of which represented multiple orthologs of Z-linked loci. We BLASTed (Altschul *et al.* 1997) sequences against the chicken genome sequence (WUGSC 2.1/galGal3) to identify orthologs on the Z chromosome. We took the physical positions of these orthologs from the genome assembly. There was one exception, ENSGALG00000004349, which does not have any annotated Z-linked orthologs in Ensembl.

Synonymous divergence estimates can be used to identify the number and boundaries of evolutionary strata, as they provide an appropriate measure of the length of time over which gametologs have differentiated from an original ancestral autosomal pair. We therefore calculated synonymous divergence (d_s) for each gametologous gene pair using whole coding sequences for chicken genes downloaded from Biomart with the exception of SPINZ and SPINW. The SPIN gametologs are poorly annotated; therefore, we identified and excluded incorrect exon annotations from the analysis by BLASTing the coding sequences against the National Center for Biotechnology Information Expressed Sequence Tag library. After translating coding sequences for Z-W orthologs to protein sequences, we used PRANK (<http://tinyurl.com/prank-msa>) to align gametologs. We checked alignments by eye, and removed poorly aligned regions where at least 50% of the amino acids were mismatched. PRANK's method of distinguishing insertions from deletions avoids overmatching of aligned sequences, sequentially producing more evolutionary accurate alignments for increasingly diverged sequences in comparison to ClustalW (Loytynoja and Goldman 2005; Tamura *et al.* 2011). However, in line with previous studies, to check for consistency we repeated alignments using ClustalW in MEGA5 (Tamura *et al.* 2011). We used CODEML in the PAML package version 4.4 (Yang 2007) to calculate maximum likelihood estimates of synonymous (d_s) and nonsynonymous (d_n) divergence in pairwise comparisons and generate standard errors with the curvature method. In one case, we removed ENSGALG00000013670 from the analysis to avoid biasing our results, as d_s was found to be greater than two for the multiple Z-linked orthologs, making accurate divergence estimates in avian coding sequence impossible due to mutational saturation and double hits (Axelsson *et al.* 2008). As problems with the use of PAML to calculate d_s values have been documented (Bierne and Eyre-Walker 2003) we also used an additional method, *K*-estimator 6.1 (Comeron 1995, 1999), to verify our synonymous divergence estimates for the 18 Z-W orthologs.

Some Z-W orthologs had multiple W or Z paralogs. To identify the true gametolog, we calculated d_s individually

for each potential orthologous gene pair and used the gene pair with the lowest d_s estimate in the final results. The surplus genes were excluded. This is different from previous methods that employed a consensus sequence (Nam and Ellegren 2008), but avoids potential problems of relaxed or diversifying selection acting on gene copies (Graur and Li 2000) likely due to gene duplications following recombination suppression, which can result in significant divergence in both gene sequence and expression (Busby *et al.* 2011). This approach and the subsequent exclusion of multicopy genes meant that while we had sequence data for 26 W-linked genes, our conclusions regarding the number and boundaries of strata were based on d_s estimates from 18 true Z-W gametologs.

Gene conversion (Slattery *et al.* 2000; Ross *et al.* 2005) between differentiated Z-W orthologs has the potential to bias our estimates of d_s by homogenizing the two independently evolving sequences. To ensure this did not influence our identification of true gametologs, we calculated the GC content at the third codon position of Z-linked genes (GC3) and their multiple candidate W orthologs using the R package seqinR (Charif and Lobry 2007). GC content and recombination rate are positively correlated in vertebrates (Galtier 2003; Meunier and Duret 2004), leading to the prediction that, in the absence of gene conversion, the GC3 content of W-linked genes should not exceed that of Z-linked gametologs. We obtained 95% confidence intervals with bootstrapping of 1000 replicates.

Additionally, we used GENECONV as an independent test of gene conversion (<http://www.math.wustl.edu/~sawyer>) between Z-W gametologs, using *Anolis carolinensis* as an outgroup. For the 18 Z-linked genes, we identified 16 *Anolis* orthologs and downloaded the longest coding transcript from BioMart. We used PRANK (<http://tinyurl.com/prank-msa>) to generate alignments and GENECONV to identify aligned global fragments for which gametologs are sufficiently similar to be suggestive of past gene conversion. GENECONV assigns *P*-values by two methods, the first based on 10,000 random permutations and the second by a method of Karlin and Altschul (1990, 1993). Both methods are corrected for multiple comparisons and sequence length when detecting global fragments.

We mapped d_s estimates to the Z chromosome and used four criteria to delineate stratum boundaries: (1) average and range of d_s in physically proximate regions, (2) *k*-means clustering of d_s estimates into a defined number of groups in R (Forgy 1965; MacQueen 1967; Hartigan and Wong 1979; Lloyd 1982; www.R-project.org), (3) Z-W ortholog density, and (4) physical location on the Z chromosome. To verify the refined strata, we used one-tailed *t*-tests to test our explicit predictions regarding the increase in d_s estimates with strata age. Although this is a biased estimate, it is useful as a means of comparison with *k*-means clustering results.

We were also able to use d_s estimates to calculate divergence dates for Z-W orthologs with a molecular clock that accounts for sex-specific mutation rates in Z and W

chromosome lineages based on male-mutation bias estimates in birds. In galliforms, the W chromosome rate is approximately half the autosomal rate (Axelsson *et al.* 2004). Based on avian and mammalian divergence dates, the autosomal mutation rate for the chicken lineage is estimated to be 2.5×10^{-9} /site/year (Dimcheff *et al.* 2002; Hedges 2002; Webster *et al.* 2006; Nam and Ellegren 2008) and correspondingly 1.25×10^{-9} /site/year for the W chromosome. Previous work in galliforms indicates the Z chromosome mutation rate is only slightly larger than the autosomal rate leading to an estimate of 2.6×10^{-9} site/year (Nam and Ellegren 2008). The Z and W mutation rates mentioned above are therefore consistent with previous studies that estimate male mutation bias in galliforms as lying between 2 and 4 (Harlid *et al.* 2003; Berlin *et al.* 2006). These estimates result in a chicken Z-W divergence rate of 3.8×10^{-9} /site/year (Z chromosome mutation rate = 2.6×10^{-9} + W chromosome mutation rate = 1.2×10^{-9}) and divergence time of $d_s \times 3.8 \times 10^{-9}$ (Nam and Ellegren 2008) years, although this has a high variance.

To enhance our understanding of the formation of avian strata through time, we used synteny with the turkey, which diverged from the chicken ~ 30 MYA (Dimcheff *et al.* 2002). Genomic rearrangements are not frequent across the avian genome and birds therefore show highly conserved synteny across vast evolutionary distances (Backstrom *et al.* 2010; Warren *et al.* 2010). For the 18 Z-linked genes used to identify chicken strata, we identified 1-1 turkey orthologs and their position along the Z chromosome from Ensembl (Turkey_2.01/ GCA_000146605.1), and plotted them against Z-linked positional information from the chicken.

Comparing expression across strata

After defining putative strata boundaries based on d_s estimates, we used fragments per kilobase of exon per million mapped reads (FPKM) values obtained from the left gonad RNA-Seq data to compare expression among strata. The assembly method is discussed above under *Identification of misassembled W-linked genes*. We filtered the expression dataset to exclude any genes not expressed in at least four individuals, and all genes within the male-hypermethylated region (MHM), located between 2.5 Mb and 3.5 Mb (Teranishi *et al.* 2001). The MHM was excluded to avoid biasing our comparison of expression between strata, as microarray analysis indicates that this region is strongly downregulated in males, a markedly different pattern from the rest of the Z chromosome (Melamed and Arnold 2007).

With the filtered dataset, we separately averaged female and male FPKM values for each stratum and calculated male-biased expression as a function of \log_2 (male FPKM/female FPKM) = \log_2 (male FPKM) – \log_2 (female FPKM). We compared average male-biased expression among strata using permutation tests with 1000 replicates and calculated 95% confidence intervals using bootstrapping with 1000 replicates.

In addition, as dosage compensation acts to equalize expression across the Z chromosome and autosomes, we calculated the Z:A expression ratio separately for males and females to test for signatures of this selective regime. We calculated average male and female expression separately for all Z-linked genes (minus the MHM) and autosomal genes and finally a Z:A expression ratio for each sex. This was also repeated separately for each stratum. The 95% confidence intervals were calculated by bootstrapping male and female Z-linked and autosomal averages with 1000 replicates.

We were also able to calculate and compare the rate of masculinization of the Z chromosome under both logarithmic and linear functions to describe the relationship between average stratum age and male-biased expression. In addition, to verify that the relationship we observe is not due to chance, we randomized the ratio of male to female gene expression across the Z chromosome (1000 replicates). Each time, we recalculated average male-biased expression for each stratum to obtain a distribution of randomized r^2 values.

Gene function analysis

To determine whether nonrandom distributions of gene function were contributing to gene expression differences, we used GOrilla to perform a gene ontology (GO) enrichment analysis between each stratum and the entire Z chromosome (minus the MHM), and between the Z chromosome (minus the MHM) and autosomes (Eden *et al.* 2007, 2009). We conducted each GOrilla analysis with 1-1 mouse orthologs, a threshold of $P < 10^{-2}$ using standard hypergeometric statistics and the Benjamini and Hochberg correction for multiple tests (Benjamini and Hochberg 1995).

Results

Resolving stratum boundaries

As synonymous estimates provide an appropriate measure of the length of time over which Z-W orthologs have differentiated from an original autosomal pair, and this method has been used in several organisms to establish the number and boundaries of evolutionary strata (Lahn and Page 1999; Ellegren and Carmichael 2001; Handley *et al.* 2004; Bergero *et al.* 2007; Nam and Ellegren 2008). We therefore estimated d_s values for 18 Z-W gametologs (Table 1), 6 of which were previously unknown, representing a 50% increase in the known number of Z-W orthologs. With the exception of the HINTW gene, which is known to have undergone adaptive molecular evolution in birds (Handley *et al.* 2004), d_N estimates ranged from 0.0002 to 0.0831, suggesting a high degree of conservation between Z-W gametologs. Our d_s estimates ranged from 0.128 to 0.513. Alignments repeated for consistency using ClustalW were extremely similar to those generated by PRANK, with the

Table 1 Sequence divergence between chicken Z-W orthologs

Gene pair (Z/W)	Ensembl ID (Z/W) ^a	Position on Z (Mb)	d_s (SE) ^b	d_N (SE) ^b	Putative stratum	Divergence (MYA)
ST8SIA3Z/ ST8SIA3W	03049/ 22039	0.317	0.184 (0.0319)	0.007 (0.0035)	3	48
ZNF532Z/ ZNF532W	02852/ 14003	0.747	0.183 (0.0171)	0.043 (0.0045)	3	48
RPL17Z/ RPL17W	02696/ 22174	0.965	0.169 (0.0212)	0.000 (0.0000)	3	44
SMAD2Z/ SMAD2W	14697/ 10056	1.290	0.183 (0.0679)	0.018 (0.0107)	3	48
8030462N17RikZ/ 8030462N17RikW	01763/ 01585	1.874	0.172 (0.0311)	0.083 (0.0126)	3	45
ATP5A1Z/ ATP5A1W	14644/ 01756	1.938	0.203 (0.0284)	0.029 (0.0061)	3	54
UBAP2Z/ UBAP2W	13809/ 05785	6.876	0.206 (0.0176)	0.056 (0.0054)	3	54
VCPZ/ VCPW	01986/ 00386	7.932	0.189 (0.0231)	0.009 (0.0017)	3	50
GOLPH3Z/ GOLPH3W	03151/ 18586	9.013	0.128 (0.0340)	0.007 (0.0052)	3	34
ZFRZ/ ZFRW	03235/ 14545	9.104	0.164 (0.0160)	0.020 (0.0031)	3	43
NIPBLZ/ NIPBLW	03605/ 22678	10.720	0.147 (0.0524)	0.018 (0.0103)	3	39
MIER3Z/ MIER3W	14721/ 00140	16.837	0.219 (0.0619)	0.044 (0.0136)	2b	58
hnRNPkZ/ hnRNPkW	12591/ 14366	39.553	0.269 (0.0350)	0.004 (0.0021)	2b	71
SPINZ/ SPINW	14916/ 14641	42.572	0.224 (0.0534)	0.012 (0.0062)	2b	59
HINTZ/ HINTW	00428/ 22685	44.169	0.513 (0.1144)	0.314 (0.0459)	1	135
CHD1Z/ CHD1W	14642/ 15278	50.156	0.500 (0.0357)	0.047 (0.0044)	1	132
KCMF1Z/ KCMF1	15391/ 14441	52.658	0.286 (0.0385)	0.040 (0.0072)	2a	75
RASA1Z/ RASA1W	15639/ 22611	59.665	0.275 (0.0597)	0.034 (0.0105)	2a	72

^a Ensgalg000000...^b Standard errors generated by taking the inverse of the second derivative of the log likelihood in Paml.

exception of 4 gametologs. This is because PRANK's treatment of insertions and deletions, in comparison to ClustalW, results in the productions of more evolutionarily accurate alignments for increasingly diverged sequences (Loytynoja and Goldman 2005; Tamura *et al.* 2011). However, for previously identified orthologs, our d_s estimates after alignment with PRANK were qualitatively identical to prior work (Nam and Ellegren 2008). To verify our synonymous divergence estimates, we used *K*-estimator 6.1 (Comeron 1995, 1999) to calculate K_s estimates for 18 Z-W orthologs. With the exception of four gene pairs, K_s was qualitatively identical (<0.03 different) to our d_s estimates, providing independent evidence for the multiple cessation of recombination across the avian Z chromosome.

Our results do differ from previous studies in some instances where multiple W-linked genes share the same Z-linked orthologs and vice versa, likely due to gene duplications following recombination suppression. This is because we used the copy with the lowest d_s estimate to

avoid problems with artificially high values due to relaxed selection acting on gene duplicates (Ridley 2003). As a consequence of this approach, our d_s estimates at three locations along the Z chromosome are somewhat lower than in previous studies (Nam and Ellegren 2008), reducing slightly our estimate of when recombination ceased to form stratum 2. Additionally, newly identified Z-W orthologs extended our analysis across a wider region of the Z chromosome, allowing a reassessment of the number and boundaries of strata.

A comparison of GC content at the third codon position (GC3) between these Z-W orthologs revealed no significant difference in GC3, indicating that the lowest d_s estimates are not a result of biased gene conversion and can be used to infer true gametologs. We were also able to use GENECONV (<http://www.math.wustl.edu/~sawyer>) to identify aligned global fragments for which gametologs are sufficiently similar to be suggestive of past gene conversion. We used *A. carolinensis* as an outgroup, for which 16 of the 18 Z-W gametologs had *Anolis* orthologs. Of the 16 gametologs, 9

have not undergone gene conversion. However, there was limited evidence of gene conversion for 7 gametologs, with 1 significant inner fragment detected for Ensgalg00000014003 (permutation P_1 value = 0.002, Karlin and Altschul P_2 value = 0.003), Ensgalg00000001756 (P_1 value = 0.007 and P_2 value = 0.012), Ensgalg00000005785 (P_1 value <0.001 and P_2 value <0.001), Ensgalg00000014545 (P_1 value = 0.032 and P_2 value = 0.040), Ensgalg00000022678 (P_1 value <0.001 and P_2 value = 0.011), and Ensgalg00000022685 (P_1 value <0.001 and P_2 value <0.001). Significant inner fragments are evidence of a possible gene conversion event between ancestors of the gametologs, but GENECONV also detects outer fragments that represent conversion events originating from outside the alignment or within the sequence but where evidence has been destroyed by later mutation or gene conversion. Three significant outer fragments were detected for the Z-linked ortholog of Ensgalg00000014003 (P_1 value <0.001, 0.003, and 0.036; P_2 value <0.001, 0.004, and 0.038) and one for Ensgalg00000014545 (P_1 value = 0.006 and P_2 value = 0.008) and Ensgalg00000000386 (P_1 value <0.001 and P_2 value <0.001). Of the 7 gametologs with evidence of gene conversion, 6 of these are located in the youngest stratum and 1 in the oldest. Genes within the youngest stratum have the largest number of multiple copies as the cumulative strength of degenerative forces are weakest, and W-linked degeneration is minimal compared to older strata. Correspondingly, we expect a certain degree of intrachromosomal recombination for these genes (Rozen *et al.* 2003; Backstrom *et al.* 2005; Davis *et al.* 2010), which might bias GENECONV's detection of conversion events between Z and W orthologs and explain the nonrandom distribution of genes with evidence of gene conversion across the strata. In line with this, four of the seven W-linked genes for which GENECONV detected significant fragments are present in multiple copies or have a W-linked paralog.

Overall, synonymous divergence estimates cluster into at least three groups, (Handley *et al.* 2004; Nam and Ellegren 2008; Suh *et al.* 2011); however, we also find evidence for a potential fourth stratum located within the previously identified stratum 2 (Figure 1). These strata correspond to 44–50 Mb (d_S = 0.500–0.513, stratum 1), 50–60 Mb (d_S = 0.275–0.286, stratum 2a), 17–44 Mb (d_S = 0.219–0.269, stratum 2b), and 0.3–11 Mb (d_S = 0.128–0.206, stratum 3). The physical separation of stratum 2a and 2b by stratum 1 suggests that recombination suppression occurred in these regions in quick succession.

Genes within stratum 1 have a significantly higher d_S value than stratum 2 (P < 0.001, *t*-test), supporting the notion that recombination ceased first in this region. Our d_S estimates also differ significantly between putative strata 2a and 2b (P = 0.050, *t*-test), indicating recombination was suppressed independently twice in close succession. Finally, recombination ceased most recently in stratum 3, where d_S values are significantly lower than strata 1 (P < 0.001, *t*-test), 2a (P < 0.001, *t*-test), and 2b (P < 0.001, *t*-test).

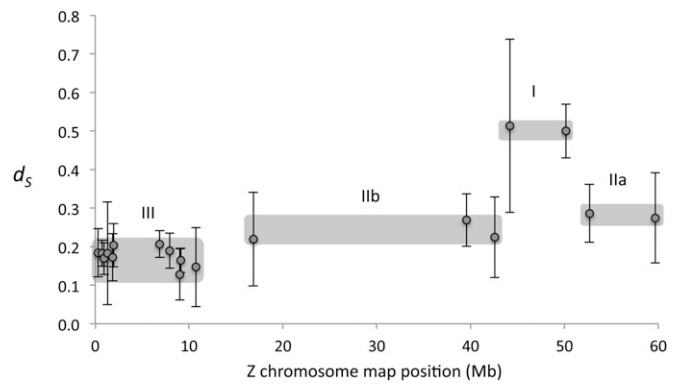


Figure 1 Evolutionary history of the chicken Z chromosome. The distribution of synonymous divergence estimates (d_S) for Z-W orthologs is shown. Physical position on the Z chromosome is based on current Ensembl genome assembly (WUGSC 2.1/galGal3). The 95% confidence intervals are based on 1000 bootstrap replicates. Synonymous divergence estimates cluster into up to four groups, which differ significantly from each other, providing support for the existence of multiple Z chromosome strata.

The results from the *k*-means analysis (Forgy 1965; MacQueen 1967; Hartigan and Wong 1979; Lloyd 1982; www.R-project.org) provide further support for multiple recombination cessation events. We used *k*-means to partition our d_S estimates into three clusters. The three groups defined by *k*-means are identical to the groups supported by the *t*-tests, providing an independent line of evidence to support the boundaries of strata 1, 2, and 3 corresponding to 44–50 Mb (stratum 1), 17–60 Mb (stratum 2), and 0.3–11 Mb (stratum 3). Due to the very short divergence interval between strata 2a and 2b, the power of *k*-means to identify these as independent clusters is weakened.

In addition to significant clustering of d_S estimates, the density of genes within each region provides independent evidence to identify strata (Lahn and Page 1999). The suppression of recombination between Z-W orthologs promotes the degeneration of W-linked genes by neutral processes, the rate of which declines with the number of functionally constrained loci (Bachtrog 2008). As recombination ceased most recently in the youngest stratum, the cumulative strength of degenerative forces are weakest, and we predict minimal W-linked degeneration compared to older stratum. Correspondingly, the largest density of identifiable gametologs is expected in the youngest stratum. Gene density across the chicken Z chromosome is consistent with this prediction (Figure 2) where the youngest stratum (1.06 genes per megabase) has a gene density of gametologs at least three times larger than the oldest (0.33 genes per megabase). The intermediate stratum has a gene density of 0.22 genes per megabase, excluding the MHM. Reasons for excluding the MHM are discussed below. Furthermore, the majority of the multigene copies excluded from the analysis were located within the youngest stratum.

As gene conversion has the potential to bias our identification of stratum boundaries, we repeated the one-tailed

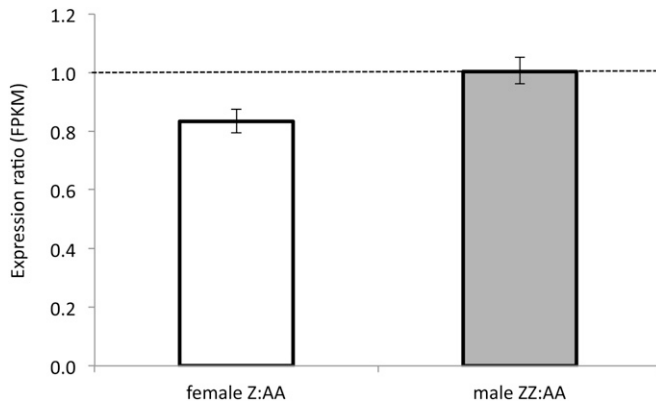


Figure 2 Comparison of gene expression across the Z chromosome and autosomes. The average Z:A expression ratio is shown for males and females separately. The 95% confidence intervals (based on 1000 bootstrap replicates) are shown. Dotted line represents the expression ratio expected under equal gene dose.

t-tests and *k*-means clustering after excluding the 7 Z-W orthologs with evidence of gene conversion. *K*-means clusters the 11 remaining gametologs into the same three groups corresponding to strata 1, 2, and 3. There is also still a significant difference between d_s estimates between strata 2 and 3 ($P < 0.001$, *t*-test), 2a and 3 ($P < 0.001$, *t*-test), 2b and 3 ($P = 0.001$, *t*-test), as with the previous analysis; however, there is now no significant difference between strata 2a and 2b ($P = 0.051$, *t*-test). As only one gene pair remains in stratum 1 after the 7 gametologs are removed, we could not conduct any tests of significance for this group. The consistency between strata boundaries defined from analyses excluding and including gametologs subject to gene conversion indicates our results are not biased by gene conversion.

Molecular clock estimates show that stratum 1 arose between 132 and 135 MYA, stratum 2a between 72 and 75 MYA, stratum 2b between 58 and 71 MYA, and stratum 3 between 34 and 54 MYA.

Sixteen of the 18 chicken Z-linked orthologs were identified on the turkey Z chromosome, of which 7 are 1-1 orthologs. Synteny of these Z-linked orthologs is conserved between both species, indicating a lack of lineage-specific genomic rearrangements.

Gene expression and the role of dosage compensation and masculinizing selection

We were able to calculate and compare average sex-biased expression for the 33 transcribed genes located within stratum 1, 154 in stratum 2 (41 genes in stratum 2a and 113 genes in stratum 2b), and the 101 genes in stratum 3 (Table S1). The three established strata show clear differences in sex-biased expression (Table 2), with a progressive stepwise relationship where stratum 1 has the greatest degree of male-biased expression, stratum 2 is intermediate in male-biased expression, and stratum 3 shows the lowest level of average male bias. Interestingly, the extent of male

bias in stratum 3 (1.51 unlogged FPKM) is consistent with previous estimates of sex-bias associated with uncompensated genes on the mammalian X chromosome (Deng *et al.* 2011; Pessia *et al.* 2012). As stratum 3 is the youngest and so the cumulative effects of degenerative forces are weakest, this sex bias likely reflects the ancestral expression level associated with incomplete dosage compensation (Table 2). This pattern is strongly indicative of masculinizing selection acting on the avian Z chromosome in a cumulative manner, with increasing male bias accruing over time. This is opposite to predictions under selection for dosage compensation, which would produce a decrease in male-biased expression as a function of stratum age.

The differences in male-biased expression between strata 1 and 2 ($P = 0.021$, permutation test with 1000 replicates), strata 2 and 3 ($P < 0.001$, permutation test with 1000 replicates), and strata 1 and 3 ($P < 0.001$, permutation test with 1000 replicates) are all statistically significant. The division of stratum 2 into two putative strata follows the same pattern of successive masculinization of the Z chromosome (Table 2), providing further support to the idea that recombination ceased independently four times. Stratum 2a has a higher average male bias than stratum 2b as predicted; however, due to the very short divergence interval we would not expect this difference to be significant ($P = 0.247$, permutation test with 1000 replicates). Despite this, the pattern of male-biased expression is still strongly indicative of masculinizing selection, with male bias in both strata 2a and 2b greater than stratum 3 ($P < 0.001$ and $P < 0.001$, permutation tests with 1000 replicates) but smaller than stratum 1 ($P = 0.03$ and $P = 0.023$, permutation tests with 1000 replicates).

We excluded the MHM from stratum 2b in the analyses described above to avoid biasing our comparison of expression between strata, as microarray analysis indicates that this region is strongly downregulated in males, a markedly different pattern from the rest of Z chromosome (Melamed and Arnold 2007). Although the expression of the MHM is not representative of the Z chromosome, when included in the analysis, average male bias in stratum 3 is still significantly lower than stratum 2 ($P < 0.001$, permutation test with 1000 replicates), leaving the significant relationship between male-biased gene expression and age largely unchanged.

Results from a GOrilla analysis (Eden *et al.* 2007, 2009) suggest the observed difference in expression between the strata is not due to a nonrandom distribution of genes based on function. A comparison of each stratum to the whole Z chromosome revealed no significant enrichment of GO terms after correction for multiple testing ($P < 10^{-2}$). Even before correction, only strata 2b and 2 were enriched for any GO terms; [stratum 2b = behavior and negative regulation of multicellular organismal processes ($P = 2.25 \times 10^{-4}$ and $P = 6.84 \times 10^{-4}$), stratum 2 = behavior ($P = 2.10 \times 10^{-4}$)]; however, none of these terms were significant after the Benjamini and Hochberg correction.

Table 2 Sex-biased gene expression across Z chromosome strata

Stratum	Estimated age (MYA)	No. of expressed genes	Male-bias (log ₂ male FPKM–female FPKM) (unlogged male FPKM–female FPKM)
1	133	33	0.86 (2.10)
2 ^a	67	154	0.64 (1.80)
2a	73	41	0.69 (1.86)
2b ^a	63	113	0.63 (1.77)
3	46	101	0.33 (1.51)

^a Genes from the male hypermethylated region excluded.

Additionally, when the Z chromosome is compared to the autosomes, there is no significant enrichment of GO terms ($P < 10^{-2}$) after the Benjamini and Hochberg correction. Before correction for multiple testing, the Z chromosome is enriched for thrombin receptor signaling pathway, oncostatin-M-mediated signaling pathway, positive regulation of peptidyl-tyrosine phosphorylation, positive regulation of Rho protein signal transduction, protein–lipid complex subunit organization, plasma lipoprotein particle organization, positive regulation of Ras protein signal transduction, regulation of biological quality, positive regulation of cell migration, positive regulation of positive chemotaxis, thrombin receptor activity, cytokine receptor activity, signal transducer activity, and transmembrane signaling receptor activity, intrinsic to plasma membrane ($P = 1.77 \times 10^{-5}$, $P = 8.42 \times 10^{-5}$, $P = 2.03 \times 10^{-4}$, $P = 3.26 \times 10^{-4}$, $P = 3.88 \times 10^{-4}$, $P = 3.88 \times 10^{-4}$, $P = 4.66 \times 10^{-4}$, $P = 5.43 \times 10^{-4}$, $P = 8.89 \times 10^{-4}$, $P = 9.47 \times 10^{-4}$, $P = 3.98 \times 10^{-5}$, $P = 8.42 \times 10^{-5}$, $P = 4.54 \times 10^{-4}$, $P = 4.54 \times 10^{-4}$, $P = 6.39 \times 10^{-4}$, and $P = 8.60 \times 10^{-4}$). However, it is not apparent how these processes would explain the unique expression pattern observed.

As dosage compensation acts to equalize expression across the Z chromosome and autosomes, calculating the Z:A expression ratio separately for males and females is essential for accurately assessing the selective forces driving expression evolution. We were able to calculate average male and female expression for 492 Z-linked genes (minus the MHM) and 10,530 autosomal genes and show that the Z:A expression ratio differs significantly between the sexes (Figure 2). The female Z:A ratio is consistent with incomplete dosage compensation observed in previous studies (Ellegren *et al.* 2007; Itoh *et al.* 2007, 2010), whereas the male ratio reflects the ZZ:AA karyotype. There is no significant difference in female and male Z:A ratio across strata, and this may be due to the variation in overall expression level among individual genes, which would be expected to have a strong stochastic effect in strata with relatively few genes.

Rate of masculinization

The degree of male bias increases across the Z chromosome as a function of time since divergence from the orthologous

W chromosome (Figure 3, A and B). We found a stronger correlation between stratum age and degree of sex bias under a logarithmic rather than a linear function for both the three strata ($r^2 = 0.93$) and four strata ($r^2 = 0.89$) models of Z chromosome evolution. Consequentially, this suggests an upper limit to the increase in masculinization of Z-linked gene expression over evolutionary time.

We randomized gene expression across the Z chromosome (1000 replicates) to assess the probability that the observed differences in sex bias are due to chance alone. Our observed r^2 values for the positive logarithmic relationship between stratum and sex-biased expression was significant ($P = 0.04$), but the linear relationship was not ($P = 0.08$), reinforcing our results that the rate of masculinization declines over time.

Discussion

The Z chromosome could potentially be subject to two different sex-specific selection regimes. Due to the unequal inheritance pattern of the chromosome, male-specific selection for dominant alleles is predicted to drive the evolution of the Z chromosome coding content (Rice 1984), leading to the accumulation of male-benefit alleles. Correspondingly, masculinizing selection acting on gene expression (Connallon and Clark 2010) is expected to increase male bias over time.

Loss-of-function mutations for W-linked genes is expected to cause negative fitness effects (Charlesworth 1978). The resulting loss of gene activity in females, and thus unbalanced gene dose with the autosomes, is predicted to be costly, especially for dosage-sensitive Z-linked genes that interact with autosomal genes in large protein complexes (Pessia *et al.* 2012). This is predicted to result in selection for hypertranscription of dosage-sensitive genes to equalize expression with the autosomes (Ohno 1967). If selection for dosage compensation were cumulative, we would expect it to result in a pattern opposite to that produced by masculinizing selection, as an indirect effect of selection for dosage compensation is to decrease sex-biased expression on the Z over time.

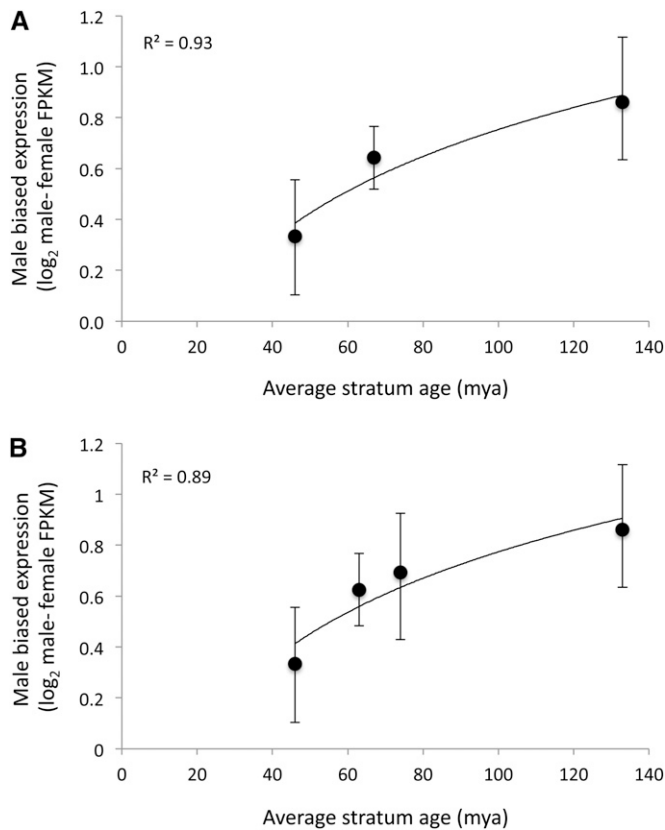


Figure 3 Rate of masculinization of the Z chromosome. The degree of male-biased expression for inferred Z chromosome strata is shown for both three strata (A) and four strata (B) models of Z chromosome evolution. The 95% confidence intervals (based on 1000 bootstrap replicates) are shown. Masculinization of Z-linked gene expression increases as a function of age, but ultimately levels out, thereby limiting the role of the Z chromosome in the evolution of sexual dimorphism.

The Z chromosome has been previously observed to show a pervasive pattern of male-biased expression (Kaiser and Ellegren 2006; Storchova and Divina 2006; Mank and Ellegren 2009a); however, the relative role of masculinizing selection vs. incomplete dosage compensation in driving this pattern has been difficult to untangle. Here we employ a novel method to detect signatures of sex-specific selection from selection for dosage compensation. Our results indicate that masculinizing selection predominates over selection for dosage compensation, and we were able to calculate the rate of masculinization using the evolutionary history of the chicken Z chromosome itself. Our analysis includes 18 Z-W gametologs; a sizable dataset in line with mammalian studies (Lahn and Page 1999) and to date the largest in birds, as well as 492 expressed genes along the Z chromosome.

Definition of Z chromosome strata

This study is reliant on accurate knowledge of the number and precise boundaries of strata, and using synonymous divergence estimates for newly identified Z-W orthologs together with gene density we were able to re-examine the

chicken strata across a wider region of the Z chromosome than in previous studies. In doing so, we found support for the existence of up to four strata along the chicken Z chromosome. Our data suggest that recombination ceased first in the 44- to 50-Mb region of the Z chromosome, between 132 and 135 MYA (stratum 1). Then, in rapid succession, d_s estimates indicate recombination ceased in the 53- to 60-Mb region between 72 and 75 MYA (stratum 2a) and the 17- to 43-Mb region ~58–71 MYA (stratum 2b). The final suppression occurred in the 0.3- to 11-Mb region most recently, between 34 and 54 MYA (stratum 3), as shown in Figure 1. The large reduction in gene density across the oldest and intermediate strata compared to stratum 3 provides additional evidence for up to four chicken strata. In line with patterns of gene density observed across the mammalian X chromosome (Lahn and Page 1999), the number of gametologs is predicted to decline with age of the strata where the cumulative strength of degenerative forces is weakest and W-linked divergence is minimal. This decay is not predicted to follow a linear progression, but instead should decline with the number of functionally constrained loci (Bachtrog 2008).

The identification of a possible fourth stratum may suggest that recombination suppression occurred on both sides of stratum 1 in close succession, rather than requiring a suppression event for stratum 2 followed by a subsequent structural rearrangement across the boundaries of strata 1 and 2 leading to a nonlinear distribution of strata (Nam and Ellegren 2008; Bergero and Charlesworth 2009). The conservation of relative gene order between turkey and chicken Z-linked orthologs argues against a chicken-specific inversion, but does not rule out the possibility of this event occurring before the two lineages diverged. Therefore, our model to describe the successive formation of avian strata does not evoke additional cessation of recombination events but instead proposes an alternative arrangement of these events over evolutionary time.

Successive masculinization of the Z chromosome

By comparing sex-biased expression among the chicken strata, we uncover a pattern consistent with cumulative masculinizing selection acting on gene expression across the Z chromosome, where the magnitude of male-biased expression correlates closely with stratum age. Masculinization may have occurred to some extent in the youngest stratum, which ceased to recombine with the W chromosome between 34 and 54 MYA; however, the effect is greater in the older strata. This logarithmic relationship holds when both three ($r^2 = 0.93$) and four strata ($r^2 = 0.89$) are considered and is supported by randomization tests ($P = 0.04$). The significance of the randomization test, although marginal, is convergent with the correlation between stratum age and male-biased expression level. The cumulative masculinization of Z chromosome expression is likely due to the effect of male-specific selection, as the Z chromosome is twice as often present in males than females; therefore,

dominant alleles are more often selected for their male-specific fitness effects (Rice 1984; Connallon and Clark 2010).

Unbalanced sex-specific selection on sex chromosomes has been suggested to explain the complex nonrandom distribution of sex-biased genes in mammals (Khil *et al.* 2004) and *Drosophila* (Parisi *et al.* 2003; Sturgill *et al.* 2007; Chen *et al.* 2011). However, it has not been clear from previous work in birds how much of the male bias on the Z chromosome is due to masculinization (Ellegren 2011) and how much to incomplete dosage compensation (Ellegren *et al.* 2007; Itoh *et al.* 2007). Previous attempts to circumvent the problems of gene dose compared embryonic and adult gene expression levels (Mank and Ellegren 2009b); however, this did not account for the fact that sex-specific selection and sex-biased gene expression shift rapidly throughout development and the adult life cycle (Mank *et al.* 2010). Our method allows male-biased selection to be detected without conducting a comparison between developmental time points, avoiding the problems encountered in previous studies of varying magnitudes of sex-specific selection. Consequentially, our results suggest that the increase in male bias over time is consistent with a cumulative effect of masculinizing selection rather than selection for dosage compensation. The older strata have been subjected to longer periods of stronger selection for dominant male-benefit alleles, and hence show a greater degree of masculinization. This finding is consistent with theoretical predictions and a recent study showing that genes expressed in the testis are overrepresented among newly emerged Z-linked genes (Ellegren 2011). The results can also explain previous findings that the extent of sex-biased expression varies across the Z chromosome (Melamed and Arnold 2007).

Determining whether sex-specific selection is responsible for the analogous feminization of the X chromosome in *Drosophila* (Parisi *et al.* 2003; Sturgill *et al.* 2007; Chen *et al.* 2011) and mice (Khil *et al.* 2004), is complicated by meiotic sex chromosome inactivation, a genetic mechanism inactivating sex-linked genes during late spermatogenesis (Vibrantovski *et al.* 2009a). MSCI has been proposed as a driving force behind the underrepresentation of spermatogenesis genes on the X chromosome; however, this is surrounded by much debate (Khil *et al.* 2004; Vibrantovski *et al.* 2009b; Meiklejohn *et al.* 2011). In birds, MSCI is ephemeral, lasting only from early pachytene to diplotene phases during oogenesis (Schoenmakers *et al.* 2009). As our samples were taken before this stage, MSCI is unlikely to drive the majority of the nonrandom pattern of sex-biased genes on the Z chromosome we observe.

The pattern of successive masculinization across the Z chromosome is in contrast to that seen on the human X chromosome, whereby more genes escape X chromosome inactivation and are therefore expressed more in females, in the younger regions of the X than the older regions (Carrel and Willard 2005). This hints at the important role of dosage compensation in shaping the pattern of sex-biased genes

on sex chromosomes, as the dosage compensation mechanism is less effective in younger regions of the mammalian X chromosome (Lin *et al.* 2007; Deng *et al.* 2011). The *Drosophila* X chromosome does not exhibit strata, possibly because the lack of recombination in males means that the Y decayed as a single unit or because the Y and X are not orthologous (Hackstein *et al.* 1996). Birds lack global sex chromosome dosage compensation (Ellegren *et al.* 2007; Itoh *et al.* 2007), and we would expect that if there were significant selection for dosage compensation, the pattern of male-biased expression would decrease as a function of stratum age. We predict that these differences should be strongly visible across the Z chromosome, as evidence in several animals suggests that dosage compensation mechanisms evolve relatively slowly. The sex chromosomes of monotremes and birds are ancient and yet still display incomplete dosage compensation (Itoh *et al.* 2007; Deakin *et al.* 2008) and while there is a current debate regarding the status of dosage compensation in eutherian mammals, evidence suggests that these chromosomes also lack complete dosage compensation (Deng *et al.* 2011; Pessia *et al.* 2012). However, limited evidence from plants indicates that in some cases selection for dosage compensation may be more rapid (Muyle *et al.* 2012). Despite this, our data are not consistent with selection for dosage compensation, as we observe male bias increasing over time in a cumulative fashion. Additionally, comparing Z-linked and autosomal expression confirms that females are not under selection for dosage compensation, as the expression ratio is consistent with a lack of hypertranscription of the single Z chromosome (Ellegren *et al.* 2007; Itoh *et al.* 2007, 2010). This suggests a trade-off between dosage compensation mechanisms and sex-specific selection on gene expression, with dosage compensation mechanisms acting against the effects of cumulative sex-specific selection on sex chromosome transcription.

Interestingly, the static architecture of the avian genome may also contribute to the pattern of male-biased expression we observe on the Z chromosome. Recently, it has been shown that there is little gene traffic on and off the Z chromosome, potentially due to a lack of active transposons within the avian genome (Toups *et al.* 2011). The unequal inheritance pattern of the Z chromosome renders it unfavorable for dominant female-benefit alleles (Rice 1984); however, the potential for these genes to move to the autosomes is severely limited. Subsequently, all Z-linked genes are subject to strong male-specific selection, favoring the evolution of male-biased expression.

Concluding remarks

A considerable body of theory focuses on the importance of sex chromosomes in facilitating the evolution of sexually dimorphic phenotypes (Rice 1984). Indeed, the Z chromosome is thought to be especially conducive to sexual selection and predicted to play an important role in encoding sexually selected traits, due to the male biased inheritance pattern (Reeve and Pfennig 2003; Kirkpatrick and Hall

2004; Albert and Otto 2005). Implicit in these predictions is that the importance of sex linkage in encoding sexually dimorphic phenotypes increases over evolutionary time with cumulative exposure to sex-specific selection; however, chromosome or stratum age is typically not considered when attempting to measure the importance of sex chromosomes in the evolution of sexually selected traits. Here we show that the cumulative effects of male-specific selection vary across the chromosome, indicating that older regions harbor more genes that may contribute to sexual dimorphic phenotypes via sex-biased expression than younger ones.

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Supporting Information

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Trade-off Between Selection for Dosage Compensation and Masculinization on the Avian Z Chromosome

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Table S1 Fold change (\log_2 male - \log_2 female FPKM) for Z-linked *Gallus gallus* genes

<i>Gallus gallus</i> Ensembl ID	\log_2 male - \log_2 female FPKM
ENSGALG00000000004	0.144214448
ENSGALG000000000145	2.563551346
ENSGALG000000000151	1.807940416
ENSGALG000000000161	0.862825974
ENSGALG000000000184	0.07049208
ENSGALG000000000189	0.768135987
ENSGALG000000000192	0.854906526
ENSGALG000000000208	1.342652937
ENSGALG000000000215	0.692398338
ENSGALG000000000218	1.382153483
ENSGALG000000000220	0.372756729
ENSGALG000000000234	-0.348308739
ENSGALG000000000236	0.775994181
ENSGALG000000000241	0.670339502
ENSGALG000000000244	1.222864394
ENSGALG000000000247	0.860096486
ENSGALG000000000253	0.51143834
ENSGALG000000000255	0.47141194
ENSGALG000000000264	0.760406708
ENSGALG000000000273	0.649324985
ENSGALG000000000276	0.363257015
ENSGALG000000000280	0.160895578
ENSGALG000000000428	0.743060433
ENSGALG000000000438	0.534075327
ENSGALG000000001668	0.598442223
ENSGALG000000001677	0.667541141
ENSGALG000000001732	0.423139336
ENSGALG000000001761	0.962319536
ENSGALG000000001763	0.704717855
ENSGALG000000001765	1.142400234
ENSGALG000000001766	-0.099745553
ENSGALG000000001782	1.133440634
ENSGALG000000001801	0.62915481
ENSGALG000000001808	0.310473336
ENSGALG000000001840	-0.775983688
ENSGALG000000001843	0.547062189
ENSGALG000000001856	0.050797749
ENSGALG000000001864	0.423745911
ENSGALG000000001865	0.662841922
ENSGALG000000001918	0.419298471
ENSGALG000000001933	0.33772121
ENSGALG000000001944	0.586726019
ENSGALG000000001947	1.107498032
ENSGALG000000001954	1.461911344
ENSGALG000000001986	0.876549107
ENSGALG000000002009	1.542753236
ENSGALG000000002014	0.998596175
ENSGALG000000002022	0.792854036
ENSGALG000000002023	0.486255463
ENSGALG000000002064	0.757698013
ENSGALG000000002069	0.07861647
ENSGALG000000002087	0.353503481
ENSGALG000000002110	0.682311961
ENSGALG000000002125	2.10409502
ENSGALG000000002142	0.17470154
ENSGALG000000002162	0.404068102
ENSGALG000000002165	-0.249179169
ENSGALG000000002183	0.198792425
ENSGALG000000002187	0.759674565
ENSGALG000000002196	-0.531567517

ENSGALG00000002227	0.580157208
ENSGALG00000002250	1.104408492
ENSGALG00000002294	-0.172500592
ENSGALG00000002308	1.23309249
ENSGALG00000002326	0.963521317
ENSGALG00000002341	0.737179364
ENSGALG00000002345	0.154821836
ENSGALG00000002358	0.78272549
ENSGALG00000002361	0.125165954
ENSGALG00000002371	0.095290291
ENSGALG00000002386	-0.128208101
ENSGALG00000002394	0.983361321
ENSGALG00000002399	0.71053722
ENSGALG00000002412	0.042181674
ENSGALG00000002413	-1.42649468
ENSGALG00000002419	0.789687641
ENSGALG00000002429	0.820735973
ENSGALG00000002430	0.75595148
ENSGALG00000002439	0.296663518
ENSGALG00000002505	0.377772191
ENSGALG00000002523	0.418782577
ENSGALG00000002529	0.176264947
ENSGALG00000002539	0.415746625
ENSGALG00000002548	0.520383539
ENSGALG00000002563	1.001962514
ENSGALG00000002578	0.20190484
ENSGALG00000002580	0.451055214
ENSGALG00000002599	0.946997848
ENSGALG00000002605	1.056184391
ENSGALG00000002613	-0.412780499
ENSGALG00000002617	-2.013077456
ENSGALG00000002677	0.430315076
ENSGALG00000002696	1.618241165
ENSGALG00000002712	-0.88247702
ENSGALG00000002777	1.142039974
ENSGALG00000002793	0.649588179
ENSGALG00000002805	0.577308475
ENSGALG00000002839	-0.079050694
ENSGALG00000002852	1.116907637
ENSGALG00000002854	0.714426019
ENSGALG00000002872	0.518245913
ENSGALG00000002917	0.797476482
ENSGALG00000002948	0.691815468
ENSGALG00000003019	0.31194661
ENSGALG00000003066	0.897301381
ENSGALG00000003087	0.606326673
ENSGALG00000003151	0.7833409
ENSGALG00000003168	0.000610865
ENSGALG00000003235	0.798062847
ENSGALG00000003248	0.512508083
ENSGALG00000003253	1.428556833
ENSGALG00000003288	1.387445366
ENSGALG00000003295	1.080551882
ENSGALG00000003326	1.193268989
ENSGALG00000003353	0.799601709
ENSGALG00000003365	1.28513858
ENSGALG00000003373	1.31154087
ENSGALG00000003387	0.876676348
ENSGALG00000003432	1.129648506
ENSGALG00000003446	0.812484167
ENSGALG00000003547	0.5894354
ENSGALG00000003558	0.847399099
ENSGALG00000003582	-2.995683678

ENSGALG00000003605	0.704647921
ENSGALG00000003622	1.809526901
ENSGALG00000003629	1.31893074
ENSGALG00000003631	1.379755087
ENSGALG00000003688	1.418906268
ENSGALG00000003708	1.098806088
ENSGALG00000003716	0.782360676
ENSGALG00000003726	0.960842014
ENSGALG00000003733	1.2507204
ENSGALG00000003747	1.253311158
ENSGALG00000003775	0.476059989
ENSGALG00000003792	0.057281068
ENSGALG00000003803	-0.94260209
ENSGALG00000004311	0.526658371
ENSGALG00000004339	-0.16142575
ENSGALG00000004350	1.119297433
ENSGALG00000004353	0.99533908
ENSGALG00000004390	0.999432389
ENSGALG00000004425	0.088783625
ENSGALG00000004437	1.895887543
ENSGALG00000004438	-0.015137823
ENSGALG00000005016	0.26568517
ENSGALG00000005316	1.207630786
ENSGALG00000005317	-0.583572836
ENSGALG00000005323	1.090868879
ENSGALG00000005330	0.950365928
ENSGALG00000005337	0.655218064
ENSGALG00000005346	0.897072262
ENSGALG00000005354	0.895198572
ENSGALG00000005368	0.728533386
ENSGALG00000005372	0.969175294
ENSGALG00000005390	0.900936042
ENSGALG00000005395	0.907372682
ENSGALG00000005416	0.638722628
ENSGALG00000005423	0.690397434
ENSGALG00000005426	0.330510137
ENSGALG00000005433	1.380543351
ENSGALG00000005437	0.896961124
ENSGALG00000005441	0.52845375
ENSGALG00000005800	1.143464857
ENSGALG00000005806	0.766200026
ENSGALG00000005810	1.051992375
ENSGALG00000005812	-0.070058068
ENSGALG00000005814	0.230083615
ENSGALG00000005821	0.351738043
ENSGALG00000005831	1.462134859
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ENSGALG00000005846	-0.124466689
ENSGALG00000005848	-0.197986878
ENSGALG00000008174	0.752073168
ENSGALG00000008188	-1.138548034
ENSGALG00000008197	0.649035922
ENSGALG00000008204	0.846062564
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ENSGALG00000008237	1.380436762
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ENSGALG00000008893	-2.181744409
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ENSGALG00000010164	0.096934815
ENSGALG00000010166	0.14697631

ENSGALG00000010178	1.110609359
ENSGALG00000010179	0.299521602
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ENSGALG00000010683	1.023433377
ENSGALG00000010693	1.846193962
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ENSGALG00000014845	1.283501262
ENSGALG00000014846	-0.088831478

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ENSGALG00000014869	0.751345481
ENSGALG00000014872	1.252650855
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ENSGALG00000014884	-2.716423629
ENSGALG00000014891	-0.429288639
ENSGALG00000014903	0.016821128
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ENSGALG00000014939	1.251708585
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ENSGALG00000014945	-0.086129213
ENSGALG00000014946	-0.012766137
ENSGALG00000014948	0.061417202
ENSGALG00000014952	0.61435107
ENSGALG00000014956	0.823070537
ENSGALG00000014960	1.12521584
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ENSGALG00000014983	0.604876902
ENSGALG00000014984	-0.768379043
ENSGALG00000014994	1.448054479
ENSGALG00000014995	1.780488889
ENSGALG00000014999	0.17160365
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