# SEXUAL SELECTION DRIVES EVOLUTION AND RAPID TURNOVER OF MALE-BIASED GENES

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***Abstract***

The profound and pervasive differences in gene expression observed between males and females, and the unique evolutionary properties of these genes in many species, have led to the widespread assumption that they are the product of sexual selection and sexual conflict. However, we still lack a clear understanding of the connection between sexual selection and transcriptional dimorphism, often termed sex-biased gene expression. Moreover, the relative contribution of sexual selection versus drift in shaping broad patterns of expression, divergence and polymorphism remains unknown. To assess the role of sexual selection in shaping these patterns, we assembled transcriptomes from an avian clade representing the full range of sexual dimorphism and sexual selection. We use these species to test the links between sexual selection and sex-biased gene expression evolution in a comparative framework. Through ancestral reconstruction of sex-bias, we demonstrate a rapid turnover of sex-bias across this clade driven by sexual selection, and show it to be primarily the result of expression changes in males. We used phylogenetically controlled comparative methods to demonstrate that phenotypic measures of sexual selection predicted the proportion of male-biased, but not female-biased gene expression. Although male-biased genes showed elevated rates of coding sequence evolution, consistent with previous reports in a range of taxa, there was no correlation between sexual selection and rates of coding sequence evolution, suggesting that expression changes may be more labile and less functionally constrained. Taken together, our results highlight the power of sexual selection to act upon gene expression differences and shape genome evolution.

***Significance statement***

Genes with different expression between males and females (sex-biased genes) show rapid rates of sequence and expression divergence in a range of taxa. This has led many to assume that sex-biased genes are the product of sexual selection and sexual conflict, and this assumption remains to be rigorously tested. Using a phylogenetically controlled analysis of birds that exhibit diverse levels of sexual selection, we show a rapid turnover in sex-biased gene expression primarily through evolution of male expression levels, and that the degree of sexual selection predicts the proportion of male-biased genes, but does not account for rates of coding sequence evolution.

Numerous studies across a range of organisms have convergently shown that the majority of variation in overall gene expression is explained by sex (1-6). These sex-biased genes have distinct evolutionary properties, namely that they show faster rates of sequence and expression divergence, as well as rapid rates of turnover, broadly consistent with sexual selection (reviewed in (7, 8)). The sizable proportion of genes exhibiting sex-biased expression suggests that sexual selection has the potential to shape many aspects of genome biology. Recent studies of intra-sexual variation in gene expression differences between males and females of the same species have revealed patterns of overall transcription consistent with the degree of phenotypic sexual dimorphism (9, 10), and experimental manipulation of sex-specific selection affects sex-biased gene expression over short time scales (11-13). These studies together suggest that increasing sexual selection across species should lead to increased turnover in sex-biased gene expression and a greater sexualization of the transcriptome over longer evolutionary timescales.

The elevated rates of coding sequence evolution often (14) but not always (15) observed for male-biased genes has been suggested to be the product of positive selection resulting from sexual selection acting primarily in males (14). This assumes an adaptive mechanism underlying gene sequence evolution, and if true, predicts that rates of evolution for male-biased genes might be higher in species under stronger sexual selection. However, recent molecular data (16) have suggested that genes with male-limited expression have elevated levels of deleterious polymorphisms. If this is true on a broader scale, it suggests that elevated rates of evolution in male-biased genes might instead be due to relaxed purifying selection. The relative role of sex-specific selection and drift in shaping broad patterns of expression, divergence and polymorphism for these genes therefore remains unclear.

In order to assess the long-term effects of sexual selection on genome and transcriptome evolution, we require a clade of organisms with a well-resolved phylogeny, known variation in sexual selection and with constituent species that can be reared in controlled conditions in order to minimize the effects of environmental variance in gene expression. These conditions are all met by the Galloanserae (the landfowl and the waterfowl), a 90 million year old clade of birds (17) that exhibit multiple independent transitions in sexually selected traits and sexual dimorphism. Moreover, the high degree of genomic stability exhibited by birds (18) means that these changes in sex-specific selection are acting on a relatively static genome.

We assembled male and female transcriptomes from gonadal and somatic tissue from multiple individuals of six species within the Galloanserae in order to assess the role of sexual selection on long-term evolutionary dynamics of gene expression, divergence and polymorphism. We deliberatively chose species with a full range of sexual dimorphism and sexual selection, ranging from the Darwinian paradigm of sexual selection, the polygynous and strikingly sexually dimorphic peafowl (*Pavo cristatus*) to monogamous and sexually monomorphic species such as the swan goose (*Anser cygnoides*). We used these data to critically test the connection between sexual selection and the evolution of sexually dimorphic transcriptomes. Our results provide a clear link between sex-biased gene expression evolution and sexual selection across phylogenetic space in a robust comparative framework.

## *Results and Discussion*

### *Transcriptome sequencing, mapping and orthology*

We sequenced mRNA from the spleen and gonads of male and female individuals from six species of Galloanserae (mallard duck, *Anas platyrhynchos*; swan goose, *Anser cygnoides*; wild turkey, *Meleagris gallopavo*; helmeted guineafowl, *Numida meleagris*; indian peafowl, *Pavo cristatus* and common pheasant, *Phasianus colchicus*) all in their first breeding season and deliberately chosen to represent the full range of sexual dimorphism and sexual selection observed in birds. This yielded 629Gb (105Gb on average per species) of 100 bp paired end reads. Following quality filtering we constructed a *de novo* transcriptome for each species using Trinity (19), and used RSEM (20) to quantify expression levels, filtering out lowly expressed and erroneous contigs from the assemblies (Table S1). One-to-one orthology was determined across the six species, identifying 2,817 autosomal orthologs shared across the phylogeny referred to hereafter as the six-species orthologs. Although orthology across all six study species is required for studies of gene sequence evolution and some of the analyses of expression evolution, it is possible that genes which are unambiguously orthologous across all our study species may not be those subject to the strongest sexual selection. Therefore, we also used a larger dataset of reciprocal best orthologs between each of our study species and the chicken for some analyses, referred to as the two-species orthologs. This approach resulted in 9,178 autosomal orthologs for mallard, 9,350 for swan goose, 9,018 for turkey, 8,995 for guineafowl, 8,777 for peafowl and 9,182 for pheasant. Chromosomal location was defined by orthology in chicken, allowing us to capitalize on the stability of avian genomes (18). Due to the incomplete Z chromosome dosage compensation in birds (21-23) and the unique evolutionary forces shaping sex

chromosomes (24-27), we focus only on the autosomal orthologs here, and have dealt with the Z-linked orthologs separately (28).

Sex-biased gene expression has been hypothesized to be the result of intra-locus sexual conflict over optimal transcription, and to be the underlying genetic mechanism for sexual dimorphisms relating to sexual selection (7, 14, 29), however a definitive link has yet to be established. We combined the gene sets above with phenotypic measures of sexual selection to assess the relationship between genomic characteristics of sex-biased genes and sexual selection regimes. We used sexual ornamentation (dichromatism, elongated feathers, wattles, caruncles, etc., see SI) as a proxy for pre-copulatory sexual selection (30, 31), either through female choice or male-male competition. We also examined residual testis weight and sperm number, widely used measures of post-copulatory sexual selection either from sperm competition among males, sexual conflict over fertilization, or sperm-loading needed for multiple mating (32-34).

***Sex-biased expression and sexual selection***

We used hierarchical clustering of expression levels for our six-species specific ortholog data set to visualize global transcriptomic patterns within and among the six species. Gonad samples cluster first by sex and then by phylogenetic relatedness (Fig. 1A), in contrast to somatic tissue, where samples cluster primarily by phylogenetic relatedness (Fig. 1B), reflecting lower levels of sex specific selection.

We defined sex bias within each species using standard measures (see SI, Table S2). The reduced sex-specific selection acting on somatic tissue is reflected by the fact that only a single locus exhibited significant female-bias, and significant male-bias was completely

absent in the somatic tissue. We therefore focused on the gonad for all analyses of sex-biased expression.

Even though roughly half of expressed genes were sex-biased in any species, sex-bias was not strongly conserved across the clade in our six-species ortholog data set, with only 198 male-biased and 203 female-biased genes with conserved patterns of sex-bias across all six study species. Genes with conserved sex-bias across all six study species had higher average expression levels than the remaining sex-biased genes (average $\log_2$ RPKM of 6.75 for universal male-biased genes and $\log_2$ RPKM of 4.95 for the remaining male-biased genes; 5.02 for universal female-biased genes and 4.47 for the remaining female-biased genes).

In the six-species ortholog data set, we inferred 555 male-biased and 607 female-biased genes to be ancestrally sex-biased, based on maximum likelihood reconstruction allowing gain and loss of sex bias across the six-species evolutionary history (Fig. 2A). Given the high proportion of species-specific sex-biased genes, it is probable that the most recent common ancestor of our study species possessed many male- and female-biased genes that are unbiased in all our assessed daughter species. Ancestral sex-bias in these specific loci will not be inferred based on extant taxa, therefore the number of sex-biased genes at the common ancestor is likely to be somewhat higher. Ancestral state reconstructions also indicate rapid turnover of sex bias across the clade, further emphasized by the high proportion of genes that are polyphyletic or species-specific in their sex-biased expression (Fig. 2, panels B and C), and by the rapid decay in rank-order of sex-bias, particularly in the testis, across species (Fig. 3).

In order to investigate male and female patterns of change underlying sex-biased expression evolution, we reconstructed ancestral expression levels across the phylogeny for the six-

species orthologs. This also made it possible to test for statistical artefacts in turnover of sex-bias. It is important to note that models of gene expression evolution are largely additive, and have not yet been functionally validated. Their utility in extrapolating evolutionary signals is important, but results must be interpreted cautiously.

Across our six-species ortholog dataset, nearly twice as many loci exhibited species-specific female bias (389) than were consistently female-biased across all six-study species (203). Species-specific female-biased genes showed on average mild female-bias in the nearest ancestral node ($\log_2$ fold change = -0.5879), but in many comparisons to more distantly related species were mildly male-biased ($\log_2$ fold change > 0). Furthermore, roughly half (49.5%) of all loci that were significantly female-biased in one species were significantly male-biased in at least one other. Similar to female-biased genes, more loci showed species-specific (474) male-bias than were consistently male-biased across all six study species (198), and were mildly male-biased at the nearest ancestral node ($\log_2$ fold change = 0.5389). Many species-specific male-biased genes also showed extensive change in sex-bias across the phylogeny, with 50.6% female-biased in at least one other species. For those male- and female-biased genes that exhibit differences in sex-bias across our study species, the likelihood of change in sex-bias in other species increased as a function of phylogenetic distance. This suggests that the high proportion of species-specific patterns of sex-bias is not a statistical artefact, but rather reflects rapid turnover of sex-bias across species.

If the rapid turnover of sex-biased genes that we observe is a product of sexual selection, we would expect it to be associated with phenotypic measures of sexual selection. To test this, we performed phylogenetically controlled regressions between phenotypic measures of sexual selection and turnover of sex-biased expression in our six-species orthologs. In line

with our prediction, we recovered a significant association between turnover of male-biased genes in terminal branches of our phylogeny with the degree of sexual ornamentation in males (Fig 2D; $P = 0.0017$). This provides the first statistical evidence for a link between gene expression evolution across species and sexual selection, and indicates that sexual selection can lead to major changes in transcriptional evolution.

Changes in sex-bias were on average due to greater changes in males than females, and this was true for both male- and female-biased loci. Among the 389 species-specific female-biased genes, 290 (74.6%) showed greater down-regulation in males than up-regulation in females from the nearest ancestral node. Similarly, among the 474 species-specific male-biased genes, 371 (78.2%) showed greater up-regulation in males than down-regulation in females, and only 4 showed significant changes in both sexes (> 2-fold change in both males and females). Significant change in both sexes was not observed for any species-specific female-biased loci.

In addition to rates of turnover for sex-biased expression, we also assessed whether the sexualization of the transcriptomes of our study species was associated with phenotypic measures of sexual selection in our two-species ortholog set, controlling for phylogenetic non-independence. The proportion of male-biased gene expression was significantly correlated with residual testis weight (Fig. 4A; $P = 0.032$), log sperm number (Fig. 4B; $P = 0.010$) and degree of sexual ornamentation (Fig. 4C; $P = 0.011$). All phylogenetically-controlled regressions for the proportion of female-biased genes (Fig. S1A-C; $P > 0.05$ in all cases) and for the proportion of all sex-biased genes (Fig. S1D-E; $P > 0.05$ in both cases) were non-significant, apart from the regression of sex-biased genes against sexual ornamentation whose significance is driven by male bias (Fig. S1F; $P = 0.016$).

This suggests that the rapid turnover of male-biased genes, as well as the proportional masculinization of gene expression, is the product of sexual selection. As such, our data provides a clear cross-species demonstration of a link between sexual selection and sex-biased gene expression, connecting the genome to the phenotype through aggregate gene expression patterns and identifying the signature of sexual selection in the genome.

Sex-biased gene expression is often analysed in the framework of intra-locus sexual conflict over optimal expression (35-37). If intra-locus conflict is the main driver of sex-biased expression, our results suggest that the targets of this conflict shift rapidly over phylogenetic distance. Alternatively, our data could suggest that inter-locus conflict between males and females over fertilization may also be important. Inter-locus sexual conflict over fertilization between males and females, driven by Red Queen dynamics where the co-evolutionary game is constantly shifting, results in selection for novelty in males and resistance to that novelty in females (38). This could result in the rapid change in sex-specific transcriptional profiles that we observe in our data. Although it is not possible to completely separate measures of pre- and post-copulatory sexual selection in shaping gene expression dimorphism in our data, the correlation between measures of post-copulatory sexual selection and the proportion of the transcriptome exhibiting male-biased expression suggests that gene expression in the gonad is at least partly shaped by conflict between males and females over fertilization.

***Coding sequence evolution***

In order to investigate the role of sexual selection on coding sequence evolution, we next calculated divergence estimates using the CODEML package in PAML (39) for the six-species orthologs. Within each species, average $d_N/d_S$ for male-biased genes was significantly higher

in comparison to unbiased genes (Fig. 5). For the majority of species, the average $d_N/d_S$ for female-biased genes was significantly higher than unbiased genes, but this difference was not significant for guineafowl or for pheasant. Highly male-biased genes and those with extreme male-bias show greater divergence than lowly male-biased genes and female-biased genes (Fig. 6). Genes that were universally male-biased in every species had higher $d_N/d_S$ levels than other male-biased genes (duck 0.1543, goose 0.1541, guineafowl 0.1821, peafowl 0.1710, pheasant 0.1709 and turkey 0.1739; in comparison the species average Table S3), but it is not possible to differentiate the influence of universality from that of expression level.

Highly female-biased genes also show an increase in divergence, but not to the same extent as male-biased genes. These results are consistent with our previous work in birds which demonstrate that sex-biased gene expression varies greatly through ontogeny and that male- and female-specific selection are ontogenetically decoupled due to sex differences in meiosis and gametogenesis (3). Although male-specific selection acts primarily on male-biased genes expressed in adults once spermatogenesis commences, female-specific selection produces a rapid rate of sequence evolution for genes that are female-biased in late development, with the onset and arrest of oogenesis before hatching (3, 13). Our samples are taken from adults in their first reproductive year, as we designed the experiment to examine the power of sexual selection in shaping rates of sequence and expression evolution of male-biased genes. Given the high rates of divergence observed for female-biased genes in late development, it would be interesting to examine the relationship between sexual selection and the female transcriptome from samples taken at this ontogenetic stage, however this beyond the scope of this study.

Rapid rates of coding sequence evolution for male-biased genes have previously been suggested to be the product of post-copulatory sexual selection (14). Despite male-biased genes showing elevated rates of sequence evolution, this was not significantly associated with phenotypic measures of sexual selection based on sexual ornamentation, sperm number or testis weight ($P > 0.5$ in all comparisons). Although it could be argued that we have insufficient power to test the association between $d_N/d_S$ and mating system, we did recover a significant association between these variables for a much smaller number of Z-linked loci due to neutral processes (28), suggesting that our analysis of a much larger dataset here is sufficiently powerful, assuming a similar effect size. These results suggest that the elevated rates of $d_N/d_S$ in male-biased genes may not, as is often assumed (14), be the direct product of post-copulatory sexual selection acting primarily on males.

The lack of association between rates of evolution for male-biased genes and post-copulatory sexual selection is initially perplexing, particularly given the assumption that sexual selection underlies positive selection for these genes. Although positive selection may still act on a subset of male biased genes, recent reports of a high rate of deleterious non-synonymous substitutions in a small set of male-limited proteins (16) hint at a possible explanation, as they suggest that selection may in fact be less effective on male-limited and strongly male-biased genes. In order to examine the relationship between sex-bias and sequence evolution, we assessed synonymous ($p_S$) and non-synonymous ($p_N$) diversity for different sex-bias categories across species. Although there are differences across species, likely due to differences in effective population size, overall diversity ($p_S$) does not differ consistently across different expression categories within species (Fig. S3A). This data also suggests that male-specific selection has not depleted the underlying male-biased genes of

functional polymorphism, and male-biased genes in each of our study species exhibit equal or higher proportions of non-synonymous polymorphisms than unbiased or female-biased genes (Table S4). Moreover, functional diversity ($p_N$) is significantly higher for strongly male-biased genes in four of our six study species (Fig. S3B). Most importantly, although there was no relationship between elevated $p_N$ and mating system, it is these classes of genes that show the highest rates of evolution. This suggests that at least some of the elevated rates of evolution observed for these gene expression categories might in fact be due to non-adaptive genetic drift rather than adaptive evolution driven by sexual selection. Male-biased genes in many animals, including birds, tend to be more tissue-specific, with more focused expression in the testis, than unbiased or female-biased genes (40, 41). This expression profile could mean that male-limited and strongly male-biased genes expressed only in the testis may simply be subject to selection only in males (42), thereby resulting in a reduced power of purifying selection in some of these genes to purge alleles with very mild deleterious effects.

***Allometric scaling and relative expression***

Although rarely discussed in the literature, allometric scaling could explain previous reports of gene expression differences among species (e.g. 4, 6,) and populations (e.g. 11). Allometric differences are particularly problematic for studies involving whole-body comparisons, as variation in the relative scaling of constituent tissues could produce a signal of gene expression variation (4,6), however allometry is also a possible confounding issue in studies of gene expression evolution of single organs and tissues (BRAWAND REF), such as the one here. Moreover, previous reports of turnover in sex-bias (4,6) could be due, at least in part, to allometric differences, particularly if one sex shows extensive variation in

allometry. It is therefore possible that allometric scaling between sub-tissues and total testis mass could result in different tissue proportions in the testis among our study species.

In our study, most of the turnover in sex-bias that we observe across our study species is due to changes in male expression, while female expression is overall relatively static. Allometric scaling could affect relative expression levels of genes that are differentially expressed among sub-tissues in males, and potentially contribute to the turnover in sex-bias that we observe. If allometry were causing the pattern of turnover we observe, it might be expected to cause similarity in overall transcription between species with similar testis mass, as although measures of testes mass typically show some phylogenetic signal (Iossa et al. 2008), the signal is low than for many other traits (Kamilar and Cooper, Moller and Briskie). The strong phylogenetic signal that we observe in both hierarchical clustering (Fig. 1) and rank order correlation (Fig. 3), as well as the fact that the likelihood of change in sex-bias increased as a function of phylogenetic distance, together suggest that allometric effects are not a major concern in this dataset. Further hierarchical clustering of sex-biased genes, which we would expect to be most affected by allometry, also showed a clear phylogenetic pattern, rather than one associated with testis mass (Fig. S2). However, these results, although suggestive, do not rule out the possibility that allometric differences among our study species cause at least some of the turnover that we observe, particularly if testes mass shows a phylogenetic signal.

In order to investigate the possible influence of allometry further, we tested for an association between relative expression level and testis mass for each locus in our six-species ortholog dataset. . It is important to note that we tested for an association between normalized expression levels and testes size. Normalization corrects for differences in read

count among samples, and produces a relative expression measure that could be influenced by allometric scaling. We identified 239 loci that showed a significant association ($p < 0.05$). Although none of these were significant after correcting for multiple testing, we removed these 239 genes from our dataset and repeated all analyses of sex-biased evolutionary properties. In all cases, the results were qualitatively identical: transcriptional clustering and rank order correlation showed similar phylogenetic signatures; there was a significant relationship between sexual ornamentation and turnover of male-biased genes ($p = 0.002$); proportion of male-biased genes showed significant associations with residual testis weight ($p = 0.028$), log sperm number ($p = 0.006$) and sexual ornamentation ($p = 0.006$); and male-biased genes exhibited significantly higher $d_N/d_S$ than unbiased genes ($p < 0.05$). The robustness of our results after removing possible allometry-associated loci suggests that allometry is not a major source of bias in our dataset

Although our results suggest that allometry is not a major contributor to turnover in sex-bias, we hasten to point out that they do not entirely rule out the possibility that allometry is a contributor to the patterns that we, and many others, observe. The allometry issue has important implications to the interpretation of sex-bias turnover, and more broadly to studies of gene expression evolution. If allometry is the major contributor to turnover observations, then it suggests that sexual selection is not acting so much on gene regulation, but rather changes in the relative size of the constituent parts of the testes results in the reordering of relative expression of testes-expressed genes. Unfortunately, it is currently not possible to truly differentiate these alternative explanations for change in sex-bias over time. However, recent advances in single-cell transcriptome analysis may be useful in

revealing the relative contributions of allometry versus regulatory evolution in cases such as these.

***Concluding remarks***

In order to assess the long-term effects of sexual selection on genome evolution, we assembled transcriptomes from six species of Galloanserae, combining gene expression, divergence and polymorphism data with phylogenetically controlled measures of sexual selection in a robust comparative framework. The species assessed were deliberately selected to encompass the full range of sexual selection and mating systems, ranging across promiscuity, polygyny and monogamy. This allows us to critically test the route by which sexual selection affects genome evolution across species.

Our results indicate that both turnover and magnitude of male-biased expression are strongly predicted by measures of sexual selection (7, 14), and also explains the feminization of gene expression observed in *Drosophila* under enforced monogamy, which effectively eliminates polyandry and sperm competition (11). Interestingly, our results suggest that although sexual selection is driving gene expression evolution, it does not explain the higher rates of sequence evolution generally observed for male-biased genes (7, 14). This is likely not due to lack of power, rather, our data suggest that selection is less effective at purging functional polymorphism for many of these loci.

Taken together, our results indicate that the focus of sexual selection shifts rapidly across lineages. Our results also suggest that sexual selection acts primarily on expression, which may be more labile and less functionally constrained than coding sequence, and therefore

more likely to be influenced by short-term mating system dynamics among related species. The lability of gene expression evolution is illustrated in recent experimental evolution approaches that found an association between sex-biased gene expression and variations in sex-specific selection (11, 13). Gene expression lability is also clearly illustrated by the rapid turnover of sex-biased genes in our phylogeny (Fig. 2), which has also been observed in other animal clades (6, 44). Furthermore, rank order correlations show that gene expression divergence increases with evolutionary time across the Galloanserae (Fig 3), again illustrating the lability of gene expression.

In summary, our results implicate sexual selection as a powerful force in shaping broad patterns of genome evolution.

***Methods summary***

Male and female gonad and spleen samples were collected from captive-reared populations of six species of Galloanserae. RNA-Seq was performed on replicate samples for each tissue and sex, and the resulting sequence was used to construct a *de novo* transcriptome assembly for each species. Reads were mapped to these *de novo* assemblies to obtain sequence, expression and polymorphism data for one-to-one orthologs between each species and the chicken genome, and for one-to-one orthologs shared across the six species. Comparisons of normalized expression counts were used to identify sex-biased gene expression using standard measures and corrected for multiple testing (3, 9, 45). Ancestral state reconstruction was performed with the APE (46) R package to predict sex-bias in the most recent common ancestors from the sex-biased genes found in each of the six species. PAML version 4.7a (39) was used on aligned orthologs (47, 48) to obtain sequence divergence information and Samtools (49) and VarScan2 (50) were used to identify valid

single nucleotide polymorphisms. Phylogenetic Generalized Least Squared regressions were performed using BayesTraits (51) with maximum likelihood and 1000 runs for each analysis to test for associations between sex-biased expression to measures of sexual dimorphism and sperm competition. Full methods and associated references are included in *SI Methods.*


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**Fig. 1.** Heatmaps and hierarchical clustering of gene expression for (A) gonad and (B) spleen. Shown is the average relative expression for autosomal genes from male (blue) and female (red) samples. Hierarchical clustering is based upon Euclidean distance for average $\log_2$ expression for each orthologous autosomal gene across both sexes of the six species. On each node, bootstrap support values are shown from 1000 replicates.

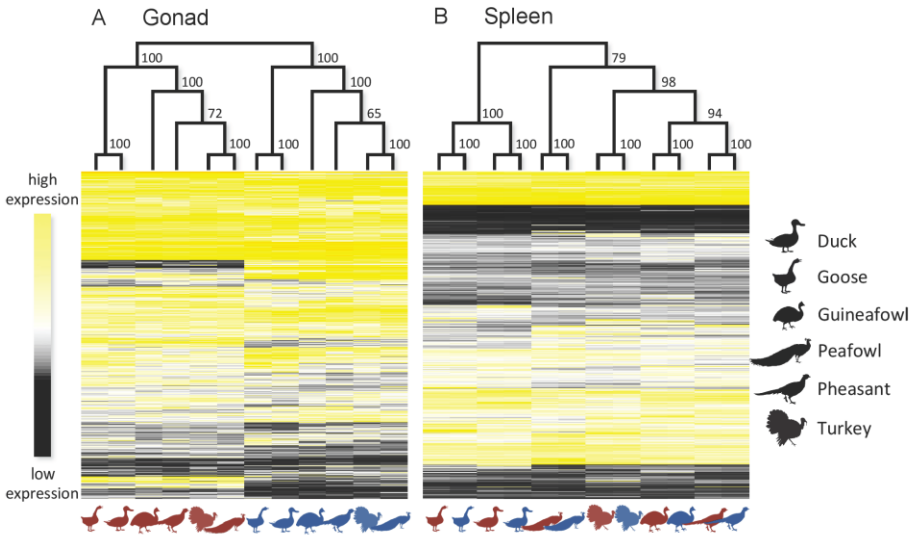

**Fig. 2.** (A) Maximum likelihood phylogeny, sex-bias for each of the six study species and inferred ancestral sex-bias. Gain and loss of sex-biased genes is displayed on each branch, based on ancestral reconstruction of male and female expression. The scale bar indicates the number of substitutions per site. The proportion of species specific, universal, monophyletic and polyphyletic (B) male-biased and (C) female-biased orthologs were calculated based on the actual sex-biased gene numbers for each species. The high proportion of species-specific sex-biased genes suggests that some sex-biased genes in the common ancestor are unbiased in all daughter species, and therefore cannot be identified using ancestral state reconstruction. (D) Phylogenetically controlled regression of the turnover of male-biased genes on the tip branch of each species against sexual ornamentation. Correlation and significance was determined using phylogenetic generalized least squares models in BayesTraits (51) with maximum likelihood and 1000 runs for each analysis.

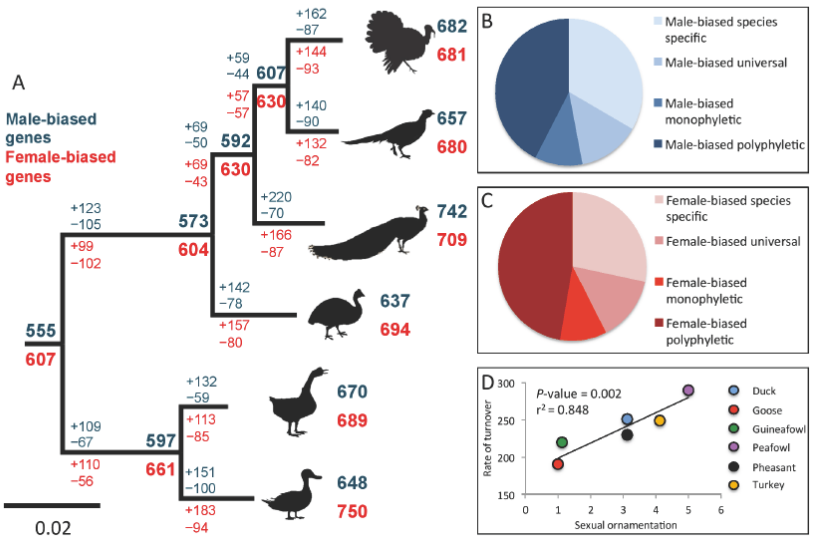

**Fig. 3.** Spearman's rho rank order correlations between pheasant and each other species for (A) average male expression in testis and spleen (B) average female expression in ovaries and spleen and (C) $\log_2$ fold change in sex-biased expression in gonad and spleen. Divergence time between pheasant and each species was based upon the maximum likelihood phylogeny (Fig. 2). Testis is shown in blue, ovaries in red, gonad in purple and spleen in black. Confidence intervals are shaded, and were calculated by bootstrapping with 1000 replicates.

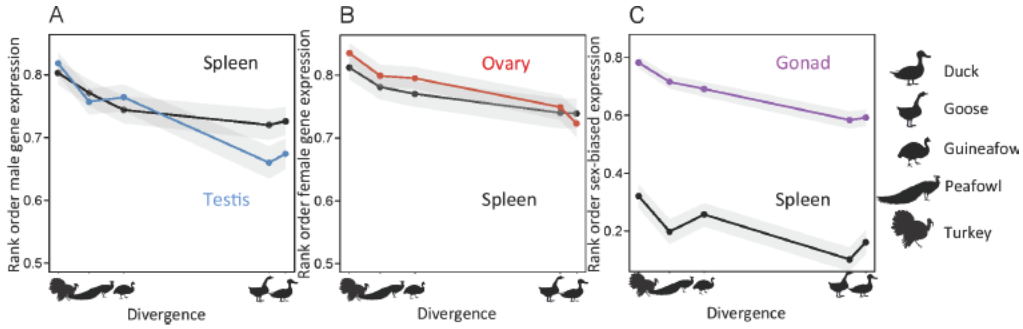

**Fig. 4.** Phylogenetically controlled regression between the proportion of male-biased genes for each species and (A) residual testis weight, (B) log sperm number and (C) sexual ornamentation. The significance was determined using phylogenetic generalized least squares models in BayesTraits (51) with maximum likelihood and 1000 runs for each analysis.

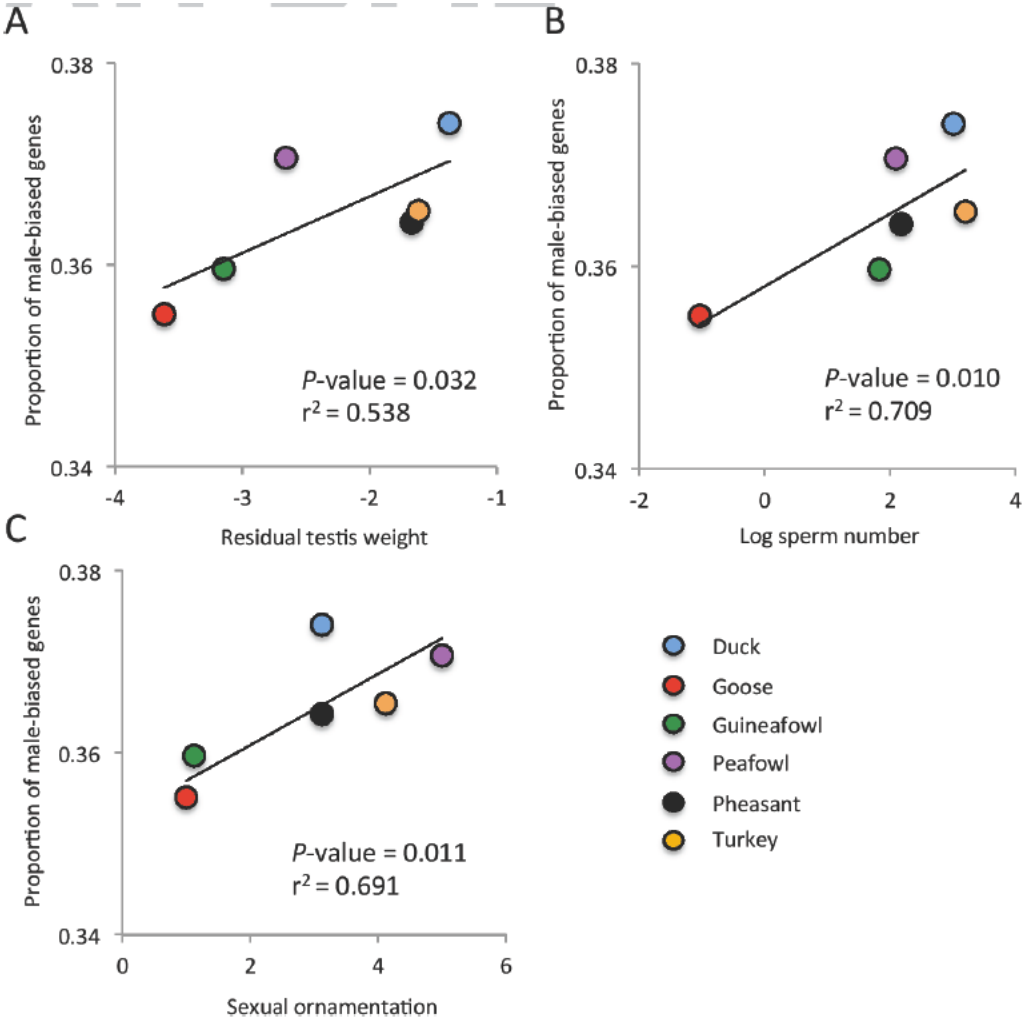

**Fig. 5.** Average ratio of non-synonymous substitutions ($d_N$) to synonymous substitutions ($d_S$) for unbiased (grey), male-biased (blue) and female-biased (red) genes. Significance values were determined by permutation tests of unbiased versus either male-biased or female-biased genes, and 95% confidence intervals were derived from bootstrapping with 1000 replicates. Displayed significance scores are *** = $P < 0.001$ and **** = $P < 0.0001$.

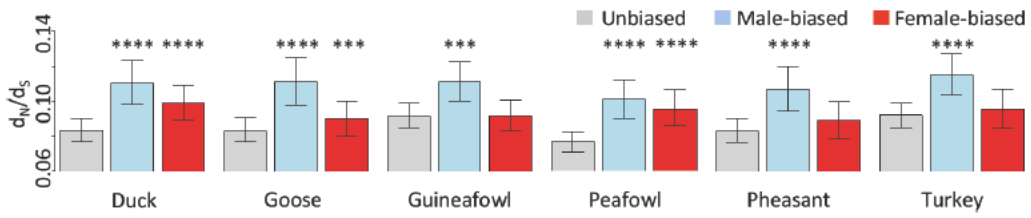

**Fig. 6.** The ratio of nonsynonymous substitutions per nonsynonymous site ($d_N$) to synonymous substitutions per synonymous site ($d_S$) is shown for male-biased, female-biased and unbiased genes, subdivided based on fold change, see SI methods. Highly male-biased genes show elevated $d_N/d_S$ ratios for all species.