Rapid Evolution of Female-Biased, but Not Male-Biased, Genes Expressed in the Avian Brain

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The powerful pressures of sexual and natural selection associated with species recognition and reproduction are thought to manifest in a faster rate of evolution in sex-biased genes, an effect that has been documented particularly for male-biased genes expressed in the reproductive tract. However, little is known about the rate of evolution for genes involved in sexually dimorphic behaviors, which often form the neurological basis of intrasexual competition and mate choice. We used microarray data, designed to uncover sex-biased expression patterns in embryonic chicken brain, in conjunction with data on the rate of sequence evolution for >4,000 coding regions aligned between chicken and zebra finch in order to study the role of selection in governing the molecular evolution for sex-biased and unbiased genes. Surprisingly, we found that female-biased genes, defined across a range of cutoff values, show a higher rate of functional evolution than both male-biased and unbiased genes. Autosomal male-biased genes evolve at a similar rate as unbiased genes. Sex-specific genomic properties, such as heterogeneity in genomic distribution and GC content, and codon usage bias for sex-biased classes fail to explain this surprising result, suggesting that selective pressures may be acting differently on the male and female brain.

Introduction

Many of the numerous behavioral, physiological, and anatomical differences between males and females originate from autosomal genes that are identical in both sexes. In order to produce distinct female and male phenotypes from the same gene complement, many coding regions are expressed at different levels in the sexes, a phenomenon referred to as sex-biased gene expression (Ellegren and Parsch 2007). Sex-biased expression is common, and microarray gene expression analyses have indicated that a large proportion of the transcriptome shows different expression between females and males in several metazoan species (Ranz et al. 2003; Parisi et al. 2004; Marinotti et al. 2006; Yang et al. 2006). Sex-biased genes are likely responsible for a great deal of intersexual variation and are often associated with various aspects of reproduction (Rinn and Snyder 2005); therefore, these genes are frequently subject to the powerful selective pressures of sexual selection, mate choice, species recognition, and sperm competition (Swanson and Vacquier 2002).

Reproductive pressures could theoretically increase the rate of sequence and protein evolution for both male- and female-biased genes, a condition that has been demonstrated primarily for coding regions associated with the reproductive tract (Civetta and Singh 1999; Swanson et al. 2001, 2004; Zhang et al. 2004; Jagadeeshan and Singh 2005). However, sex-biased genes are not limited to the reproductive tract and are present in somatic tissues as well (Ranz et al. 2003; Parisi et al. 2004; Yang et al. 2006), giving rise to behavioral, physiological, and anatomical sexual dimorphism. Studies of sex-biased somatic genes have suggested that primarily male-biased genes undergo faster rates of functional evolution (Meiklejohn et al. 2003; Pröschel et al. 2005); however, these studies have been primarily limited to *Drosophila melanogaster*, which may not be an appropriate generalized model for sex-biased gene evolution (Metta et al. 2006). Sex-biased genes expression in brain are the probable cause of many, if not most of the behavioral differences between males and females (Dauwalder et al. 2002; Drapeau et al. 2003; Kadener et al. 2006), and sex-biased brain genes are therefore important for various aspects of premating sexual selection, including courtship and breeding behavior, as well as mate choice and species recognition (Andersson 1994). This situation warrants an examination of the relationship between sex-biased gene expression in brain and rate of molecular evolution in organisms in which sexual selection is pronounced. Many avian species show elaborate morphological and behavioral dimorphisms, many of which have been linked to sexual selection, making birds an ideal clade for such an investigation.

It is possible to estimate the rate of sequence evolution for different classes of genes by comparing the ratio of nonsynonymous (or coding) substitutions to synonymous (or silent) substitutions in the alignment of orthologous coding regions for 2 or more species. This ratio corrects for neutral mutation rate variation and gives an indicator of how much functional change a given gene has undergone since those species diverged. We used this approach to compare the divergence of sex-biased and unbiased brain-expressed genes in birds in order uncover the pattern of molecular evolutionary properties of the coding regions that underlie behavioral dimorphisms. We utilized microarray expression data from chicken (*Gallus gallus*) brain tissue, designed to reveal differences in expression between male and female embryos (Ellegren et al. 2007). We then used the annotated chicken genome sequence information (ICGSC 2004) for roughly 4,000 identified brain-expressed genes, in conjunction with orthologous zebra finch (*Taeniopygia guttata*) brain expressed sequence tag (EST) data from the 'Songbird Neurogenomics Initiative' (Clayton 2004) to estimate the rate of sequence evolution that has occurred since the chicken and zebra finch diverged, roughly 100 MYA (van Tuinen et al. 2000). We also examined other genomic properties, such as chromosome location, Gene Ontology terminology, GC content, and bias in codon usage that have been linked to rate of sequence evolution in birds and other organisms (ICGSC 2004; Meunier and Duret 2004; Zhang et al. 2004; Axelsson et al. 2005; Hambuch and Parsch 2005; Spencer et al. 2006).

Key words: gene expression, sexual selection, sex bias, d_N/d_S, chicken.

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Materials and Methods

Expression Data Collection

RNA was collected from the brain tissue of 4 female and 3 male 18-day chicken embryos, and each sample was analyzed individually using the Chicken Affymetrix GeneChip microarray platform. Further details regarding sample preparation and methodology are found in Ellegren et al. (2007) and are also available in the supplementary material online. All preprocessing and statistical analysis of microarray data was performed in R version 2.4.1 using Bioconductor packages release 1.9 (Ihkaka and Gentleman 1996). CEL files were processed with GC Robust Multi-array Average (Wu and Irizarry 2004), a background adjustment method that corrects for the GC content of probes when assessing nonspecific binding, followed by quantile normalization and median polish summarization of probe intensities into probe set intensities. A linear model was then fitted to the $\log_2$ of the expression levels based on all probe sets using the Limma package (Smyth 2004). After preprocessing and linear model fitting, the probe sets were filtered on expression; an expression threshold was set on both average expression level and absent/present calls from the R implementation of the Affymetrix MAS 5.0 algorithm. Only probe sets with average expression over a defined threshold and present in more than half of the samples within each sex were considered as significantly expressed. Average relative fluorescence was calculated among replicates of each sex, and sex-bias estimates were calculated as $\log_2$ (average male expression) – $\log_2$ (average female expression). Data were then averaged across replicate probe sets that corresponded to the same coding region. These data are available at the NCBI Gene Expression Omnibus repository under the accession number GSE8693.

For brain-expressed coding regions, we identified both male-biased and female-biased genes according to a range of fold-change cutoff criteria. Previous studies of reproductive tract genes have used a 2-fold cutoff to define sex-biased genes (Zhang et al. 2004; Zhang and Parsch 2005); however, the testis and the ovary are the most sexually dimorphic of all tissues and would therefore have much higher levels of sex-biased gene expression than other, less dimorphic tissues (such as brain). We therefore used lower thresholds to delineate sex-biased coding regions. Three categories were used based on expression in one sex compared with the other: genes were classified as sex biased if they were upregulated by at least 125%, 133%, or 150% in one sex compared with the other, with a *t*-test–adjusted *P* value corresponding to a 10% false discovery rate. Genes that did not meet these fold-change cutoffs were classified as unbiased; genes with sex-biased expression over the defined threshold but with insufficient adjusted *P* values were removed from the analysis at appropriate fold-change cutoffs. This accounts for the slight variation in total coding regions at different fold-change cutoffs. Results are unchanged if these ambiguous genes are included in the unbiased expression category (supplementary tables S1 and S2, Supplementary Material online); however, sex-biased genes are inherently more variable in expression than unbiased genes, which directly effects significance statistics. This implies that it may not be fair to assume that genes exceeding fold-change cutoffs but lacking significant *P* values are unbiased.

Alignment and Estimation Gene-Specific Substitutions

Detailed methodology for the estimation of d_N and d_S estimates for the coding regions in this study has been previously described (Mank et al. 2007). Briefly, for all significantly expressed genes, we searched for corresponding zebra finch (*T. guttata*) EST contig data generated by the Songbird Neurogenomics Initiative, available at http://titan.biotec.uiuc.edu/songbird/ (Clayton 2004). The transcripts, all derived from multiple cDNA libraries made from telencephalon of embryonic, juvenile, and adult birds of both sexes, were aligned with their chicken orthologues, as defined by BlastN search to all known and ab initio predicted protein-coding chicken genes identified in Ensembl (www.ensembl.org/Gallus_gallus/index.html). Orthology was established using the principle of best reciprocal hit, given a minimum *e* value of 10^{-30} for a match. Because *e* scores are influenced by sequence length, we excluded all alignments less than 100 bp from further analysis in order to reduce the possibility of false homology between short alignments.

DNA sequences were subsequently translated into amino acid sequences and aligned using DIALIGN2 (Morgenstern 1999), and the CODEML PAML package, version 3.15 (Yang 1997) was used to estimate the nonsynonymous (d_N, defined as the number of nonsynonymous substitutions per nonsynonymous site) and synonymous (d_S, the number of synonymous substitutions per synonymous site) divergence for each set of orthologues. The maximum likelihood estimation was performed separately for each alignment, which allows for variation in codon usage, a factor implicated in *Drosophila* sex-biased gene expression (Hambuch and Parsch 2005). For all analyzed coding regions, we inferred the chromosomal location from the May 2006 Ensembl galGal3 assembly of the chicken genome.

We excluded 41 coding regions from our data set with $d_S > 3$ as divergence estimates are not reliable for saturated sites. The expression pattern of these 41 genes (3 female biased, 5 male biased, and 33 unbiased) did not significantly differ from the expectation given the overall expression pattern ($\chi^2 = 2.45$, $P = 0.29$, 2 degrees of freedom [df]); therefore, we have no reason to suspect that the removal of these genes causes a bias in our analysis. Additionally, all transcripts with premature stop codons were removed as these either represent pseudogenes or errors introduced by reverse transcription. Finally, we screened all zebra finch sequences for high complexity contamination using the *Gallus*-derived repeat library in RepeatMasker (Smith et al. 2002; http://www.repeatmasker.org) As the Ensembl gene annotation methodology automatically removes repetitive DNA elements via RepeatMasker (Curwen et al. 2004), it was not necessary to screen the orthologous chicken coding regions for retroviral contamination. No contamination in the zebra finch sequences was identified.

Estimation of Rates of Molecular Evolution for Sex-Biased Genes

For the entire genomic complement of genes in our data set, mean categorical values of d_N and d_S for biased and unbiased categories, as well as the genomic average, were calculated by dividing the sum of the number of substitutions over genes by the sum of the number of sites over genes. This avoids problem of infinitely high d_N/d_S values arising from genes with no synonymous substitutions and has the added advantage that data for individual genes are weighted by alignment length. We assessed the 95% confidence intervals (CIs) for d_N/d_S ratio estimates with bootstrapping (1,000 repetitions). We also compared the various sex-biased categories to unbiased genes for all brain-expressed categories with a permutations test (1,000 repetitions).

Regardless of expression bias, sex-linked genes in birds have been shown to evolve more rapidly (Mank et al. 2007), a likely consequence of the so-called Fast-Z evolution resulting from the hemizygous exposure in the heterogametic sex (Charlesworth 1991). They also undergo higher levels of mutation than autosomal genes (Axelsson et al. 2004). Additionally, the lack of complete dosage compensation in chicken and other birds means that the majority of genes on the Z chromosome are expressed at higher levels in males (ZZ) than in females (ZW) (Ellegren et al. 2007; Itoh et al. 2007). Together, these observations suggest that an increased d_N/d_S ratio for male-biased genes on the Z chromosome may not be due to sex-biased expression, but rather Z linkage. We therefore removed Z-linked genes from the data set and compared the d_N, d_S, and d_N/d_S estimates for different sex-biased expression categories on the autosomes as described above for the full genomic data set. This data set was used for all subsequent analyses unless explicitly noted.

Tissue of Maximal Expression

Many genes are expressed in multiple tissues and organs throughout the body (Waxman and Peck 1998; Ericson et al. 2006). Microarray studies reveal whether a coding region is expressed in a given tissue, but they do not generally reveal the relative expression of that coding region throughout the organism. This means that a brain microarray data set such as the one we employ here is a blend of brain-specific genes, genes that exhibit maximal expression levels in the brain but are expressed at lower levels in other tissues, genes that are expressed in many tissues at similar levels, and genes that are expressed at low levels in the brain but achieve maximal expression in other organs. Although expression in other tissues does not mean that a given coding region is not important in brain function and development, a hodgepodge of expression characteristics can obscure evidence of sequence evolution as genes with maximal expression in tissues other than brain may be responding to selection for extraneurological functions.

We therefore identified genes that are confined to, or primarily expressed in, the central nervous system using data from the UniGene EST assemblage (ftp://ncbi.nlm.nih.gov/repository/unigene/). This database is a repository of EST libraries from a variety of organisms across a wide array of tissues. We selected EST libraries from chicken that represented a single defined tissue or organ, and the resulting data set comprised nonnormalized chicken EST libraries for blood, cartilage, central nervous system, digestive tract, epiphyseal growth plate, eye, genitourinary tract, heart, limb, and the lymphoreticular system. Data for each coding region were standardized to number of transcripts per million (TPM) for each EST library. This metric is a measure of the number of transcripts detected for a given gene per million transcripts analyzed from tissue i and is defined as

$$\mathrm{TPM}_i = \frac{\mathrm{EST}_i \times 10^6}{\mathrm{Library}_i}.$$

N is the number of tissues examined, and TPM_{max} is the highest expression level detected for a given gene over all tissues examined. We then determined which tissue possessed the highest TPM for each coding region in our study, detailed in supplementary table S3 (Supplementary Material online) and identified those coding regions where expression was highest in, or confined to, the central nervous system. We assigned the resulting 313 coding regions into sex-biased expression categories, using only the 125% cutoffs and adjusted P values in order to maximize sex-biased sample sizes for statistical power and compared d_N, d_S, and d_N/d_S for each category with permutation tests (1,000 repetitions).

Expression Level

We calculated the relationship between expression level and rate of evolution for all the genes in the 125% expression bias class. We assessed the correlation with linear regression between d_N/d_S and the $\log_2$ average expression, based on normalized relative fluorescence of the microarray data, for each sex separately. We also calculated the average the expression level for unbiased, male-biased, and female-biased genes and determined the 95% CIs based on bootstrapping (1,000 repetitions).

Gene Ontology

In order to investigate whether different functional classes of genes could be influencing our results, we tested for overrepresentation of Gene Ontology (GO) terms (Gene Ontology Consortium 2000) using ONTOLOGIZER 2.0 (Robinson et al. 2004). We first tested whether there was an overabundance of GO terms for Z-linked genes using all mapped coding regions as the population. We then tested for overrepresentation of GO terms in both autosomal female- and male-biased expression classes (at the 125% fold-change level) using the full complement of autosomal genes as the population set. For all these analyses, we used term-for-term comparisons and implemented the Bonferroni correction for multiple comparisons.

Heterogeneity Estimation

Chickens have a wide range of chromosome sizes, from the large macrochromosomes (chromosomes 1–5),

the intermediate chromosomes (chromosomes 6–10), and the small microchromosomes (chromosomes 11–38) (ICGSC 2004). These chromosomal classes have distinctive properties, with microchromosomes generally being more gene dense, having higher rates of recombination and higher GC content than the other chromosomal classes (ICGSC 2004). These properties have been shown to influence the rate of sequence evolution in birds (ICGSC 2004; Axelsson et al. 2005); therefore, in order to investigate whether genomic distribution influences our observed rates of evolution, we compared the distribution of sex-biased genes to the distribution of all brain-expressed genes for macrochromosomes and microchromosomes with a χ^2 test (1 df).

GC and third position GC (GC3) content has been shown to influence diversity and substitution (Williams and Hurst 2000; Smith et al. 2002). Therefore, we calculated GC and CG3 content for sex-biased classes, based on Ensembl annotation of the chicken genome sequence, and calculated the statistical significance of the difference among sex-bias classes using the 125% cutoff only in order to maximize statistical power through large sex-biased sample size, with permutation testing (1,000 repetitions). We also assessed the role of GC and GC3 on d_N/d_S among 125% sex-biased expression categories using both linear regression and analysis of covariance (ANCOVA) (GC and GC3 as independent variables, 2 df).

Male-biased genes in flies have been shown to exhibit less codon bias than other expression categories (Hambuch and Parsch 2005), which, if such a phenomenon were present in birds as well, could explain the high d_S values in male-biased brain genes. We therefore calculated the effective number of codons (ENC) for each gene (Wright 1990) based on the Ensembl annotation of the chicken genome. ENC can take values from 20, in the case of extreme bias where only one codon is used for each amino acid, to 61, where all alternative codons are equally likely. We then tested ENC among expression categories for statistical difference at the 125% threshold using analysis of variance (ANOVA) (2 df).

Results

The microarray experiments yielded gene expression data for 8,612 Ensembl-annotated chicken coding regions. We found orthologous sequence data in zebra finch for 4,251 of those genes, though 96 genes were removed due to premature stop codons, 37 had total aligned sequences of >100 bp and were removed, and a further 41 resulted in a $d_S > 3$ and were removed due to potential problems with saturation. Our data set is therefore comprised of 4,077 chicken–zebra finch orthologous coding regions, corresponding to 2.36 Mb of sequence data. Of these, 176 genes were mapped to the Z chromosome.

For the entire genomic data set, there were more male- than female-biased genes across the range of cutoff values (table 1), though this disappeared after sex-linked coding regions were removed (see below). For all genomic coding regions, female- and male-biased genes showed a significantly higher d_N/d_S ratio than unbiased genes expressed in brain across the range of cutoff values (125%, 133%, and 150% higher expression in either sex, table 1), though only female-biased expression categories showed consistent significant differences across all fold-change cutoffs. In every case, this was due to an increased d_N for sex-biased categories. Bootstrap testing of expression categories indicated that female-biased genes showed both higher d_N/d_S ratios as well as wider 95% CIs than male-biased genes (fig. 1).

Table 1
Mean Divergence Values for Sex-Biased Brain Genes for All Nuclear-Coding Regions

Fold-change cutoff (%)	Unbiased (*n*)	Female Biased (*n*)	Male Biased (*n*)
d_N/d_S			
125	0.0789 (3327)	0.1011** (155)	0.0901* (286)
133	0.0795 (3542)	0.1146** (107)	0.0888 (240)
150	0.0803 (3845)	0.1145** (44)	0.0938 (142)
d_N			
125	0.0365	0.0441*	0.0446*
133	0.0369	0.0477*	0.0433*
150	0.0371	0.0516*	0.0467*
d_S			
125	0.4630	0.4363	0.4947*
133	0.4641	0.4159*	0.4878
150	0.4625	0.4502	0.4997

NOTE.—Sex-biased values that significantly differ from the unbiased mean, based on permutation tests with 1,000 replicates, are indicated (*$P < 0.05$, **$P < 0.01$). The number of genes in each category (*n*) is shown.

There are sound reasons to focus on the autosomal set of genes as sex-linked genes may evolve unusually fast due to the Fast-Z effect (Mank et al. 2007), an evolutionary phenomenon completely independent of sex-biased expression (Charlesworth 1991). Additionally, recent data suggest that birds lack dosage compensation, therefore, most Z-linked genes are male biased in expression due solely to gene dose (Ellegren et al. 2007; Itoh et al. 2007). These 2 characteristics create the possibility that the Fast-Z effect (analogous to Fast-X, Charlesworth 1991) could be mistakenly taken for rapid evolution of male-biased genes if the Z chromosome is included in the analysis. Consistent with this reasoning, confining further analysis to the 3,901 autosomal coding regions comprising 2.24 Mb significantly altered the picture of molecular evolution for some sex-biased categories. Although female-biased genes maintained a consistently higher d_N/d_S ratio in the autosomal data set (fig. 1 and table 2, permutation test with 1,000 repetitions, $P \leq 0.05$), male-biased autosomal genes did not have a d_N/d_S ratio that differed statistically from the unbiased average. For example, for genes with a 150% difference in expression level between the sexes, d_N/d_S was roughly 43% higher for female-biased genes (0.1145) than for unbiased (0.08) and male-biased (0.0654) genes. As in the full genomic data set, the high d_N/d_S of female-biased genes was due to significantly higher d_N than unbiased genes. Male-biased genes, though generally exhibiting higher d_S than unbiased genes, lacked significantly higher d_N values.

Microarray studies such as the one employed here detect gene expression in given tissues, but they do not differentiate between broadly or narrowly expressed genes.

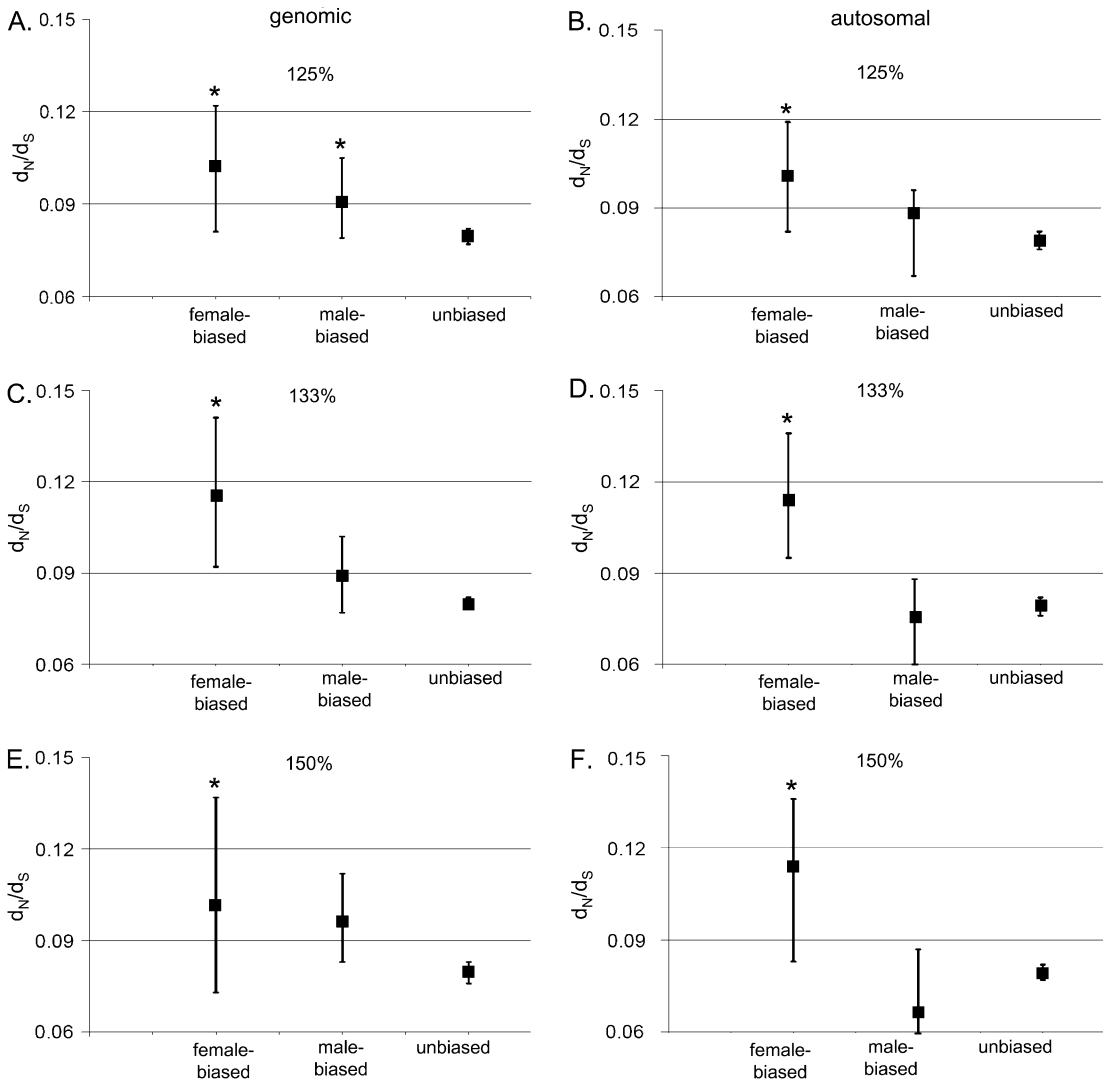

FIG. 1.—d_N/d_S ratios for unbiased-, female-, and male-biased genes for both genomic (*A*, *C*, and *E*) and autosomal(*B*, *D*, and *F*) coding regions. Data from 125% (*A* and *B*), 133% (*C* and *D*), and 150% (*E* and *F*) fold-change cutoff values are shown. The 95% confidence whiskers for sex-biased genesets are based on bootstrap values (1,000 repetitions). Sex-biased values that differ significantly ($P < 0.05$) from the unbiased gene class in each fold-change category are marked (*).

Many genes expressed in brain are also expressed in other tissues throughout the organism (detailed in supplementary table S3, Supplementary Material online) and may be responding to selective pressures for extraneurological function. In general, pleiotropic genes are constrained in the evolution of sex-biased expression (Mank et al. 2008). In order to focus on genes potentially involved in cognitive and behavioral processes, we analyzed the abundance of EST transcripts in chicken cDNA libraries from a variety of tissues from the UniGene EST assemblage. The EST data indicated that 313 coding regions from our autosomal data set were either limited to or primarily expressed in the central nervous system. This subset of data exhibited the same substitution profile as the full autosomal data set, with female-biased d_N/d_S (0.123 at 125% fold change) genes nearly 2-fold higher than our estimate for unbiased genes (0.066). Similar to the complete autosomal data set, male-biased genes (0.069) did not have a higher overall d_N/d_S compared with unbiased genes (table 3).

Expression level has been tied to rates of evolution, with highly expressed genes generally showing decreased rates of functional change than genes with lower expression levels (Pal et al. 2001; Subramanian and Kumar 2004; Liao and Zhang 2006). Expression level in our data, averaged separately over male and female samples, was weakly though significantly associated with d_N/d_S for both sexes ($r^2 = 0.0289$ for female expression level, $r^2 = 0.0308$ for male expression levels; $P < 0.001$ in both cases), with both relationships showing that more highly expressed genes evolve more slowly. However, although female- and male-biased classes had lower average expression levels than unbiased genes, they did not differ from each other (fig. 2).

Different functional classes of genes can show different rates of evolution in response to stronger or weaker selection pressures; therefore, we tested for overrepresentation of GO terms in our gene categories. After correction for multiple comparisons, there were no significant differences

Table 2
Mean Divergence Values for Autosomal Sex-Biased Brain Genes

Fold-change cutoff (%)	Unbiased (*n*)	Female Biased (*n*)	Male Biased (*n*)
d_N/d_S			
125	0.0786 (3293)	0.1011** (155)	0.0880 (161)
133	0.0792 (3500)	0.1146*** (107)	0.0758 (119)
150	0.0800 (3767)	0.1145* (44)	0.0654 (46)
d_N			
125	0.0364	0.0441*	0.0425
133	0.0368	0.0477*	0.0399
150	0.0371	0.0516*	0.0395
d_S			
125	0.4636	0.4363	0.5316**
133	0.4648	0.4159*	0.5269*
150	0.4635	0.4502	0.6039**

NOTE.—Sex-biased values that significantly differ from the unbiased mean, based on permutation tests with 1,000 replicates, are indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The number of genes in each category (*n*) is shown.

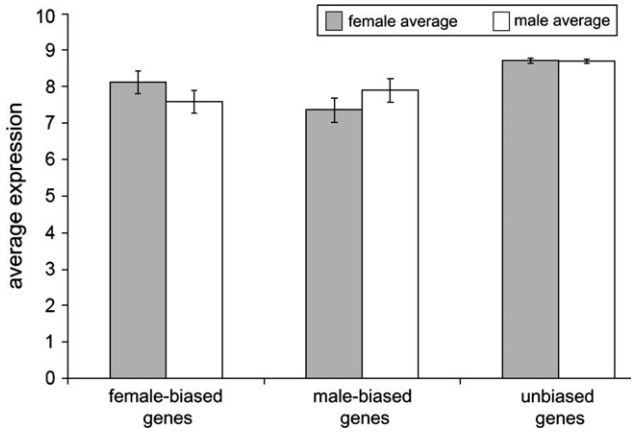

FIG. 2.—Average expression of expression categories (125% fold-change level). Shown are the average relative expression levels averaged across within-sex samples, for female-, male-, and unbiased gene expression categories. The 95% confidence whiskers are shown, based on bootstrapping (1,000 replicates).

in the occurrence of GO terms between sex-biased gene classes and the full autosomal gene set. In all cases, Bonferroni-adjusted *P* values exceeded 0.1.

Substitution rates as well as selective constraint in avian genomes have been shown to relate to several genomic parameters in birds. Specifically, genes on the microchromosomes have generally lower d_N/d_S and higher d_S than macrochromosome genes (ICGSC 2004; Axelsson et al. 2005). Given the d_N/d_S differences among female-biased, male-biased, and unbiased brain genes described above, it is important to test whether these differences can be explained by genomic parameters known to influence substitution patterns. There is no observed heterogeneity in the genomic distribution of sex-biased categories (supplementary table S4, Supplementary Material online) as no chromosome class deviated from random expectation regarding the number of male- or female-biased genes (χ^2 tests, $P > 0.07$ in all cases, 1 df for each test).

GC and third position GC (GC3) content varied by expression category according to permutation testing (tables 4 and 5). In birds, regions of high GC, and corresponding high incidence of mutable CpG sites, have a corresponding elevated d_S level (Webster et al. 2006). Our data are consistent with these as female-biased genes in general have lower GC and GC3 content, which is consistent with their low d_S, than brain-expressed genes. Male-biased GC and GC3 were significantly higher, consistent with their high d_S. The relationship between GC content and d_N/d_S was less clear. There was an overall negative correlation between GC content and d_N/d_S in the different expression categories (for all correlations <0.001), and we detected no significant heterogeneity among best-fit lines between GC and d_N/d_S (ANCOVA $F = 0.30$, $P = 0.74$, 2 df). Statistically different d_N/d_S levels among expression classes remained after accounting for variance in GC (ANCOVA $F = 5.45$, $P = 0.004$), suggesting that the differences in d_N/d_S we observed for male-biased, female-biased, and unbiased expressed categories are, at least in part, independent of GC content. Similarly, the relationship between GC3 and d_N/d_S did not differ among expression categories (ANCOVA $F = 0.10$, $P = 0.901$, 2 df); however, GC3 did not differ after accounting for d_N/d_S differences in expression classes (ANCOVA $F = 0.64$, $P = 0.529$, 2 df). Although GC and GC3 can indicate bias in codon usage, we observed no difference in codon bias among female-, male-, and unbiased genes (ANOVA $F = 2.264$, $P = 0.104$, 2 df) (data provided by Matthew Webster).

Table 3
Mean Divergence Values for Sex-Biased Genes Limited to or Primarily Expressed in the Chicken Central Nervous System Based on UniGene EST Data

	Unbiased	Female Biased	Male Biased
n	273	15	25
d_N/d_S	0.0660	0.1229**	0.0693
d_N	0.0282	0.0454*	0.0385
d_S	0.4270	0.3699	0.5561*

NOTE.—Sex bias was determined by a 125% fold-change cutoff and sex-biased categories that significantly differ from the unbiased mean (permutation test, 1,000 replicates, *$P < 0.05$, **$P < 0.01$) are indicated by asterisk.

Discussion

Sex-biased genes expressed in the avian brain showed a higher d_N/d_S ratio, suggesting that they have a faster functional rate of evolution than unbiased genes. The autosomes

Table 4
Average GC Content for Sex-Biased Expression Categories

Fold-change cutoff (%)	Unbiased (*n*)	Female Biased (*n*)	Male Biased (*n*)
125	0.4316 (3293)	0.4137*** (155)	0.4703*** (161)
133	0.4319 (3500)	0.4132** (107)	0.4694*** (119)
150	0.4321 (3767)	0.4089** (44)	0.4722* (46)

NOTE.—Sex-biased values that significantly differ from the unbiased mean, based on permutation tests with 1,000 replicates, are indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The number of genes in each category (*n*) is shown.

Table 5
Average GC3 for Sex-Biased Expression Categories

Fold-change cutoff (%)	Unbiased (*n*)	Female Biased (*n*)	Male Biased (*n*)
125	0.4969 (3293)	0.4858* (155)	0.5407*** (161)
133	0.44979 (3500)	0.4809** (107)	0.5388*** (119)
150	0.4983 (3767)	0.4816* (44)	0.5542*** (46)

NOTE.—Sex-biased values that significantly differ from the unbiased mean, based on permutation tests with 1,000 replicates, are indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The number of genes in each category (*n*) is shown.

and Z chromosome showed somewhat different patterns however, which merit separate discussion. For autosomal genes, only female-biased genes evolve faster than unbiased genes.

The lack of dosage compensation on the chicken Z chromosome (Ellegren et al. 2007; Itoh et al. 2007) results in male-biased expression for most Z-linked coding regions, but this does not mean that selection is acting explicitly on male-biased expression for genes located on the Z, as has been reported in other species (Swanson et al. 2001; Meiklejohn et al. 2003; Parisi et al. 2004; Zhang et al. 2004; Pröschel et al. 2005). Z-linked genes in birds, regardless of expression bias, exhibit a faster rate of functional change than autosomal genes (Mank et al. 2007). This may be either due to the hemizygous exposure of Z-linked genes in the heterogametic sex, which facilitates the fixation of nondominant advantageous mutations, or due to the reduction in effective population size of the Z compared with autosomes, which reduces the effectiveness of selection against weakly deleterious alleles (Charlesworth 1991). Both these phenomena, which are independent of sex-biased expression, could result in elevated d_N/d_S and a spurious correlation between expression bias and rate of evolution in this study. The fact that male-biased autosomal genes do not show the elevated ratios of synonymous to nonsynonymous substitution in our analysis suggests that the increased d_N/d_S ratio we observed on the Z chromosome is more in-line with the Fast-Z evolution than to the effects of male bias per se.

Focusing our analysis on autosomal genes results in a pattern that is somewhat contradictory to previous studies of reproductive genes on other organisms where male-biased categories showed higher rates of functional change (Swanson et al. 2001; Torgerson et al. 2002; Zhang et al. 2004; Panhuis et al. 2006). By contrast, our data indicate that female-biased autosomal categories exhibit the highest d_N/d_S ratios, a pattern for which there are several possible explanations that can be divided into selectionist (either natural or sexual) and neutral nonselectionist categories.

We investigated a series of neutral nonselectionist genomic properties that could theoretically have produced the pattern of evolution we observed here. First, we tested whether heterogeneity in the distribution of sex-biased genes among the different chromosome classes could have produced our observations. Size differences in the avian karyotype result in different rates of recombination and mutation, as well as different GC content, for their associated gene complements (ICGSC 2004; Axelsson et al. 2005). However, as there was no significant bias in expression category distribution, the avian karyotype alone cannot account for the pattern we observed.

Next, we examined whether expression level could account for the elevated rate of evolution for female-biased genes. In general, highly expressed genes evolve more slowly than genes with low expression (Pal et al. 2001; Subramanian and Kumar 2004; Yang et al. 2005; Liao and Zhang 2006). Although sex-biased genes exhibit a lower expression level than unbiased genes (fig. 2), the expression between female- and male-biased genes was too similar (accounting for inversion of sex bias) to explain the difference between female- and male-biased d_N/d_S levels. GC and GC3 levels were significantly higher in male-biased genes (tables 4 and 5) and significantly lower in female-biased genes compared with unbiased gene classes. As GC correlates with recombination rate (Chen et al. 2006; Khelifi et al. 2006; Webster et al. 2006), this may indicate that recombination is more common in male-biased genes. Unfortunately, the lack of a high-density sex-specific recombination map in chicken prevents further testing of this possibility. Finally, despite the difference in GC and GC3, there was no significant difference among expression categories with regards to codon usage; therefore, it is not appropriate to invoke codon bias as a possible explanation for the elevated d_N/d_S levels for female-biased genes.

This leaves sexual and natural selection as viable alternatives to explain the observed pattern, and we can offer several possible alternatives. One possibility is that the force of sexual selection is stronger on female-specific behaviors, something that could potentially result in a faster rate of evolution for female-biased brain genes. However, this seems unlikely as the rigors of female choice and male–male competition, common selective pressures in birds that involve sex-specific behaviors (Gould and Gould 1997), would exert a powerful evolutionary force on male breeding behaviors and therefore male-biased brain genes. Sexual selection could still be acting on male-biased brain genes that are expressed at a different time in the life cycle. Little is known about the temporal aspects of sex-biased expression differences through an organism's life span, nor how this would affect our results. We collected tissue for microarray analysis at day 18 of development, well after circulating sex hormones initiate somatic sexual differentiation in chickens (Bruggeman et al. 2002). However, it is possible that the genes involved in male-specific reproductive behaviors, which would presumably be subject to sexual selection, are simply expressed at a different life stage than that which forms the data set for this analysis.

Additionally, we do not yet have sufficient gene expression data for zebra finch to determine the extent to which sex-biased gene expression is conserved between these study species or among birds in general. Gene expression in general in the brain shows a high degree of conservation (Strand et al. 2007). Additionally, the experimental designs of both this microarray study and the source for the zebra finch orthologs (Clayton 2004) ensures that the genes employed here are expressed in the brains of both chicken and zebra finch. However, sex-biased expression for some genes has been shown to alter relatively rapidly among species in response to changing selective regimes (Ranz et al. 2003; Metta et al. 2006). We used data here on sex-biased

expression differences in chicken, but the pattern of sex bias may be somewhat different for some subset of our genes in zebra finch.

Natural selection, as opposed to sexual selection, may be acting differently on the male and female brain to produce the d_N/d_S patterns described here. For instance, natural selection could be acting on female-specific behaviors involved in brood care or other aspects of reproduction. Additionally, a high d_N/d_S is not necessarily a consequence of adaptive evolution but could also reflect relaxed selective constraint. Male-biased and unbiased genes may, for some reason, be subject to stronger purifying selection, which would result in lower d_N/d_S values. A similar discussion is made for brain genes of humans and chimpanzee, where elevated rates of sequence evolution could either be due to adaptive evolution or relaxed constraint (Dorus et al. 2004; Khaitovich et al. 2005; Shi et al. 2006; Wang et al. 2006).

In summary, this study represents an attempt to look at the rate of molecular evolution of neurological sex-biased genes that are likely contributors to sexually dimorphic behaviors. Taken as a whole, our results suggest that sexually dimorphic behaviors result from strong evolutionary pressures acting on the avian brain, primarily in females. Future work, involving both gene expression and avian sequence data, will be helpful in further clarifying sex-specific evolutionary processes acting on avian neurology.

Supplementary Material

Supplementary tables S1–S4 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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