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Positive Selection Underlies Faster-Z Evolution of Gene Expression in Birds

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Abstract

The elevated rate of evolution for genes on sex chromosomes compared with autosomes (Fast-X or Fast-Z evolution) can result either from positive selection in the heterogametic sex or from nonadaptive consequences of reduced relative effective population size. Recent work in birds suggests that Fast-Z of coding sequence is primarily due to relaxed purifying selection resulting from reduced relative effective population size. However, gene sequence and gene expression are often subject to distinct evolutionary pressures; therefore, we tested for Fast-Z in gene expression using next-generation RNA-sequencing data from multiple avian species. Similar to studies of Fast-Z in coding sequence, we recover clear signatures of Fast-Z in gene expression; however, in contrast to coding sequence, our data indicate that Fast-Z in expression is due to positive selection acting primarily in females. In the soma, where gene expression is highly correlated between the sexes, we detected Fast-Z in both sexes, although at a higher rate in females, suggesting that many positively selected expression changes in females are also expressed in males. In the gonad, where intersexual correlations in expression are much lower, we detected Fast-Z for female gene expression, but crucially, not males. This suggests that a large amount of expression variation is sex-specific in its effects within the gonad. Taken together, our results indicate that Fast-Z evolution of gene expression is the product of positive selection acting on recessive beneficial alleles in the heterogametic sex. More broadly, our analysis suggests that the adaptive potential of Z chromosome gene expression may be much greater than that of gene sequence, results which have important implications for the role of sex chromosomes in speciation and sexual selection.

Key words: **female heterogamety, gene expression divergence, selection, drift, Fast-X, sex chromosomes.**

Introduction

The unique properties of the sex chromosomes are thought to influence rates of evolution for the genes they contain, and comparisons between the sex chromosomes and autosomes are important for understanding the role that dominance, effective population size and recombination play in adaptive evolution. For both X and Z chromosomes, hemizygosity and lower relative effective population size (N_E) of sex chromosomes can lead to an increased rate of functional change in comparison to autosomes (Charlesworth et al. 1987; Vicoso and Charlesworth 2006), a process termed Fast-X or Fast-Z evolution.

In female heterogametic sex chromosome systems, the single copy of the Z chromosome in females means that recessive beneficial alleles are always exposed to selection when expressed in this sex, leading to greater rates of fixation of recessive advantageous alleles. This would result in the Fast-Z effect due to adaptive evolution. Alternatively, Fast-Z can occur as a result of the reduced N_E of the Z compared with the autosomes. When male and female reproductive success are equal, there are only three Z chromosomes for every four autosomes ($N_{EZ} = \frac{3}{4} N_{EA}$). The reduction in N_{EZ} leads to a reduction in the efficacy of purifying selection on the Z chromosome (Caballero 1995; Laporte and Charlesworth 2002) and drift has greater potential to fix mildly deleterious alleles (Vicoso and Charlesworth 2009). Differentiating the role of hemizygosity versus reduced N_E in rates of evolution for sex chromosomes is essential for determining the relative role of adaptive evolution versus genetic drift in sex chromosome evolution, with important implications for sexual selection and speciation (e.g., Haldane 1922; Kirkpatrick and Hall 2004).

Fast-Z evolution has been broadly detected in studies of coding sequence in birds (Mank et al. 2007; Mank, Nam, et al. 2010; Corl and Ellegren 2012; Wright et al. 2015), snakes (Vicoso et al. 2013), and moths (Sackton et al. 2014). Most examples of Fast-Z sequence evolution have mainly been attributed to drift (Mank, Nam, et al. 2010; Vicoso et al. 2013; Wright et al. 2015) although evidence from silk moths suggests positive selection (Sackton et al. 2014). Moreover, drift may be particularly strong on Z chromosomes due to sexual selection. Increasing variance in male reproductive success, such as that produced by sexual selection (Wade 1979; Andersson 1994), reduces relative N_{EZ} below $\frac{3}{4}$ N_{EA}, unlike male heterogametic systems (Mank, Vicoso, et al. 2010). Recent estimates of N_{EZ} in birds have been significantly less than $\frac{3}{4}$ N_{EA} (Corl and Ellegren 2012; Wang et al. 2014; Wright et al. 2015) potentially resulting in elevated levels of genetic drift for Z-linked genes.

Studies of Fast-Z evolution have so far focused on coding sequence data of orthologous genes to compare rates of change on the Z chromosome versus the autosomes (Mank et al. 2007; Mank, Nam, et al. 2010; Corl and Ellegren 2012;

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Vicoso et al. 2013; Sackton et al. 2014; Wright et al. 2015). Genes that are orthologous across species tend to be under high purifying selection (Wang et al. 2007) and as such this may limit the ability of gene sequence studies to detect adaptive signals of Fast-Z. Although gene expression studies also use orthologous genes, sequence and expression can show different patterns of evolution, even for the same locus. For example, purifying selection may act more weakly on expression of conserved orthologous genes if the regions regulating gene expression are less conserved, thus allowing greater capacity for adaptive evolution of gene expression. Additionally, gene expression evolution may also be influenced by *trans*-regulation from different chromosomes (Meisel et al. 2012; Meisel and Connallon 2013; Meiklejohn et al. 2014). Studies of expression evolution on sex chromosomes may therefore be particularly informative for understanding the nature of gene expression evolution (Kayserili et al. 2012; Meisel et al. 2012; Meisel and Connallon 2013), and for identifying the adaptive potential of sequence versus expression evolution (Stern and Orgogozo 2008).

In order to perform the first test of Fast-Z evolution of global gene expression, we built de novo transcriptome assemblies from somatic and gonadal tissue from captive males and females of six species of the Galloanserae, including turkey (*Meleagris gallopavo*), pheasant (*Phasianus colchicus*), peafowl (*Pavo cristatus*), guinea fowl (*Numida meleagris*), swan goose (*Anser cygnoides*), and mallard duck (*Anas platyrhynchos*) (Harrison et al. 2015; Wright et al. 2015). Our data indicate that gene expression on the Z chromosome evolves more rapidly than that on the autosomes, consistent with previous studies of Fast-Z in coding sequence. However, we observe more pronounced Fast-Z in females than males, suggesting that unlike protein coding sequence, Fast-Z in avian gene expression is primarily adaptive in nature. Together, our results suggest that gene expression on the Z chromosome may have a greater adaptive potential than coding sequence, a finding with important implications for sexual selection and speciation.

Results

Faster-Z Evolution of Gene Expression

We calculated the pairwise similarity in expression separately for each sex, using Spearman's rho correlation coefficient (ρ) (Brawand et al. 2011; Meisel et al. 2012). We used pheasant as the reference point (i.e., comparing expression of each of the other five species to pheasant) in order to achieve even phylogenetic spacing of taxa, which maximizes our power to test for differences in the slope of ρ between the Z and autosomes. Other focal species result in clustering of the data into two groups, thereby making comparisons of the slope meaningless. Therefore, we calculated ρ between each species and the pheasant, and then plotted ρ for autosomal and Z genes for each expression class by divergence time. Our results show a greater rate of decline in ρ over time for the Z chromosome compared with the autosomes, consistent with Fast-Z evolution of gene expression; however, the effect was primarily observed in females (fig. 1). For genes expressed in the female spleen, ρ decreased more rapidly over time (resulting in a significantly steeper slope) for the Z chromosome compared with the autosomes (fig. 1*A*, supplementary fig. S1 and table S1, Supplementary Material online). In the male spleen, the effect was marginally nonsignificant (fig. 1*B* and table S1, Supplementary Material online). Similarly, in the gonad the slope was greater for the Z chromosome than the autosomes in females (fig. 1*C* and table S1, Supplementary Material online), but not in males (fig. 1*D* and table S1, Supplementary Material online). In the female and male spleens, ρ was significantly lower on the Z chromosome than on the autosomes in the majority of comparisons (fig. 1). In the female gonad, there was a significantly lower ρ on the Z chromosome compared with the autosomes only in comparisons between waterfowl and pheasant (fig. 1).

We also looked for signatures of Fast-Z evolution using expression divergence in all pairwise comparisons between the six species (Meisel et al. 2012). In the female spleen, we detected higher gene expression divergence for the Z chromosome than autosomes in 14 of 15 comparisons (fig. 2). In the male spleen, gene expression divergence for the Z chromosome was significantly greater than that of the autosomes for 7 of 15 pairwise comparisons (fig. 2).

In the female gonad, 14 of 15 pairwise comparisons showed higher divergence on the Z chromosomes than autosomes (fig. 3). In the male gonad, only 4 of the 15 pairwise comparisons showed higher divergence on the Z (fig. 3), and interestingly all of these comparisons with higher gene expression divergence on the Z involved divergence from duck.

Correlation of Gene Expression between Males and Females (C_{mf})

These results suggest that Fast-Z evolution of expression occurs primarily in females. Interestingly, the Fast-Z effect is weakly detectible in the male spleen, but not at all evident in the male gonad. This difference in Fast-Z evolution of expression in males may be the result of different levels of intersexual correlation in expression in somatic versus gonadal tissues. To explore the differences in intersexual correlation, and its possible effects on Fast-Z evolution, we measured the correlation in gene expression between males and females (here termed C_{mf}) across our six avian species. In order to control for phylogeny, we used Phylogenetic Generalized Least Squares (PGLS) in the R package Caper (R-Core-Team 2012); therefore, the strength of the correlation in expression across the six species (C_{mf}) was measured using r^2. In the spleen, expression levels between males and females are highly correlated across the clade both for genes on the autosomes and Z chromosome (fig. 4*A*, median $C_{mf\ (autosomes)} = 0.91$, median $C_{mf\ (Z\ chromosome)} = 0.86$; Wilcoxon rank sum, $P < 0.0001$). This suggests that most expression variation selected in females will also be expressed in males and may explain why both females and males show Fast-Z expression evolution in the spleen.

In contrast, the correlation between gene expression in males and females is much lower in the gonad (fig. 4*B*,

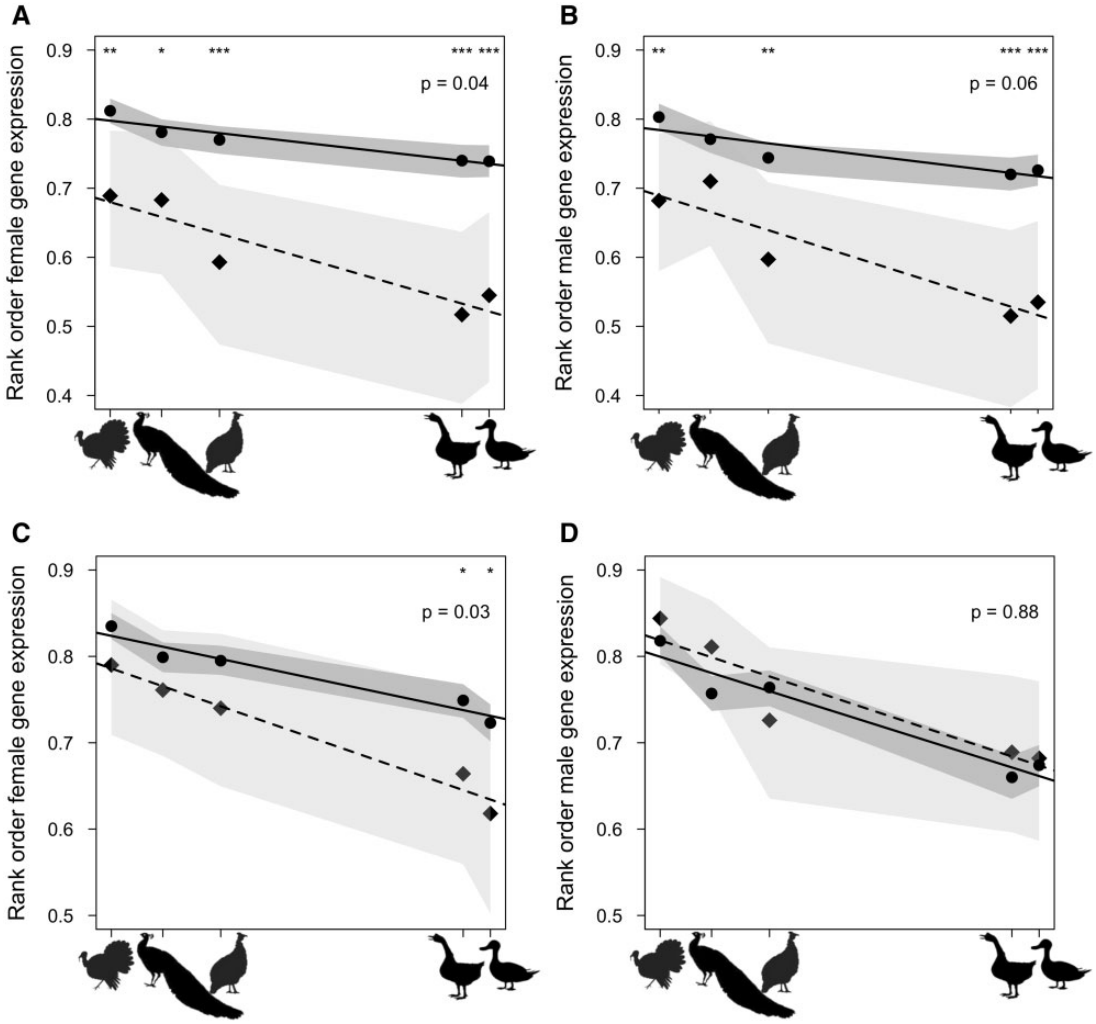

FIG. 1. Spearman's rho correlations for pairwise similarity between pheasant and each other species in the (*A*) female spleen, (*B*) male spleen, (*C*) female gonad, and (*D*) male gonad. Regression for genes on autosomes shown by solid line (and circles) and Z chromosome by dashed line (and diamonds). Shaded areas represent the 95% confidence intervals calculated through 1,000 bootstrap replicates. *P* values are for interactions between chromosome × divergence time. Significant differences between the Z chromosome and autosomes denoted by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

$C_{mf\ (autosomes)} = 0.28$, median $C_{mf\ (Z\ chromosome)} = 0.24$; Wilcoxon rank sum, $P = 0.263$). The reduction in C_{mf} in the gonad compared with the spleen implies that most adaptive expression variation in the female gonad will not be similarly expressed in males, and may explain why Fast-Z expression evolution was only observed in females in this tissue.

Fast-Z Expression Evolution in Females Is Consistent with an Adaptive Process

The stronger signature of Fast-Z in females than males is consistent with an adaptive process driving Fast-Z evolution of gene expression due to hemizygous exposure of recessive beneficial expression variation. If Fast-Z is indeed a result of fixation of recessive beneficial alleles in females, we would expect to see greater rates of Fast-Z evolution for female-biased genes than male-biased genes. Consistent with this, we find indications of Fast-Z evolution for female-biased genes in the female gonad but not for male-biased genes in the male gonad (supplementary fig. S1, Supplementary Material online). Interestingly, both Z and autosomal female-biased loci expressed in the male gonad exhibit greater variation in divergence, with a high overall average, a pattern not observed for male-biased genes expressed in the female gonad (supplementary fig. S1, Supplementary Material online).

Additionally, if purifying selection acting on coding sequence constrains adaptive Fast-Z evolution in coding sequence, we might expect greater signatures of adaptive Fast-Z expression evolution for highly expressed genes than lowly expressed genes, as the sequence of highly expressed genes has been shown to be subject to stronger purifying selection (Resch et al. 2007). Consistent with this prediction, we find more pronounced Fast-Z expression evolution in females for genes that are highly expressed compared with those with lower expression, although we do detect signatures of Fast-Z expression evolution for both expression categories (supplementary fig. S2, Supplementary Material online). As expected, highly expressed genes in general tend to be more constrained and generally show overall lower divergence than genes that have low expression across the

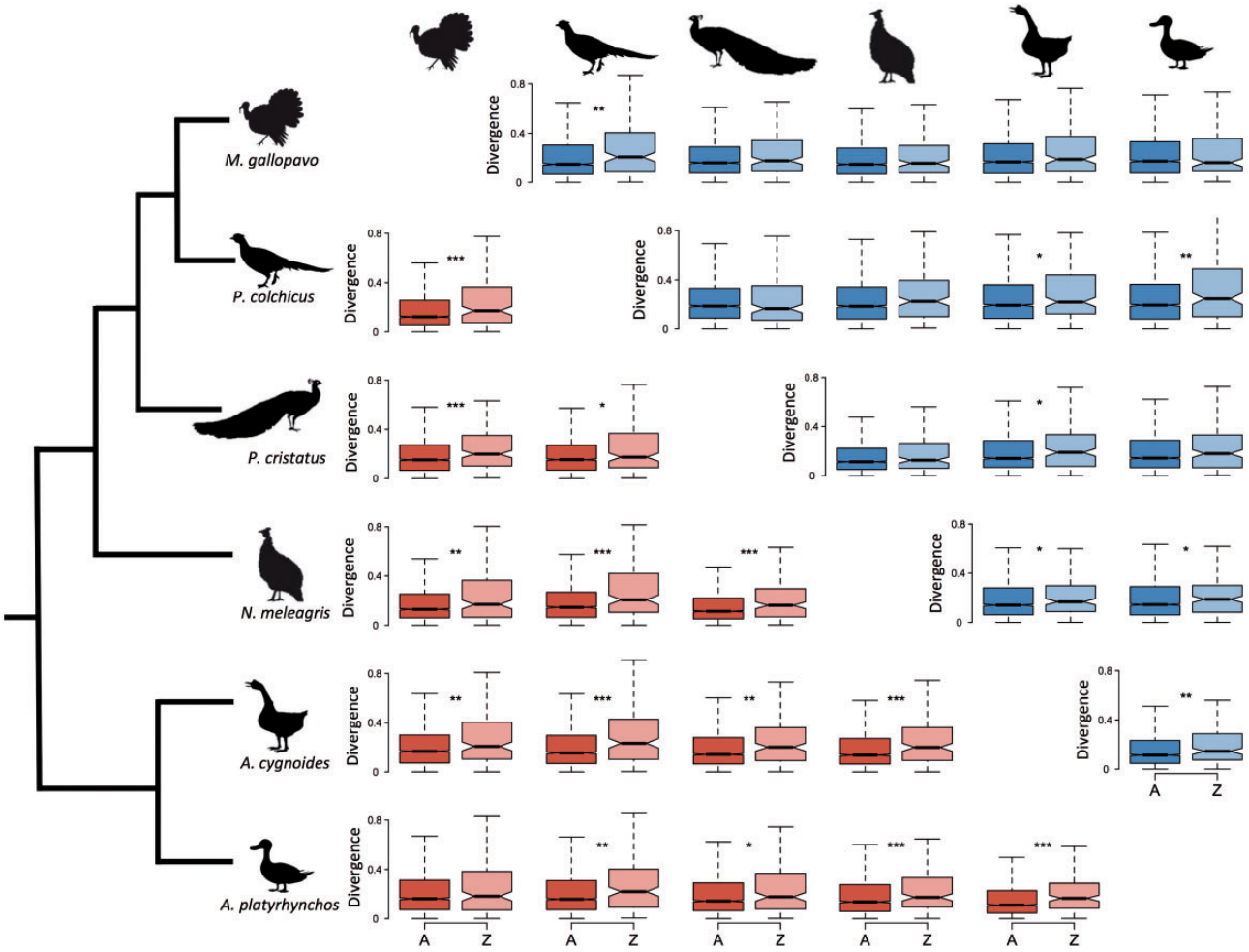

Fig. 2. Branch-specific pairwise gene expression divergence for female and male spleens. Gene expression divergence in female spleen shown below the diagonal (in red) and male spleen above the diagonal (in blue). Genes on autosomes are shaded darker and genes on Z chromosome shaded lighter. Two-sided Wilcoxon tests for significant differences between autosomal and Z chromosome divergence denoted by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

genome (supplementary fig. S2, Supplementary Material online).

Expression Variance Indicates Fast-Z Is due to Adaptive Evolution

In order to test more directly whether gene expression changes are due to adaptive versus nonadaptive processes, we used Δx, a measure of adaptive change in expression evolution (Moghadam et al. 2012) which incorporates both divergence and polymorphism (expression variance). We reconstructed ancestral expression levels using a maximum-likelihood (ML) estimator of Brownian Motion (Schluter et al. 1997; Paradis et al. 2004; Harrison et al. 2015). It is important to note that models of gene expression evolution are largely additive, and are not yet possible to functionally validate. Their utility in extrapolating evolutionary signals is important, but results must be interpreted cautiously. More importantly, error increases over phylogenetic space; therefore, we confine our analyses using ancestral reconstructions to the internal nodes nearest to each of our study species (nearest ancestor).

We measured gene expression divergence between each of our species and the reconstructed gene expression of the nearest ancestor (nearest internal node). In the spleen, higher gene expression divergence on the Z chromosome was in general detected for both males and females (fig. 5*A*). Consistent with the pairwise species comparisons in the gonad (fig. 3) we found higher expression divergence for genes on the Z than for autosomal genes in all six species comparisons in females, but not in males (fig. 6*A*). We calculated the proportion of genes on the Z and autosomes where divergence exceeds polymorphism ($-1 > \Delta x > 1$), a signal of positive selection (Moghadam et al. 2012). In the female spleen there was a higher proportion of genes on the Z chromosome showing putative positive selection in only one of the species, and a significantly lower proportion for one species in males (fig. 5*B*). However, in the female gonad in three of the six species we found a higher proportion of genes on the Z chromosome showed evidence of putative positive selection in gene expression compared with autosomes (fig. 6*B*). In contrast, there was no significant difference in the proportion of genes under positive selection on the Z or autosomes in any species in the male gonad (fig. 6*B*).

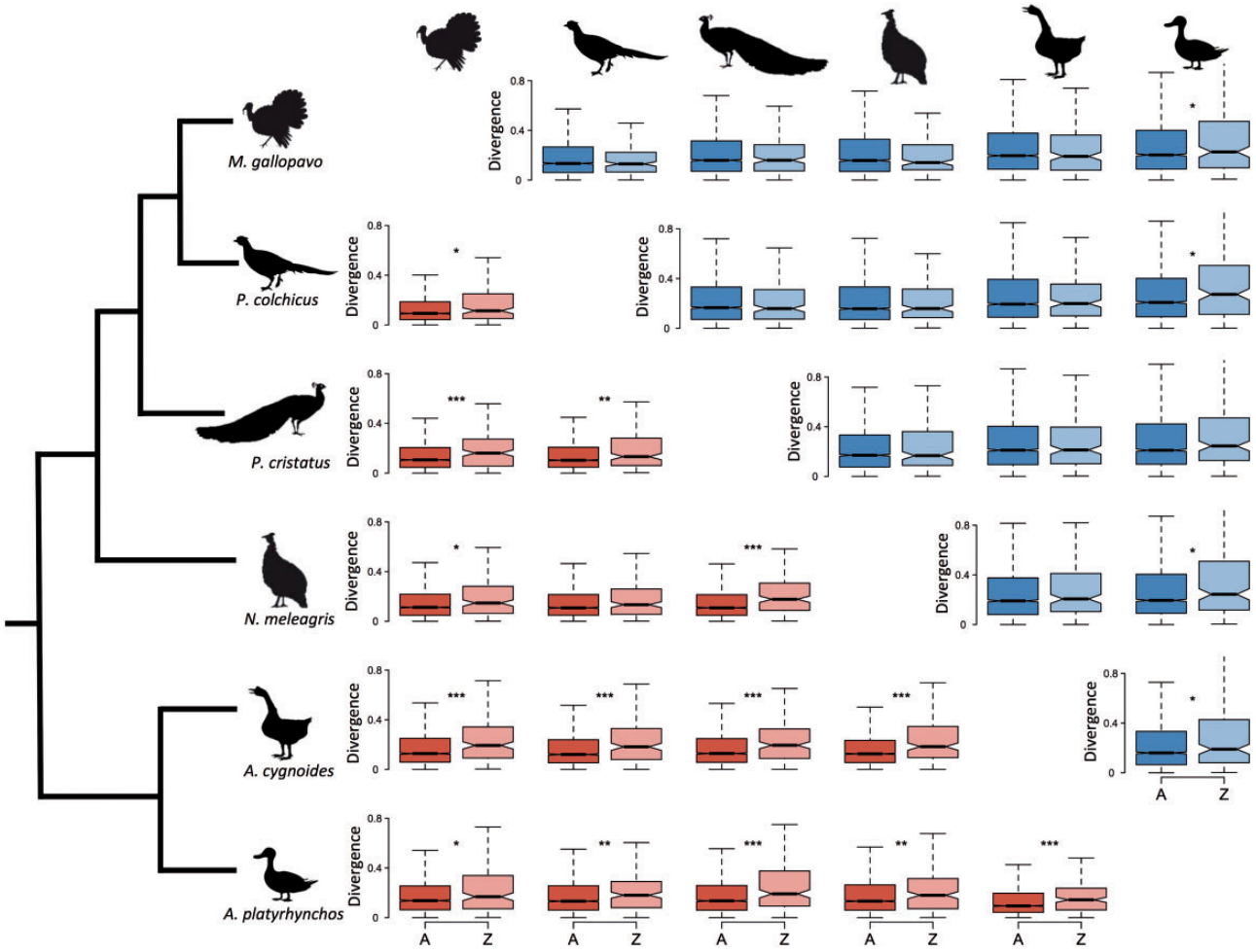

FIG. 3. Branch-specific pairwise gene expression divergence for female and male gonad. Gene expression divergence in female gonad shown below the diagonal (in red) and male gonad above the diagonal (in blue). Genes on autosomes are shaded darker and genes on Z chromosome shaded lighter. Two-sided Wilcoxon tests for significant differences between autosomal and Z chromosome divergence denoted by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Discussion

Our study finds clear signatures of Fast-Z evolution of gene expression in both the somatic and gonadal tissues, similar to a recent study on Fast-Z in gene sequence (Wright et al. 2015). However, in contrast to previous studies of protein coding data, which support a predominant role of drift in Fast-Z (Wright et al. 2015), our data indicate that Fast-Z in gene expression is primarily the result of positive selection acting in females due to hemizygous exposure of recessive beneficial variation.

Fast-Z in Gene Expression Is Largely the Result of Adaptive Evolution in Females

Our results provide several lines of evidence that support the role for positive selection in driving Fast-Z evolution of gene expression. First, the Fast-Z effect in expression is stronger in females than males, consistent with hemizygous exposure of beneficial variation. In gonadal tissue, we find strong signatures of Fast-Z in females but not males (fig. 3), and the Fast-Z effect is stronger for females than males in the spleen (fig. 2). Additionally, we find tentative support that female-biased genes show stronger Fast-Z expression evolution than male-biased genes (supplementary fig. S1, Supplementary Material online), consistent with the assumption that female-biased genes encode female phenotypes (Connallon and Clark 2011). Different methods to identify sex-biased gene expression can yield different results (Assis et al. 2012). However, our method of defining sex-bias was broadly consistent with the EdgeR method (Robinson et al. 2010), producing an overlap in sex-biased expression of 89–96% between both approaches (Wright et al. 2015). This means that our analyses of gene expression divergence for sex-biased genes are unlikely to be affected by different methods of classifying sex-biased gene expression. Finally, in females, a higher proportion of genes on the Z in several of the six species studied show evidence of positive selection for expression (figs. 5*B* and 6*B*), but we find no such difference in males. Although differences in gene function between the autosomes and Z chromosome could contribute to Fast-Z, Gene Ontology analysis for the orthologous genes across these six species suggests no difference in gene function across the autosomes and Z chromosome (Wright et al. 2015). These results taken as a whole are consistent with an adaptive explanation of Fast-Z.

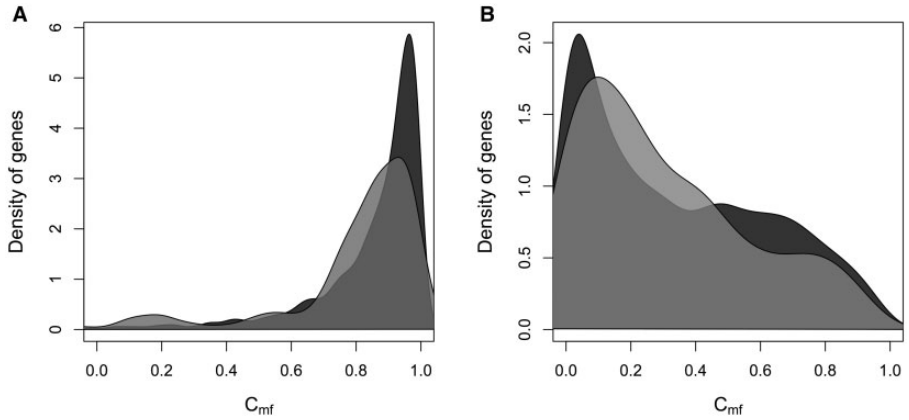

Fig. 4. Density distribution of correlations in gene expression between males and females (C_{mf}) for orthologous genes expressed in (*A*) spleen and (*B*) gonad. Correlations are r^2 values from phylogenetically controlled generalized least square models. Genes on autosomes are dark gray and on Z chromosome are light gray.

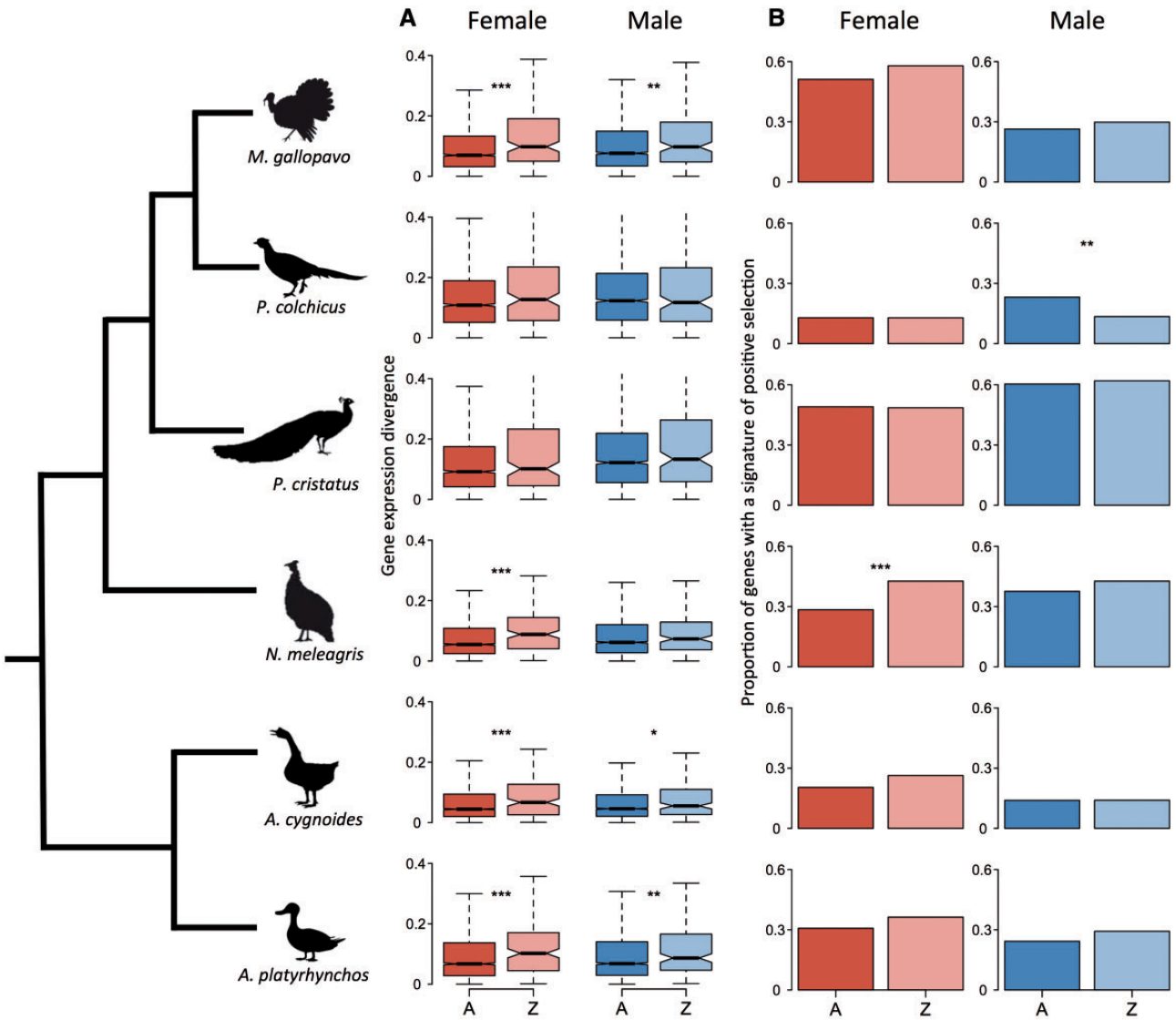

Fig. 5. (*A*) Pairwise gene expression divergence between each focal species and the estimated ancestral gene expression levels at the nearest node in female and male spleens. Two-sided Wilcoxon tests denote significant differences between autosomal and Z chromosome divergence. (*B*) Proportion of genes on the autosomes and Z chromosome with a signature of positive selection ($-1 > \Delta X > 1$) for the female and male spleens. Pearson's chi squared tests denote significant differences in the proportion of genes positively selected on Z chromosomes and autosomes. Females are on left (in red) and males on right (in blue). Autosomes are shaded dark and Z chromosome shaded light. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

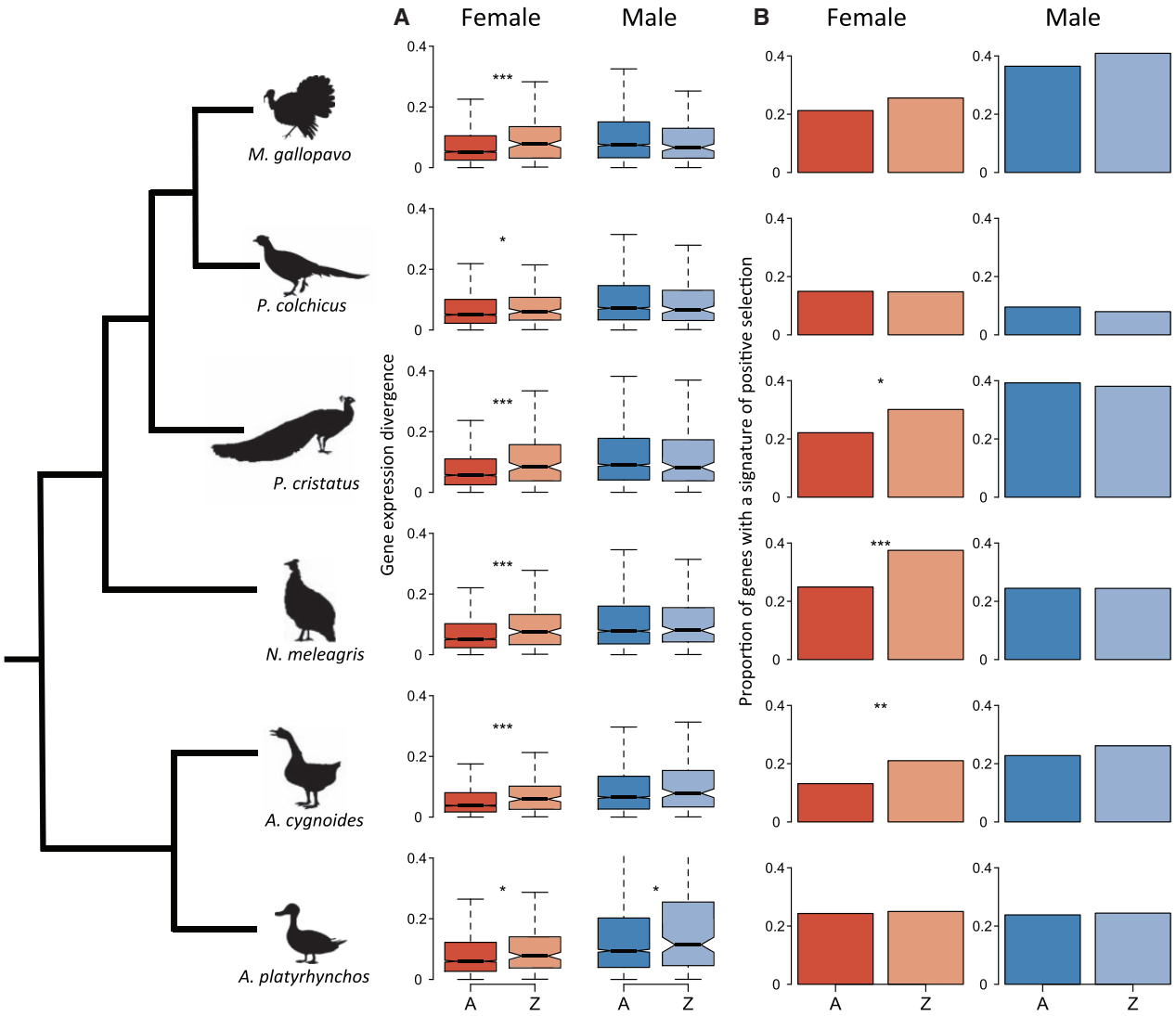

FIG. 6. (*A*) Pairwise gene expression divergence between each focal species and the estimated ancestral gene expression levels at the nearest node in the female and male gonad. Two-sided Wilcoxon tests denote significant differences between autosomal and Z chromosome divergence. (*B*) Proportion of genes on the autosomes and Z chromosome with a signature of positive selection ($-1 > \Delta x > 1$) for the female and male gonad. Pearson's chi-squared tests denote significant differences in the proportion of genes positively selected on Z chromosomes and autosomes. Females are in red and males in blue. Autosomes are shaded dark and Z chromosome shaded light. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

The role of selection in driving Fast-Z evolution of gene expression is perhaps surprising given that drift has been shown to be the primary cause of Fast-Z evolution in birds (Wright et al. 2015). This suggests that gene sequence and gene expression are subject to different evolutionary forces. The alternative reasons for Fast-Z gene sequence and gene expression evolution are likely to be linked to how selection acts on *cis*-regulatory regions. Mutations in *cis*-regulatory regions of genes are thought to be particularly important for evolutionary change (Wray 2007), and *cis*-regulated expression may be subject to stronger positive selection (Emerson et al. 2010) even in the face of pleiotropic constraint imposed on genes with conserved expression (Wray 2007). In contrast, the sequence for conserved orthologs may be largely shaped through purifying selection, limiting adaptive potential. Together, this suggests that the adaptive potential of Z chromosome gene expression may be greater than that of coding sequence, which may be important for studies of speciation and sexual selection, where the Z chromosome is often theoretically implicated as a major contributor (Haldane 1922; Kirkpatrick and Hall 2004).

Differences between the Spleen and Gonad in Fast-Z Evolution of Male Expression

Expression data are arguably more useful for studies of Fast-X or Fast-Z evolution because they can be used effectively to compare the sexes, as opposed to coding sequence data, which are the same in both males and females. Our analysis shows that Fast-Z expression evolution is consistent in the female gonad and soma, but is only weakly detectible in the male spleen and is absent from the male gonad (figs. 1–3). The difference in male Fast-Z expression evolution between the spleen and the gonad may be a consequence of the

difference in the strength of the genetic correlation between the sexes in these different tissues.

In the spleen, expression in males and females is highly correlated across the phylogeny (fig. 4*A*); therefore, the fixation of expression variation on the Z chromosome in females will often also result in the same expression pattern in males, producing a weaker, but still detectible, signature of Fast-Z expression evolution in the male spleen. In contrast to the spleen, the genetic correlation (C_{mf}) in expression between males and females is much lower in gonadal tissue (fig. 4*B*). This suggests that the majority of expression variation in the gonad is sex-specific in its effects, and therefore fixation of expression variants that are beneficial to females on the Z chromosome would not necessarily result in the same pattern when expressed in males. We also note that differences in the intersexual genetic correlation for genes on the Z chromosome and autosome are unlikely to contribute to our patterns of Fast-Z expression evolution because there were only small but significant differences in C_{mf} between the autosomes and Z chromosome in the spleen and there was no significant difference in the gonad.

Another important difference between somatic and gonadal tissue is the extent of dosage compensation. In birds, there is generally a lack of dosage compensation in the gonad, whereas the spleen tends to exhibit a degree of incomplete dosage compensation (Ellegren et al. 2007; Itoh et al. 2007). Differences in the extent of dosage compensation are thought to affect Fast-Z sequence evolution due to beneficial mutations (Mank, Vicoso, et al. 2010). When dosage compensation is more complete, Fast-Z sequence evolution due to positive selection is thought to be more pronounced, potentially because the selection coefficients in the heterogametic sex are expected to be smaller (Charlesworth et al. 1987). However, contrary to this, our data show similar patterns of Fast-Z expression evolution in the female gonad and spleen. As we do not see variation in the magnitude of dosage compensation across the six species studied, selection for dosage compensation is unlikely to drive the Fast-Z effect that we detect.

Fast-X versus Fast-Z

Faster rates of gene expression evolution on sex chromosomes have been detected in mammals and *Drosophila*, both male heterogametic systems. In mammals, the evidence suggests that Fast-X evolution of gene expression occurred as an adaptive burst on the newly formed therian X (Brawand et al. 2011). Similarly, we also find signatures of Fast-Z in expression over short evolutionary timescales (i.e., between closely related species, figs. 2 and 3), and at the tips of the phylogenetic tree (figs. 5*A* and 6*A*). However, in contrast to the mammalian study, we also find Fast-Z across more distantly related species (figs. 2 and 3), and the level of Fast-Z is correlated with phylogenetic distance (fig. 1), suggesting that the effect is cumulative over time.

Studies on Fast-X in *Drosophila* have shown that Fast-X is more strongly detected, but not limited to, male-biased genes expressed in male reproductive tissue (Meisel et al. 2012), although another study showed that Fast-X was restricted to *Drosophila* embryonic stages (Kayserili et al. 2012). Both studies are consistent with Fast-X driven by the adaptive fixation of mutations that affect gene expression in *cis*. Our data on Fast-Z provide further support that mutations affecting gene expression of genes on sex chromosomes are also primarily regulated in *cis*, and that the fitness consequences of these mutations are in general recessive (Meisel et al. 2012). Furthermore, *Drosophila* exhibits complete X chromosome dosage compensation and Z chromosome dosage compensation in birds is incomplete (reviewed in Mank 2013). The similarity between expression Fast-X in *Drosophila* and Fast-Z in birds suggests that faster rates of gene expression evolution are not restricted to a particular mode of dosage compensation (Meisel et al. 2012).

Models of Gene Expression Divergence

We note that measuring Fast-Z using gene expression rather than gene sequence may present a few caveats. First, current models of gene expression evolution assume additivity (Brawand et al. 2011; Ometto et al. 2011; Moghadam et al. 2012; Rohlfs et al. 2013), which has yet to be validated (Khaitovich et al. 2006). Second, in species with incomplete dosage compensation such as birds (Mank 2013), genes on sex chromosomes in the heterogametic sex will often have lower expression than genes on autosomes, which may affect measures of Fast-Z. However, our measure of gene expression divergence takes into account expression level and so this should not affect our ability to detect Fast-Z. Furthermore, we detect Fast-Z for both highly and lowly expressed genes, and our results are robust to different measures of Fast-Z, such as Spearman's rho correlation coefficient and gene expression divergence calculations.

Final Remarks

We detect Fast-Z evolution in gene expression across six avian species spanning 90 My of evolutionary history, and our results indicate that, in contrast to studies of coding sequence, Fast-Z in expression is primarily due to adaptive evolution of female-benefit variation. Together, this suggests that the adaptive potential of Z chromosome gene expression may be greater than that of coding sequence, which may be important for studies of speciation and sexual selection, where the Z chromosome has been theoretically shown to play a major role (Haldane 1922; Kirkpatrick and Hall 2004).

Materials and Methods

Transcriptome Assembly

Spleen and gonad samples were collected from captive-reared males and females at the start of their first breeding season for *Anas platyrhynchos* (mallard duck), *Meleagris gallopavo* (wild turkey), *Phasianus colchicus* (common pheasant), *Numida meleagris* (helmeted guineafowl), *Pavo cristatus* (Indian peafowl) and *Anser cygnoides* (swan goose), with permission from institutional ethical review committees and in accordance with national guidelines.

The left gonad and spleen were dissected separately from five males and five females for *A. platyrhynchos*, *N. meleagris*,

P. cristatus and *A. cygnoides*, and from six males and five females for *P. colchicus*. In *M. gallopavo*, four male and two female spleens were collected and five male and female gonads were collected. Samples were homogenized, stored initially in RNAlater, and RNA was then prepared with the Animal Tissue RNA Kit (Qiagen). mRNA was subtracted and individual samples barcoded by The Wellcome Trust Centre for Human Genetics, University of Oxford using Illumina's Multiplexing Sample Preparation Oligonucleotide Kit with an insert size of 280 bp. RNA was sequenced on an Illumina HiSeq 2000 resulting in on average 26 million 100-bp paired-end reads per sample.

Quality control, de novo assembly, and ortholog detection have been described previously (Harrison et al. 2015; Wright et al. 2015). Reads were mapped to de novo assemblies to obtain expression levels. Comparisons of normalized expression counts were used to identify sex-biased gene expression using standard measures and corrected for multiple testing (Pointer et al. 2013; Perry et al. 2014).

Genes used in all subsequent analyses were restricted to reciprocal 1–1 orthologs across all six study species that were expressed in either sex. We filtered out any sex-limited gene with expression less than 2 rpkm in the sex in which it was expressed, then removed any genes that were not expressed in all six of the species, resulting in 2,428 autosomal genes and 171 Z-linked genes for the spleen, and 2,729 autosomal and 184 Z-linked genes for the gonad. Analyses of gene expression similarity and divergence were done for males and females separately in R v.2.15.1 (R-Core-Team 2012).

Divergence and Phylogeny Estimation

In order to estimate divergence time as well as phylogenetic distance, nucleotide sequences for reciprocal orthologous genes were aligned with PRANK v.130820 (Löytynoja and Goldman 2008) using ML-derived guide trees, with the zebra finch as an outgroup. Reciprocal orthologs were used to construct an ML phylogeny for our six species with a GBLOCKS 0.91b (Castresana 2000) filtered alignment using RaxML (Stamatakis 2014) version 7.4.2. The gene set was filtered with Repeatmasker (http://www.repeatmasker.org) to remove retrotransposons and tandem repeats. Genes were also checked for in-frame internal stop codons and SWAMP version 1.0 (Harrison et al. 2014) was used with a threshold of four in a window size of five bases to check for regions with poor alignment and to set a minimum sequence length of 75 bp. PAML version 4.7a (Yang 2007) was used to estimate divergence for orthologous genes, and orthologous genes with $d_S > 2$ were removed from further analyses as this represents the point of mutational saturation in avian sequence data (Axelsson et al. 2008). The resulting molecular divergence was measured as root-to-tip branch length between pheasant and each species.

Measures of Fast-Z Evolution of Gene Expression

Spearman's Rho

Spearman's rho correlation coefficient (ρ) can be used to estimate the decay in similarity between species over time, and the comparison between the slope of ρ across phylogenetic distance for the Z and autosomes is a measure of Fast-Z evolution of gene expression. Spearman's rho correlation between pheasant and all other species was calculated for all genes on the autosomes and Z chromosome (Kayserili et al. 2012; Harrison et al. 2015). We used linear models to test for a significant difference between the slope of the decay in similarity across molecular divergence time for autosomes and Z chromosome (supplementary table S1, Supplementary Material online). For each pairwise comparison, we tested whether the Z chromosome ρ was significantly different from the autosomal ρ using 1,000 bootstrapped replicates consisting of the number of Z-linked genes sampled from the pool of autosomal genes. The 95% confidence intervals of the autosomal distribution were used to denote a significant difference between the Z chromosome and the autosomes.

Gene Expression Divergence

For each pairwise species comparison, expression divergence was calculated as the difference in gene expression between the two species divided by the average gene expression (Meisel et al. 2012) for each locus. Gene expression divergence was calculated separately for male and female expression in the spleen and gonad. Two-sided Wilcoxon tests were used to test for differences in gene expression divergence on autosomes and Z chromosomes.

Correlation in Gene Expression between Males and Females

The correlation in gene expression between males and females (C_{mf}) was calculated separately for each gene for expression in the spleen and gonad. PGLS models were used in the Caper package (Orme et al. 2012) (R v2.15.1) using the ML phylogeny for our six species to correct for phylogeny. The r^2 value was used as the estimate of the strength of the correlation in gene expression between males and females for each gene. Sex limited genes were removed from these analyses as the models cannot account for low variance in expression across the phylogeny.

Ancestral State Gene Expression Divergence and Directional Selection

Ancestral state reconstruction of expression was conducted with the APE package (Paradis et al. 2004) using the Brownian motion-based ML estimator (Schluter et al. 1997) using the ML phylogeny of the six species described above. Gene expression divergence was calculated between each species and their most recent ancestor (i.e., their nearest internal node in the phylogenetic tree).

Models exist to test for positive selection in gene expression (Brawand et al. 2011; Roux et al. 2014). Δx (Moghadam et al. 2012) is particularly useful in this case because it corrects for expression level, which is important in comparisons between diploid and haploid chromosomes, and in systems lacking complete sex chromosome dosage compensation. We calculated Δx (Moghadam et al. 2012) between each species and their most recent ancestor (i.e., their nearest

internal node in the phylogenetic tree). Δx incorporates expression variance as an indicator of polymorphism, and values for $\Delta x > 1$ or < -1 indicate that divergence from the point estimate of the ancestral state is greater than standing genetic variation in gene expression within the species, a typical indicator of positive selection.

Sex-Biased Gene Expression

Sex-biased gene expression rapidly changes across the phylogeny (Harrison et al. 2015) and few genes remain sex-biased in all six species (Harrison et al. 2015). Genes whose ancestral reconstruction was sex-biased at the ancestral node to all six species were therefore classified as sex-biased. Male- and female-biased genes were identified using log 2-fold change gene expression between males and females. This resulted in 24 female-biased genes and 54 male-biased genes on the Z chromosome and 589 female-biased genes and 554 male-biased genes on the autosomes.

Expression Level

Genes were broadly divided into highly and lowly expressed. Average gene expression across the six species was calculated for each gene and then expression was averaged across males and females. Genes with expression above the median (4.55 rpkm) were classified as highly expressed and below were classified as lowly expressed. Significant differences in gene expression divergence on the Z chromosome and autosomes between the ancestral state and each species were analyzed as before.

Supplementary Material

Supplementary figures S1 and S2 and table S1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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