Ontogenetic Complexity of Sexual Dimorphism and Sex-Specific Selection

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Abstract

Sex-biased gene expression is becoming an increasingly important way to study sexual selection at the molecular genetic level. However, little is known about the timing, persistence, and continuity of gene expression required in the creation of distinct male and female phenotypes, and even less about how sex-specific selection pressures shift over the life cycle. Here, we present a time-series global transcription profile for autosomal genes in male and female chicken, beginning with embryonic development and spanning to reproductive maturity, for the gonad. Overall, the amount and magnitude of sex-biased expression increased as a function of age, though sex-biased gene expression was surprisingly ephemeral, with very few genes exhibiting continuous sex bias in both embryonic and adult tissues. Despite a large predicted role of the sex chromosomes in sexual dimorphism, our study indicates that the autosomes house the majority of genes with sex-biased expression. Most interestingly, sex-specific evolutionary pressures shifted over the course of the life cycle, acting equally strongly on female-biased genes and male-biased genes but at different ages. Female-biased genes exhibited high rates of divergence late in embryonic development, shortly before arrested meiosis halts oogenesis. The level of divergence on female-biased late embryonic genes is similar to that seen in male-biased genes expressed in adult gonads, which correlates with the onset of spermatogenesis. These analyses reveal that sex-specific selection pressure varies over the life cycle as a function of male and female biology.

Key words: sex-biased gene expression, sexual selection, spermatogenesis, oogenesis, development, sperm competition.

Introduction

Recent interest in sex-biased gene expression has increased as global transcription profiling has revealed large proportions of the genome with markedly different expression levels in males and females for a wide array of animals (Ellegren and Parsch 2007). Many aspects of male- and female-specific phenotypes are the product of different expression levels of the same genes. Indeed, sex often explains the majority of gene-expression differences between samples, and this has important implications in a wide array of biological (Pröschel et al. 2006; Mank, Axelsson, and Ellegren 2007; Mank, Hultin-Rosenberg, et al. 2007; Zhang et al. 2007) and medical (Vandenbroeck et al. 2003; Ivakine et al. 2005; Naugler et al. 2007) disciplines.

Because sex-biased genes form a large proportion of the molecular basis of sexually dimorphic phenotypes, they offer a method to study sex-specific selection at the genetic level (Mank 2009b), and several recent studies have shown that sex-biased genes have a suite of characters that are consistent with sexual selection. Sex-biased genes exhibit accelerated rates of evolution consistent with sexual selection pressures (Meiklejohn et al. 2003; Ranz et al. 2003; Zhang et al. 2004; Mank, Axelsson, and Ellegren 2007; Mank, Hultin-Rosenberg, et al. 2007). Sex-biased expression also varies among species (Metta et al. 2006; Zhang et al. 2007; Reinius et al. 2008), presumably as a result of species-specific sex-specific selection regimes and shows a genomic distribution (Khil et al. 2004; Mank and Ellegren 2009b) consistent with theoretical predictions of sexual antagonism (Rice 1984).

Much of this work has focused explicitly on the gonad, and sex-biased genes associated with the gonad exhibit higher rates of functional evolution, usually for loci that are male-biased in adults (Zhang et al. 2004; Jagadeeshan and Singh 2005; Ellegren and Parsch 2007; Haerty et al. 2007). This has been interpreted to be the result of the powerful selection pressures related to sperm competition (Ellegren and Parsch 2007) or other male-specific evolutionary forces. However, a few studies have not been consistent with the pattern, indicating that sperm competition may not be the only major factor shaping sex-specific selection pressures (Metta et al. 2006; Mank, Axelsson, and Ellegren 2007; Mank, Hultin-Rosenberg, et al. 2007) and hinting at a greater complexity than has been assumed. Moreover, most of these studies have focused on adult samples, and this strategy means that we lack a global picture of the temporal aspects of sex-biased transcription and sex-specific selection. Clearly, many genes are required to make different male and female phenotypes from an underlying genome that is, aside from the sex chromosomes, identical. However, we do not know how early in development generalized patterns of sex bias emerge nor how long sex-biased transcription lasts. Are sexual dimorphisms encoded by short bursts of sex-biased gene expression, or is

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a prolonged period of steady sex-biased expression more common? More importantly, how does the different biology of males and females affect gene-expression patterns as well as sex-specific selection pressures throughout the life cycle?

Birds are particularly useful for addressing these questions. The chicken (International Chicken Genome Sequencing Consortium 2004) and zebra finch (Warren et al. [76 co-authors] in preparation) genomes have been sequenced and assembled and encompass roughly 100 My of avian evolutionary history between them. Much is also known about the developmental stages and progression of avian ontogeny, particularly for the chicken (Brown et al. 2003). Sexual dimorphism and ornamentation are clearly evident in adult chickens (Parker and Garant 2004; Cornwallis and Birkhead 2008; Wright et al. 2008), and there is also evidence of sex-specific selection pressures, including sperm competition (Froman et al. 2002; Pizzari et al. 2003, 2007). These characterizations combined make chicken a useful organism with which to study sex-biased gene expression in the context of development.

Materials and Methods

Sample Collection, Preparation, and Microarray Analysis

All animals were purchased from OVA Productions (Morgongåva, Sweden), and derived from the same white leghorn line with a controlled genetic background. The collection, preparation, and expression data analysis methods have been previously described (Mank and Ellegren 2009a, 2009b), and only a brief overview of relevant details will be presented here.

Fifteen to 20 embryos were collected from eggs at embryonic day (ed) 15 and ed19 of incubation. Ed15 was chosen as it represents the earliest point at which gonad tissue is of sufficient size for standard total RNA preparation and microarray analysis. Ed19 represents the point just before hatching. From each sample, the left gonad was placed in RNAlater (Qiagen) prior to homogenization. Adults were collected at week 26 posthatching, after the onset of sexual maturity, which was visually confirmed for all individuals during dissection. Adult left gonads were minced, placed in the recommended amount of RNAlater (Qiagen), and subsequently handled in the same fashion as embryonic samples.

Samples were subsequently mechanically homogenized, and 1 ml of the homogenate was used for RNA preparation (RNEasy Lipid Tissue Mini Kit, Qiagen), according to the manufacturer's protocol. Prepared RNA was pooled from four to five individuals, with three nonoverlapping pools for each sex at each time point. Biotinylated fragmented cRNA was prepared from RNA pools, and this material was hybridized to Affymetrix Chicken GeneChip expression arrays, which were then processed according to standard microarray procedures. All microarray hybridizations passed Affymetrix quality control criteria.

The CEL files (cell library) for all 18 hybridizations were RMA preprocessed (Irizarry et al. 2003), with quantile normalization and median polishing. Following quality control to confirm that there were no outlier slides in our data set, we normalized the preprocessed replicates arrays and probe sets and filtered the data to determine significant expression above background noise levels in at least half the time point replicate slides (three of the six slides per time point treatment). Significantly expressed probe sets were annotated with the Ensembl map of the chicken genome (WASHUC2 May 2006, www.ensembl.org), and we retained only those probe sets that mapped definitively to chicken autosomes 1–28 for further analysis.

Identification of Sex-Biased Expression

For autosomes, we first identified sex-biased genes based on overall expression (modified *t*-testing, with a Benjamini–Hochberg false discovery rate [FDR] correction of 5%), performing this analysis for each time point separately. We then employed $\geq$2-, $\geq$5-, and $\geq$10-fold thresholds of fold change (otherwise known as fold difference), employing a 5% FDR correction to delineate sex bias. In all subsequent analysis and graphic displays, we employed a $\log_2$ fold-change convention that results in even scaling of female- and male-biased genes. This metric results in negative values for female-biased loci, positive values for male-biased regions, and values near zero for those genes with similar expression in both sexes.

We then investigated the correlation in overall expression and fold change between neighboring time points with standard regression and assessed correlation. For overall expression, we averaged normalized expression level across male and female replicates in each time-point treatment. We also investigated the expression patterns of genes that were sex biased at various time point with *k*-means clustering. We focused on adult gonad, as the greater degree of sex bias in this tissue makes it easier to determine overall temporal patterns. We identified autosomal sex-biased genes with a $\geq$2-fold, 5% FDR threshold in adult samples. We then clustered these probe-set lists separately in order to determine temporal patterns that produced sex bias, using five initial clusters based on Pearson correlation, and testing 100 additional clusters with 1,000 iterations. We then identified the clusters with the greatest number of member probe sets.

Divergence Estimates and Sex-Specific Selection Estimation

We downloaded annotated coding sequence from the chicken (WASHUC2) and zebra finch genomes (TeaGut3.2.4) from biomart (www.biomart.org) and determined 1:1 orthology between the two species using Inparanoid3.0 (O'Brien et al. 2005) with reciprocal basic alignment search tool (Blast) searches and bootstrapping, employing 90% support cutoffs. For genes with more than one coding sequence, we selected the longest coding sequence for this analysis. Selected coding sequences were then translated into protein sequences prior to alignment, and aligned amino acid sequences were then translated

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back into DNA using MUSCLE (Edgar 2004) and custom-made PERL scripts. Using this method, we obtained 11,176 pairwise 1:1 orthologs, and for each of these, we calculated the number of nonsynonymous substitutions per nonsynonymous site (d_N) and the number of synonymous substitutions per synonymous site (d_S) for each ortholog pairwise using codeml in PAML4.1 (Yang 1997). For this calculation, codon frequency was calculated by F3X4 model, and kappa (transition–transversion ratio) was estimated by maximum likelihood method together with d_N and d_S, simultaneously. From these data, we estimated the number of nonsynonymous substitutions per nonsynonymous site (d_N) and the number of synonymous substitutions per synonymous site (d_S). The ratio of these two metrics (d_N/d_S) can be used as a measure of functional evolutionary change in the protein structure, correcting for underlying variation in mutation rate.

From this data set, we removed all alignments <100 bp, as maximum likelihood estimates of divergence can be problematic for short transcripts. Additionally, we filtered the data to remove all orthologs where $d_S > 2$, as it has been previously shown for the chicken–zebra finch comparison that the transition–transversion ratio at 4-fold degenerate sites is linear for $d_S \leq 2$, and is nonlinear for values > 2 (Axelsson et al. 2008), suggesting $d_S = 2$ is the threshold over which mutation rates become saturated with double hits. Orthologs with significant expression in gonad tissue were identified and parsed for each time point into female-biased, male-biased (in both cases $>$ 2-fold expression differences, corrected for 5% FDR), and unbiased genes. For each of these categories, mean d_N and d_S were calculated by dividing the sum over all genes in a genomic category of the number of substitutions by the sum of the number of sites. This has the advantages of weighting data by alignment length and circumventing the problem of infinitely high d_N/d_S values arising from genes with no synonymous substitutions. Ninety-five percent confidence intervals were determined via bootstrapping (1,000 repetitions), and a permutation test with 1,000 repetitions was used to assess significant differences for each metric (d_N, d_S, and d_N/d_S) between expression categories.

Gene Ontology (GO)

We used the GO for chicken (Gene Ontology Consortium 2000) to determine whether sex-bias patterns we observed could be related to functionality. We used Ontologizer 2.0 (Robinson et al. 2004; Bauer et al. 2008) to test for overrepresentation of GO terms in autosomal sex-biased (≥2-fold threshold with 5% FDR in gonad) and unbiased gene lists when compared with all significantly expressed genes for each time point separately. We used parent–child analysis, correcting for multiple comparisons with the Westfall–Young Single Step method (Grossman et al. 2007). We also report all GO terms that were significantly different after correction ($P < 0.05$). However, any multiple testing correction is going to be overconservative given the vast array of tests we performed; therefore, we also report P values that were significant before multiple comparison correction.

Table 1. Degree of Sex-Biased Gene Expression for Gonad-Expressed Autosomal Loci.

Time Point	Cut-Off Threshold	Significantly Expressed[a]	Female Biased (%)	Male Biased (%)
Ed15	10% FDR[b]	12,030	1,045 (8.7)	564 (4.7)
	≥2[c]		341 (2.8)	201 (1.7)
	≥5[d]		55 (0.5)	28 (0.2)
	≥10[e]		25 (0.2)	8 (0.1)
Ed19	10% FDR[b]	11,982	1,432 (12.0)	1,136 (9.5)
	≥2[c]		460 (3.8)	257 (2.2)
	≥5[d]		92 (0.8)	35 (0.3)
	≥10[e]		29 (0.2)	12 (0.1)
Adult	10% FDR[b]	12,078	4,088 (33.5)	3,405 (28.2)
	≥2[c]		2,657 (22.0)	1,915 (15.9)
	≥5[d]		750 (6.2)	797 (6.6)
	≥10[e]		278 (2.3)	448 (3.7)

NOTE.—For each time-point comparison, the number and percent of sex-biased genes are shown for various fold-change cut-off thresholds.

[a] Defined as significantly expressed above background in three of six tissue/time-point samples.

[b] Based on modified t-test alone with FDR correction.

[c] Male-biased $\geq \log_2$ m/f = 1, female-biased $\leq \log_2$ m/f = −1.

[d] Male-biased $\geq \log_2$ m/f = 2.3, female-biased $\leq \log_2$ m/f = −2.3.

[e] Male-biased $\geq \log_2$ m/f = 3.3, female-biased $\leq \log_2$ m/f = −3.3.

Results

After data filtering, we had significant expression for 36,682 probe sets in at least one time point, corresponding to 12,827 Ensembl-identified protein-coding sequences in the chicken genome. Of those, we focused on the autosomal loci in the current chicken-genome assembly, as the avian Z chromosome is predominantly male biased due to the lack of dosage compensation (Ellegren et al. 2007; Itoh et al. 2007; Mank 2009a), and theoretical predictions relating to unbalanced sex-specific selection pressures (Rice 1984; Charlesworth et al. 1987) have been documented on the avian Z chromosome (Mank, Axelsson, and Ellegren 2007; Mank and Ellegren 2009b).

We created three nonoverlapping pools of four to five individuals for each sex at every time point (ed15, ed19, and adult), and these pools represent within-sex biological replicates. The replicates were highly correlated, and R^2 ranged from 0.983 to 0.996 for the autosomal targets considered here. Averaged over all significantly expressed probe sets, the $\log_2$ fold change for tissue/time-point treatments ranged from −0.0491 to 0.00924, which was not statistically different from zero, or equal between females and males. Interestingly, there were more female-biased than male-biased genes after normalization, as defined by nearly all thresholds (table 1).

The percentage as well as the magnitude of sex-biased expression increased as a function of age (table 1 and fig 1), and both metrics were greatest in adults. This reflects the progressive divergence of the ovary and the testes from the same precursor tissue (Bruggeman et al. 2002), and this is grounded in the biological reality of the different gametes that they must produce and deliver once reproductively mature. Viewed in terms of sexual antagonism, the adult tissue is the most sexually antagonistic, as this is when

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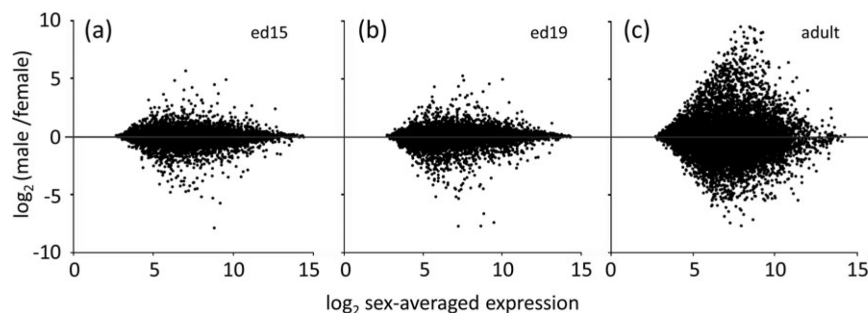

Fig. 1. Autosomal sex-biased expression in chicken gonad is greatest in adult tissue. Shown is the sex-biased ($\log_2$ m:f) expression versus normalized ($\log_2$) expression intensity for each time point. Expression is relative in one-color arrays, with zero representing the minimum. Male-biased genes are >0 and female-biased genes <0 according to the $\log_2$ (male/female) data treatment. Shown are ed15 (*a*), ed19 (*b*), and adult (*c*) values.

the reproductive interests of males and females originate and diverge (Chippindale et al. 2001).

Averaged across all replicates, regardless of sex, the relative expression between adjacent time points was highly correlated, with $R^2 = 0.984$ between ed15 and ed19 and $R^2 = 0.809$ for ed19 and adult gonad. Most interestingly, the temporal pattern of sex-biased expression, in contrast to the sex-averaged expression, is surprisingly ephemeral (fig. 2), with $R^2 = 0.574$ for ed15 and ed19, and $R^2 = 0.001$ for ed19 and adult tissues. This suggests that the pattern of sex-biased expression is fleeting, turning on or switching directionality relatively quickly during development, and sharing little correlation with adult patterns. This is also the case when statistical fold-change cut-offs were used to designate sex-bias categories (fig. 3), as very few genes that were sex biased at any single time point were also similarly biased at any other time point.

We examined our data to determine whether upregulation or downregulation was more important in creating sex-biased expression patterns, as evolutionary theory predicts that downregulation of sexually antagonistic genes may be more common in the sex suffering harm (Rice 1984), and this pattern may play out over the course of development. For this analysis, we focused on genes with sex-biased expression in the adult gonad, as the stronger sex-biased expression patterns make clustering more effective (supplementary fig. 1, Supplementary Material online). It is evident from looking at these clusters that gonad genes are sex biased for several different reasons. These different temporal clusters indicate that upregulation and

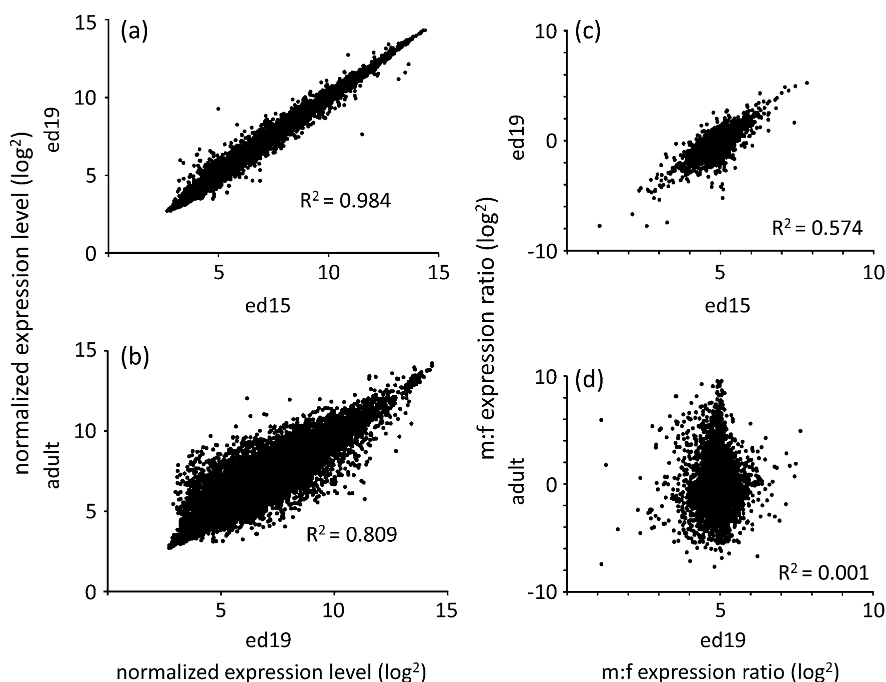

Fig. 2. Sex-biased expression changes, even though sex-averaged expression is conserved over adjacent time points. Relative (sex-averaged) expression for autosomal genes (*a*,*b*) between adjacent time points shows a high degree of correlation. Sex-biased expression ($\log_2$ m:f) between adjacent time points for all significantly expressed autosomal genes is somewhat conserved during embryonic development (*c*) and much less so in adults (*d*). Correlation coefficients are shown in each panel.

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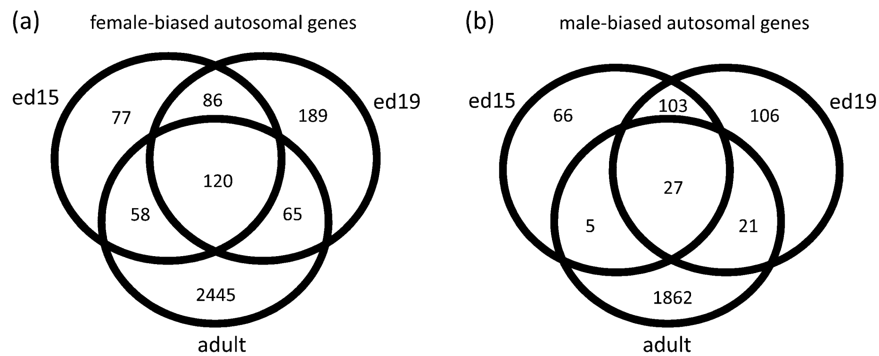

Fig. 3. Venn diagrams of autosomal sex-bias continuity among time points for female-biased (*a*) and male-biased (*b*) genes. Sex bias was defined as >2-fold expression change with an FDR correction of 5%.

downregulation are both common in the production of sex-biased gene expression.

In order to determine whether the complex patterns of expression change can result in genes that shift from sex biased in one direction to sex biased in the other through the life cycle, we filtered the significantly biased genes based on fold change. Additionally, sex-specific gene expression patterns are thought to indicate different types of sexual antagonism (Rice 1984; Mank and Ellegren 2009b), and it is important to test the assumptions underlying this association. We searched for genes that were both female and male biased at some point in the life cycle, using a $\geq$2-fold threshold and a 5% FDR, resulting in 299 genes, with 165 genes shifting from female to male biased, and 134 loci shifting from male to female biased. In all cases, the change in directionality occurred between embryonic and adult time points, rather than between ed15 and ed19.

We examined divergence data for 1:1 chicken–zebra finch orthologs in order to identify how sex-specific selection shifts over the course of the life cycle. After filtering, we had divergence data for 3,623 chicken–zebra finch orthologs expressed at ed15, 5,868 expressed at ed19, and 5,924 expressed in adult gonad, for which we also had expression data. With these divergence data, we estimated rates of functional change (d_N/d_S) for sex-biased gene categories at the three stages of the life cycle. There was no statistical difference in d_N/d_S among the different expression classes for ed15 gonad. This changed by ed19, with female-biased genes showing a 31% increase in d_N/d_S compared with unbiased genes ($P = 0.029$, permutation testing with 1,000 replicates), although there was no difference between male-biased and unbiased genes. The pattern reversed in the adult gonad, with male-biased genes showing collectively a 25% increase in d_N/d_S compared with unbiased genes ($P < 0.001$), as in figure 4, whereas divergence for female-biased genes expressed at this time point was not statistically different than that for unbiased genes. These divergence estimates show that sex-specific selection pressures shift over the life cycle, revealing previously unknown complexity.

Divergence rate has been shown to be correlated with maximal expression level (Roux and Robinson-Rechavi 2008; Artieri et al. 2009). In our data, although overall average relative expression for 1:1 orthologs with quantified divergence level was higher for female- and male-biased genes compared with unbiased genes in both ed19 and adult gonad (table 3), the sex-specific patterns in these two time points do not reveal a specific association with expression level. Specifically, for sex-specific expression in the sex with highest expression, male-biased genes were expressed more in males than were female-biased genes in

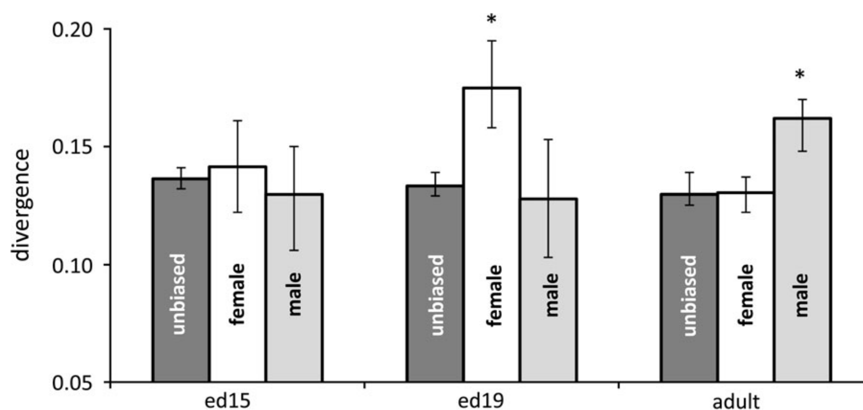

Fig. 4. Divergence (d_N/d_S) estimates of autosomal sex-biased gene categories over the life cycle. Divergence estimates are based on chicken–zebra finch 1:1 ortholog alignments. Female-biased genes are shown in white, male-biased in light gray (both with >2-fold expression thresholds, 5% FDR) and unbiased genes in dark gray. Whiskers represent 95% confidence intervals based on bootstrapping (1,000 replicates), and significant difference among categories is indicated (*), based on permutation testing (1,000 replicates).

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Table 2. Divergence Estimates for Sex-Biased Gene-Expression Categories at Different Time Points.

Time Point	Expression	N (kb)	d_N (p)	d_S (p)	d_N/d_S (p)
Ed15	Unbiased	3,375 (42,044.8)	0.0684	0.5019	0.1363
	Female biased	155 (173.6)	0.0669 (n.s.)	0.4735 (n.s.)	0.1414 (n.s.)
	Male biased	93 (103.6)	0.0541 (0.011)	0.4173 (<0.001)	0.1296 (n.s.)
Ed19	Unbiased	5,581 (8,176.4)	0.0649	0.4870	0.1332
	Female biased	188 (215.7)	0.0830 (0.035)	0.4747 (n.s.)	0.1748 (0.029)
	Male biased	99 (103.7)	0.0551 (n.s.)	0.4312 (0.015)	0.1277 (n.s.)
Adult	Unbiased	3,686 (5,533.6)	0.0638	0.4921	0.1296
	Female biased	1,336 (1,693.8)	0.0632 (n.s.)	0.4849 (n.s.)	0.1304 (n.s.)
	Male biased	902 (1,391.8)	0.0745 (<0.001)	0.4603 (<0.001)	0.1619 (<0.001)

NOTE.—Shown are the numbers of genes (N) in each expression category with divergence data from 1:1 chicken–zebra finch orthologs, as well as the total alignment length for those orthologs (kb). Where divergence (d_N, d_S, and d_N/d_S) estimates differed statistically from the unbiased pool of genes, the P values (based on permutation testing with 1,000 replicates) are given.

females in both time points, despite the fact that female-biased genes at ed19 show the highest d_N/d_S level of the sex-bias categories at this developmental stage.

The GO is a way to explore the functional differences among gene lists (Gene Ontology Consortium 2000), and though the results should always be interpreted with caution, overrepresentation of ontology terms can be informative in some situations. We identified sex bias with a $\geq$2-fold change for gonad (both with a 5% FDR). In each case, we tested the list of female-biased, male-biased, and unbiased genes against the list of all significantly expressed genes. Several GO terms were significant ($P \leq 0.01$) based on Fisher's exact tests (supplementary table 1, Supplementary Material online), though the vast majority did not remain significant after correction for multiple comparison. Those that did remain ($P_{adj} \leq 0.05$) include "nucleotide metabolic process, organelle organization and biogenesis" and "oxygen and reactive species metabolic process" for adult male-biased genes, as well as "deoxyribonucleotide metabolic process" for ed19 male-biased genes.

Discussion

The transcriptome-level time-series experiments presented here build on previous avian time-series analysis of early embryonic development for a limited set of genes (Scholz et al. 2006) and global transcription profiling for single time points (Ellegren et al. 2007; Itoh et al. 2007; Mank, Axelsson, and Ellegren 2007; Mank, Hultin-Rosenberg, et al. 2007). It is the first time series to our knowledge in any animal specifically designed to study temporal patterns in sex-specific selection pressure and presents a complex picture of the development and evolutionary pressures shaping sexual dimorphism.

The analysis presented here suggests that gene expression underlying differences between males and females begins early in development, and this is in agreement with what is known about chicken ontogeny. Gonadal differences begin early, with morphological sex differentiation of the gonads in chicken embryos histologically visible at ed6.5 (Carlon and Stahl 1985; Ebensperger et al. 1988). Sex differences in plasma levels of estrogen and testosterone appear shortly thereafter (Woods et al. 1975; Tanabe et al. 1979, 1986; Woods and Brazzill 1981).

It is difficult to know the implications and possible biological mechanisms that determine what percentage of the transcriptome is male versus female biased, but it is interesting to note that there are more female-biased than male-biased genes that map to the autosomes at most tissue/time-point treatments in our data set. This is somewhat unusual compared with comparative studies in *Drosophila* (Zhang et al. 2007) that showed either a predominately male-biased or relatively equal distribution of sex bias in most species. Male-biased genes also predominate in transcription profiling of *Xenopus* species (Malone et al. 2006), which have independent but analogous female heterogametic sex chromosomes. Mice have a mild excess of female-biased genes in some tissues (Yang et al. 2006), though overall they have a more equal distribution of male- and female-biased genes than the birds examined here. It is possible that mating system and sexual antagonism influence the degree and directionality of sex-biased gene expression, especially in the gonad; however, the exact nature of the relationship between mating system and gene expression, or even whether the differences noted are simply a statistical artifact, is not yet known.

The sex-biased expression signal from the autosomes is one of complexity. Sex-biased expression is highly variable among time points (figs. 2 and 3 and Supplementary Material online), and this indicates that the process of building and maintaining female versus male phenotypes is complex, varies through over the life cycle, and requires enormous specificity in expression timing. In contrast to previous quantitative genetic studies of ontogenetic complexity of size dimorphism (Parker and Garant 2005), our study indicates significant ontogenetic complexity in both gene expression and sex-specific selection pressures related to sexual dimorphism.

Although we focus here on the autosomes, the sex chromosomes are also thought to play a role in sexual dimorphism (Rice 1984; Mank 2009b). In birds, like mammals, the sex chromosomes harbor a relatively small number of genes, with less than 750 known or predicted protein-coding loci, or less than 5% of the transcriptome. Our expression data indicate that thousands of autosomal genes are sex biased at some point in the life cycle, even at conservative statistical thresholds. This means that, even with the inherent male bias of Z-linked genes and

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Table 3. Overall Relative Expression Averaged by Sex and Over All Replicates for 1:1 Orthologs for Ed19 and Adult Gonad.

Developmental Stage	Average Female Relative Expression	Average Male Relative Expression	Total Average Relative Expression
Ed19			
Female-biased genes	7.375	6.771	7.073
Male-biased genes	6.543	8.223	7.383
Unbiased genes	6.770	6.762	6.677
Adult			
Female-biased genes	8.528	6.503	7.516
Male-biased genes	6.385	8.873	7.629
Unbiased genes	6.377	6.282	6.379

a disproportionately large role of the Z relative to its overall size, the autosomes are by far the largest source of sex-biased gene expression.

Sex-Bias and Time

In general, the degree of sex bias increases over time (fig. 1), as reproductively mature animals require the largest contingent of sex-biased genes (Chippindale et al. 2001). In the gonad, the increase of sex bias at adulthood is most likely correlated to the onset of spermatogenesis in males and follicular maturity in females. Genes that are sex biased in embryonic time points are the likely engines driving the development of sex-specific reproductive structures in both females and males, as well oogenesis in females (Hughes 1963; Smith and Sinclair 2004).

Few autosomal genes are constitutively sex biased throughout our time points (supplementary fig. 1, Supplementary Material online), and a minority of genes change the direction of sex bias. For example, 50 genes change sex-biased expression pattern between ed19 and adulthood, going from female biased to male biased, whereas a comparable number (35) switch from male biased at ed19 to female biased in adulthood. These changes in sex-biased expression illustrate the previously unknown gene-expression complexity underlying sexually dimorphic phenotypes. The high d_N/d_S for female-biased genes in ed19 is not, however, due to these 50 genes, as the rate of evolution is qualitatively the same if they are omitted from the divergence calculation.

The time-series design of these experiments shows the role of both upregulation and downregulation in producing sex-biased expression. In general, sex bias is thought to result from the evolution of some mechanism to decouple the expression level for those genes that are advantageous to one sex and detrimental to the other (Connallon and Knowles 2005), and this regulatory decoupling allows for the attainment of separate male and female transcriptional optima. However, changes in the expression pattern can be used to infer sex-specific evolutionary effects (Mank and Ellegren 2009b), and studying the expression patterns that produce sex bias may provide much needed answers to the question of how sexual antagonism influences gene expression.

Signatures of Sex-Specific Selection Pressures

Sex-specific divergence estimates can be used as a proxy for the sex-specific evolutionary pressures acting on the loci underlying dimorphic traits (reviewed in Ellegren and Parsch 2007). Our analysis indicates that in addition to strong selection pressures exerted on adult male gonads (Meiklejohn et al. 2003; Ranz et al. 2003; Zhang et al. 2004; Pröschel et al. 2006), there is an equally powerful selection pressure on female reproductive proteins during late embryonic development (table 2 and fig. 4). This has not previously been shown and has several important implications. Most obvious, our study shows that studies of rates of evolution (Meiklejohn et al. 2003; Zhang et al. 2004; Pröschel et al. 2006; Haerty et al. 2007; Mank, Axelsson, and Ellegren 2007; Mank, Hultin-Rosenberg, et al. 2007) of sex-biased genes identified from a single time point are likely to result in a limited picture. Clearly, sex-biased gene expression patterns throughout the life cycle are complex, with distinct suites of genes controlling sexual differentiation in embryonic stages and sexual dimorphisms in the adult phase. Equally complex are the sex-specific selection pressures, which shift in intensity from one sex to the other over the life cycle.

More subtle, but no less important from an evolutionary ecology perspective, is that sperm competition is not the only major sex-specific selective force acting on the gonad. It is likely that the accelerated rates of divergence for adult male-biased genes are due, at least in part, to the powerful pressures of sperm competition, and this makes intuitive sense given the prevalence of multiple mating and extrapair copulation in many bird species (Bennett and Owens 2002; Westneat and Stewart 2003). However, sex-specific selection appears to be acting equally powerfully on females in late development, and this may be because it is at this point in the life cycle that oogenesis is initiated and arrested (Hughes 1963; Smith et al. 2008). It is during this critical period that selection on female reproduction is most powerful, as these ova form the entire gametic complement for the female and will not increase in number.

Supplementary Material

Supplementary figure 1 and supplementary table 1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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