Rapid Evolution of Ovarian-Biased Genes in the Yellow Fever Mosquito (*Aedes aegypti*)

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ABSTRACT Males and females exhibit highly dimorphic phenotypes, particularly in their gonads, which is believed to be driven largely by differential gene expression. Typically, the protein sequences of genes upregulated in males, or male-biased genes, evolve rapidly as compared to female-biased and unbiased genes. To date, the specific study of gonad-biased genes remains uncommon in metazoans. Here, we identified and studied a total of 2927, 2013, and 4449 coding sequences (CDS) with ovary-biased, testis-biased, and unbiased expression, respectively, in the yellow fever mosquito *Aedes aegypti*. The results showed that ovary-biased and unbiased CDS had higher nonsynonymous to synonymous substitution rates (dN/dS) and lower optimal codon usage (those codons that promote efficient translation) than testis-biased genes. Further, we observed higher dN/dS in ovary-biased genes than in testis-biased genes, even for genes coexpressed in nonsexual (embryo) tissues. Ovary-specific genes evolved exceptionally fast, as compared to testis- or embryo-specific genes, and exhibited higher frequency of positive selection. Genes with ovary expression were preferentially involved in olfactory binding and reception. We hypothesize that at least two potential mechanisms could explain rapid evolution of ovary-biased genes in this mosquito: (1) the evolutionary rate of ovary-biased genes may be accelerated by sexual selection (including female–female competition or male–mate choice) affecting olfactory genes during female swarming by males, and/or by adaptive evolution of olfactory signaling within the female reproductive system (*e.g.*, sperm-ovary signaling); and/or (2) testis-biased genes may exhibit decelerated evolutionary rates due to the formation of mating plugs in the female after copulation, which limits male–male sperm competition.

KEYWORDS ovary-biased genes; gonads; *Aedes aegypti*; molecular evolution; protein sequence divergence; Genetics of Sex

SEX-BIASED genes, that is, genes whose expression is upregulated in one sex relative to the other, are believed to underlie sexual dimorphism and have been extensively reported in anisogamous eukaryotes including mammals, birds, fish, insects, worms, fungi, higher plants, and algae (Meiklejohn *et al.* 2003; Ranz *et al.* 2003; Zhang *et al.* 2004; Cutter and Ward 2005; Yang *et al.* 2006; Ellegren and Parsch 2007; Haerty *et al.* 2007; Whittle *et al.* 2007; Small *et al.* 2009; Assis *et al.* 2012; Parsch and Ellegren 2013; Whittle and Johannesson 2013; Lipinska *et al.* 2015; Wang *et al.* 2015). The number of genes exhibiting sex bias can represent a majority of the genome, with estimates from 50 to >75% of the genome being sex-biased in insect genera such as *Drosophila* and *Nasonia* (Ranz *et al.* 2003; Ellegren and Parsch 2007; Assis *et al.* 2012; Wang *et al.* 2015). The sex biases in expression appear to have molecular evolutionary consequences. For example, in animals studied to date, male-biased genes typically evolve more rapidly at the protein sequence level, exhibiting elevated ratios of nonsynonymous to synonymous substitutions (dN/dS), than female-biased or unbiased genes [reviewed by Ellegren and Parsch (2007); see also Mank *et al.* (2007) and Parsch and Ellegren (2013)]. Evidence suggests that sex biases in expression between whole individuals might largely result from the sex-limited organs, namely gonads and their contained sex cells (Arbeitman *et al.* 2002, 2004; Parisi *et al.* 2004; Ellegren and Parsch 2007; Small *et al.* 2009; VanKuren and Vibranovski 2014). This is consistent with the fact that the gonads comprise highly differentiated organs, with extremely divergent

Copyright © 2017 by the Genetics Society of America
doi: https://doi.org/10.1534/genetics.117.201343
Manuscript received February 21, 2017; accepted for publication May 30, 2017; published Early Online June 19, 2017.
Supplemental material is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.117.201343/-/DC1.
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transcriptomes, between males and females (Parisi *et al.* 2004; Harrison *et al.* 2015). In this regard, gonadal gene expression may largely shape the evolution of many protein-coding genes.

Genes expressed in the gonads are believed to play a central role in evolution, contributing toward reproductive success and fitness, reproductive isolation, and speciation. These genes are also most apt to be influenced by major evolutionary processes such as sexual conflict, mate-choice, and intrasexual competition (Civetta and Singh 1998; Swanson and Vacquier 2002; Jagadeeshan and Singh 2005; Ellegren and Parsch 2007; Turner and Hoekstra 2008). With respect to molecular evolution of reproductive and gonad genes, studies in animals including mammals and insects have shown that certain seminal protein genes (Swanson *et al.* 2001), testis (Good and Nachman 2005; Khaitovich *et al.* 2005; Ellegren and Parsch 2007; Haerty *et al.* 2007), and sperm genes (Torgerson *et al.* 2002; Good and Nachman 2005; Ellegren and Parsch 2007; Haerty *et al.* 2007) typically evolve rapidly as compared to the rest of the genome, and thus agree with the global pattern of rapid sequence evolution of male-biased genes. Specific testis and ovary genes directly involved in fertilization have also been demonstrated to evolve rapidly in various animal systems (Civetta and Singh 1998; Swanson and Vacquier 2002). Nonetheless, observed effects of expression within sex and reproductive genes varies among studies and with the type of tissue examined. For example, seminal fluid protein sequences evolve fast in numerous models including *Drosophila* species (Haerty *et al.* 2007), but not in others such as *A. albopictus* (Boes *et al.* 2014), and while spermatogenesis genes typically evolve rapidly (including in *Drosophila*) (Ellegren and Parsch 2007; Haerty *et al.* 2007), some fly research suggests that sperm genes may be more conservative in their rates of evolution (Dorus *et al.* 2006). In this regard, while male reproductive genes typically evolve rapidly, this may not be universal to all male sexual tissues, and the signal varies to some extent among organisms and studies.

At present, studies in animals that specifically compare whole testis- *vs.* ovary-biased expression and its effect on the evolution of protein-coding DNA remain few, with the exception of some examples from organisms such as guppies (*Poecilia*: Sharma *et al.* 2014), various birds (Harrison *et al.* 2015), sea urchins (*Allocentrotus* and *Strongylocentrotus*: Oliver *et al.* 2010), and the greatest focus on the insect genus *Drosophila* (Jagadeeshan and Singh 2005; Ellegren and Parsch 2007; Haerty *et al.* 2007; Meisel 2011; Assis *et al.* 2012). In each of these example taxa, higher dN/dS was reported for the testis-linked genes than for the ovary-linked genes [with the exception of guppies, where testis- and ovary-biased genes both evolved rapidly compared to unbiased genes (Sharma *et al.* 2014)]. Such research is an important step in deciphering the role of gonads in evolution (Jagadeeshan and Singh 2005), and particularly in DNA sequence evolution.

In addition to protein sequence divergence (Ellegren and Parsch 2007; Parsch and Ellegren 2013), another facet of protein-coding DNA that may be related to sex-biased expression is optimal codon usage (Hambuch and Parsch 2005; Ellegren and Parsch 2007; Whittle *et al.* 2007), that is, those codons that promote efficient and/or accurate translation (Sharp *et al.* 1986; Duret and Mouchiroud 1999; Akashi 2001). Synonymous codon usage appears to often be non-arbitrary, and is believed to arise either from natural selection promoting biochemically efficient and accurate protein synthesis (Duret and Mouchiroud 1999; Duret 2000; Stoletzki and Eyre-Walker 2007) or from mutational bias (Osawa *et al.* 1988; Sueoka 1988; Sharp *et al.* 1995). A role of natural selection concurs with data showing that tRNA abundance and tRNA gene copy number match the most common codons in the genome (Ikemura 1981, 1985; Duret 2000), and that a subset of codons are preferentially used in highly transcribed genes (*e.g.*, Sharp *et al.* 1986; Stenico *et al.* 1994; Duret and Mouchiroud 1999; Cutter *et al.* 2006; Ingvarsson 2008; Whittle *et al.* 2011; Whittle and Extavour 2016a). Optimization of codon usage has been shown in diverse eukaryotes including fungi, plants, worms, and insects such as *Tribolium* and *Drosophila* [Duret and Mouchiroud 1999; reviewed by Hershberg and Petrov (2008); Behura and Severson (2011, 2013); and Williford and Demuth (2012)]. However, signals have been weak or absent in other taxa, including the insect *Bombyx* (Jia *et al.* 2015; Whittle and Extavour 2016b). As optimal codons typically have been linked to selective pressures, the level of optimal codon usage may be influenced by sex-biased expression (Hambuch and Parsch 2005; Whittle *et al.* 2007), including selective pressures arising from the testes and ovaries.

Available data to date suggests that protein-coding sequence (CDS) and optimal codon usage might evolve in concert in some eukaryotes. For instance, in *Drosophila*, wherein male- (or testis-) biased genes evolve rapidly (Zhang *et al.* 2004; Ellegren and Parsch 2007; Haerty *et al.* 2007), it has been reported that male-biased genes exhibit lower frequency of optimal codons as compared to female-biased and unbiased genes (Hambuch and Parsch 2005). In addition, fast evolving sex-biased genes have been suggested to have reduced codon usage bias in other groups outside the metazoans. For example, in the hermaphrodite fungus *Neurospora*, a rare case of a fungal species wherein protein-coding genes with biased expression in female-limited organs (protoperithecia) evolve rapidly, the female genes also exhibit reduced optimal codon usage as compared to male organ- (conidia-) biased and unbiased genes (Whittle and Johannesson 2013). Similarly, rapidly evolving sex-biased genes show less optimal codon use in brown algae (*Ectocarpus*: Lipinska *et al.* 2015), and male-biased genes from the sex organs (anthers) in certain plants exhibit lower optimal codon usage than ovary-biased genes (Whittle *et al.* 2007). This frequently reported inverse correlation between rates of protein sequence evolution and optimal codon usage in sex-biased genes might result from adaptive evolution in

proteins driving fixation of linked nonoptimal codons (selective sweeps) and/or from relaxed selection (Betancourt and Presgraves 2002; Kim 2004; Hambuch and Parsch 2005; Whittle and Johannesson 2013). In this regard, an understanding of the dynamics shaping the evolution of DNA coding for sex-biased gonad genes should include its relationship to both protein divergence and optimal codon usage.

To gain further insight into the evolution of sex-biased gonadal genes, research needs to be expanded to nontraditional models, including within insects, which comprise the largest and most diverse class of animals on earth, representing up to 90% of all known animal species (Erwin 1982). The yellow fever mosquito *Aedes aegypti* has gained momentum as a model in biology, and extensive large-scale genomic and transcriptome resources have recently been made available (Nene *et al.* 2007; Akbari *et al.* 2013; Chen *et al.* 2015), making it suitable for molecular evolutionary research. *A. aegypti* is a holometabolous insect that serves as a vector for human pathogens including yellow fever (Jentes *et al.* 2011), Dengue (Simmons *et al.* 2012), and chikungunya (Leparc-Goffart *et al.* 2014), which are transmitted by a female bite (Hall *et al.* 2015). Originating from Africa, over the last 40 years this insect has spread across all continents excluding Antarctica (Kraemer *et al.* 2015). It is a short-lived organism with a typical life cycle of 15–30 days (Christophers 1960). The reproductive biology of *A. aegypti* has been well-established. Mating competency typically begins in males that are at least 24-hr old, and mating usually results during male swarming, but also by single-pair mating (Oliva *et al.* 2014). While males are polygynous, they limit fertilization by other males both by transferring sufficient sperm to fertilize all eggs a female will lay in her lifetime, and by transmitting accessory gland secretions that act as physical and chemical barriers to insemination by other males [reviewed by Oliva *et al.* (2014)]. After mating, egg maturation generally occurs after blood feeding, but can occur in its absence in a small portion (3–4%) of females (Ariani *et al.* 2015). *A. aegypti* does not have heteromorphic sex chromosomes among its three pairs of chromosomes (Juneja *et al.* 2014). Instead, sex determination is governed by a dominant male-determining factor (M-factor) located on a homomorphic (sex-determining) chromosome (with a genotype of Mm in males and mm in females). In fact, sex may largely depend on a single gene in this region named *Nix*, which is found only in males and initiates male development, and its absence causes feminization (Hall *et al.* 2015). With respect to the genome, the complete sequence for *A. aegypti* is ~1.38 GB, a relatively large size for an insect, and five-fold larger than the model mosquito *Anopheles gambiae* (Nene *et al.* 2007). The large genome size of *A. aegypti* is thought to result from an abundance of repetitive sequences (Nene *et al.* 2007; Juneja *et al.* 2014). Together, given its genomic and trancriptomic resources and its well-understood sexual biology, *A. aegypti* comprises a suitable system for the study of the molecular evolution of sex-biased gonad genes.

In the present study, we investigate the connection between sex-biased gonad expression and protein-CDS evolution in *A. aegypti*. First, using large-scale embryo transcriptomes, we identify a list of optimal codons in this mosquito, and observe clear signals that natural selection favors codons ending in G or C (GC3). Second, using testis and ovary transcriptomes, we identify CDS with sex-biased gonad expression. The data show that, as compared to testis-biased CDS, ovary-biased and unbiased CDS exhibit higher dN/dS and lower frequency of optimal codons (Fop; Wright 1990) in this insect. Surprisingly, these trends are opposite to the faster evolution of male-biased genes (and low Fop) reported in most organisms studied to date (Ellegren and Parsch 2007; Parsch and Ellegren 2013). Evaluation of ovary- and testis-specific gene expression as compared to embryos reveals that female gonad expression is consistently linked to higher dN/dS, even when these genes are additionally coexpressed in other tissues, and those patterns are connected to positive selection. Further, ovary genes were preferentially involved in olfactory functions. We compare sex-biased expression in nonreproductive male and female carcasses (defined as whole bodies without the gonads) and find that somatic female-biased genes evolve faster than somatic male-biased genes. It is shown that this effect largely results from coexpression of ovary-biased genes in the female soma. Together, we propose that the fast evolution of ovary-biased genes may result from sexual selection acting to accelerate evolution of female gonadal genes, including those involved in olfactory functions, and/or by reduced male–male sperm competition due to the formation of mating barriers in the female reproductive tract by male seminal fluids in *A. aegypti*.

Results and Discussion

Of the 15,796 protein-coding genes described for *A. aegypti* (Table S1 in File S1, assembly v3.29), we assessed expression in those CDS that started with an ATG start codon, had no unknown or ambiguous nucleotides, and comprised the longest CDS per gene when multiple isoforms were described. These filters yielded a total of 14,678 CDS for analysis. In addition to dN/dS, we wished to study if sex-biased gonadal expression shaped codon usage. For this, we first had to determine whether *A. aegypti* was a species where selection shaped its codon usage, which we assessed using comparative analysis of the large-scale transcriptome data set from embryos (see File S1 and specifically Table S1 contained within it).

Identification of optimal codons in A. aegypti

We report that *A. aegypti* exhibits strong signals showing that natural selection shapes optimal codon usage in this taxon (Table 1). As described in detail in Supplemental Note 1 in File S1, for the identification of optimal codons in this mosquito, we conducted contrasts of codon usage in highly (highest 5%) *vs.* lowly expressed (lowest 5%) genes, which

included assessment of changes in ΔRSCU (Sharp *et al.* 1986) and correspondence analysis. Both of these approaches have precedent for the identification of codons preferentially used under high expression (optimal codons) (Duret and Mouchiroud 1999; Cutter *et al.* 2006; Ingvarsson 2008; Wang *et al.* 2011; Whittle *et al.* 2011; Whittle and Extavour 2016a). For details, see Supplemental Note 1 in File S1. The codon with the largest statistically significant and positive ΔRSCU per amino acid was taken as the primary optimal codon per amino acid. As shown in Table 1, we identified 18 primary optimal codons in *A. aegypti*, which ended in C or G.

The strong link observed between expression level and C- and G-ending codons, as found here, is a good indicator that selection drives the use of those codons (Sharp *et al.* 1986; Stenico *et al.* 1994; Duret and Mouchiroud 1999; Cutter *et al.* 2006; Ingvarsson 2008; Whittle *et al.* 2011). Nonetheless, for an additional layer of stringency, we wanted to fully exclude that mutational bias may have caused differences between expression classes (Beletskii and Bhagwat 1996; Comeron 2004). For instance, it is possible that highly transcribed genes could have a mutational bias (such as reported for C to T mutations) or a transcription-coupled mutational asymmetry (Beletskii and Bhagwat 1996; Green *et al.* 2003) causing disproportionately greater usage of C3 or G3 codons in *A. aegypti*. To further confirm optimal codon usage was due to natural selection and not mutational bias, one can compare the GC content of synonymous GC3s to selectively neutral regions such as introns (GCi), where the latter should reflect mutational pressures [note that GC3s is an effective proxy of optimal codon usage, as the frequency of optimal codons (Fop, Ikemura 1981) correlates strongly to GC3s: $R = 0.93$ and $P < 2.0 \times 10^{-7}$; Figure S1 in File S1]. For this, as described in detail in File S1, we examined 36,272 introns and GC3 content in the genes under study. The introns were divided by length [short (≤130 bp) and long introns (>130 bp) (Farlow *et al.* 2012)], and for the long introns, 50 bp were removed from the ends, which may contain regulatory regions (Williford and Demuth 2012). The results showed that the GC3s for the CDS with the highest 5% expression in embryos (average GC3s = 0.618 ± 0.005), intermediate 5th to 95th expression level (0.564 ± 0.001), and the CDS with lowest 5% expression (GC3s = 0.536 ± 0.003), were each markedly and statistically significantly higher than the GCi content of their associated introns [for both short and long introns], which averaged between 0.340 and 0.380 (Figure 1; Mann-Whitney U (MWU)-test $P < 0.001$ for all contrasts). Further, unlike GC3s, which increased with expression, GCi was not elevated with respect to transcription level (and slightly decreased for short introns, Figure 1), also showing that mutational biases could not explain optimal codon usage in this taxon.

Together, we conclude that the preferential use of 18 optimal codons under high expression in *A. aegypti* (Table 1) is caused by natural selection. Thus, in addition to dN/dS, we were able to study how sex-biased gonadal expression affects selection on optimal codon usage (see below).

Expression profiles of testis- and ovary-biased genes in A. aegypti

We identified CDS with sex-biased expression in testes (plus accessory glands; henceforward denoted as testes) and ovaries in *A. aegypti* using large-scale transcriptome data sets (Akbari *et al.* 2013) and studied their molecular evolution, including protein sequence divergence and optimal codon usage. To measure interspecies divergence (Yang 2007), we used a species from the same genus, *A. albopictus* (*cf*. Zhang *et al.* 2004; Proschel *et al.* 2006; Assis *et al.* 2012), whose genome sequence has recently become available (Chen *et al.* 2015). A total of 9389 putative orthologs were identified between *A. aegypti* and *A. albopictus* (see *Materials and Methods*). For consistency across all results, this CDS list was used for all of our subsequent sex-biased expression and molecular evolutionary analysis. Sex-biased genes were defined as those with FPKM > 1 in at least one tissue, at least a twofold difference between testis and ovary expression, and $P < 0.05$ using differential expression analysis in *A. aegypti* (see *Materials and Methods*). All CDS not matching these criteria were defined as unbiased. A total of 2927, 2013, and 4449 CDS were identified for study as testis-biased, ovary-biased, and unbiased, respectively, suggesting a greater propensity for CDS to be involved in testis than ovary function.

Fold sex bias in the gonads is linked to expression level

We assessed the relationship between the degree of sex-biased gonadal expression (fold bias) and expression level in *A. aegypti*. The results showed that greater ovary and testis bias were each linked to elevated expression levels in *A. aegypti*. For this analysis, the testis- and ovary-biased CDS were each classified into three nonoverlapping fold bias classes: (1) ≥2-fold bias and <5-fold bias; (2) ≥5-fold bias and <10-fold bias; and (3) ≥10-fold bias. This classification approach is comparable to previous male/female sex bias studies, and allows observations of clear demarcations with respect to bias level (Lipinska *et al.* 2015; Perry *et al.* 2015). In addition, for rigor, given that prior data in eukaryotes indicate that expression level can be inversely related to CDS length, and particularly elevated for the shortest CDS in eukaryotic genomes (Akashi 2001, 2003; Urrutia and Hurst 2003; Comeron 2004; Lemos *et al.* 2005; Williford and Demuth 2012; Whittle and Extavour 2016a), the testis- and ovary-biased genes were divided into short (≤250 aa) and long (>250 aa) length classes. The CDS classes were separated using the 25th percentile approximated across all 9389 CDS under study (*cf*. Bloom *et al.* 2006).

As shown in Figure 2, an increase in testis-fold bias resulted from both elevated testis expression and reduced ovary expression. Specifically, as testis-fold bias progressively increased from ≥2-5 fold bias, to ≥5-10 fold bias, and to the ≥10-fold bias classes, there was elevated average (and

Table 1 The ΔRSCU between highly (high) and lowly (low) expressed genes in *A. aegypti*

Amino acid	Codon	RSCU (High)	RSCU (Low)	ΔRSCU	*P* value	Correspondence analysis
Ala	GCT	0.9704	0.9909	−0.020		
Ala	**GCC**	1.8235	1.2438	+0.579	**	GCC
Ala	GCA	0.6085	0.9658	−0.357	**	
Ala	GCG	0.5976	0.7994	−0.201	**	
Arg	CGT	1.4934	1.0031	+0.490	**	
Arg	**CGC**	1.6334	0.9633	+0.670	**	CGC
Arg	CGA	0.9562	1.3965	−0.440	**	
Arg	CGG	0.9879	1.1382	−0.150	*	
Arg	AGA	0.5099	0.8639	−0.354	**	
Arg	AGG	0.3541	0.6256	−0.271	**	
Asn	AAT	0.6486	0.9013	−0.252	**	
Asn	**AAC**	1.3266	1.0987	+0.227	**	AAC
Asp	GAT	1.0194	1.1458	−0.126	**	
Asp	**GAC**	0.9433	0.8542	+0.089	**	GAC
Cys	TGT	0.5477	0.9489	−0.401	**	
Cys	**TGC**	1.1112	1.0227	+0.088	*	TGC
Gln	CAA	0.6219	0.9189	−0.297	**	
Gln	**CAG**	1.3192	1.0811	+0.238	**	CAG
Glu	GAA	1.0727	1.1491	−0.076	**	
Glu	**GAG**	0.9118	0.8509	+0.060	**	GAG
Gly	GGT	1.095	1.0235	+0.071	*	
Gly	**GGC**	1.1659	0.8714	+0.294	**	GGC
Gly	GGA	1.4366	1.5836	−0.147	**	
Gly	GGG	0.2838	0.5214	−0.237	**	
His	CAT	0.7198	0.9272	−0.207	**	
His	**CAC**	1.1469	1.0602	+0.086	*	CAC
Ile	ATT	0.9168	1.076	−0.159	**	
Ile	**ATC**	1.8039	1.3602	+0.443	**	ATC
Ile	ATA	0.2421	0.5638	−0.321	**	
Leu	TTA	0.2848	0.4383	−0.153	**	
Leu	TTG	1.1941	1.4717	−0.277	**	
Leu	CTT	0.5594	0.7806	−0.221	**	
Leu	CTC	0.8132	0.7709	+0.042		
Leu	CTA	0.4033	0.6167	−0.213	**	
Leu	**CTG**	2.708	1.922	+0.786	**	CTG
Lys	AAA	0.6329	0.9424	−0.309	**	
Lys	**AAG**	1.3515	1.0419	+0.309	**	AAG
Phe	TTT	0.4731	0.7328	−0.259	**	
Phe	**TTC**	1.499	1.2673	+0.231	**	TTC
Pro	CCT	0.461	0.7075	−0.246	**	
Pro	**CCC**	0.9911	0.6824	+0.308	**	CCC or CCG
Pro	CCA	1.0912	1.1852	−0.094	*	
Pro	CCG	1.4071	1.4248	−0.017		
Ser	TCT	0.5852	0.6641	−0.078	*	
Ser	**TCC**	1.6615	1.0864	+0.575	**	TCC or TCG
Ser	TCA	0.4453	0.7651	−0.319	**	
Ser	TCG	1.5813	1.3423	+0.238	**	
Ser	AGT	0.6007	1.0136	−0.412	**	
Ser	AGC	1.098	1.1286	−0.030		
Thr	ACT	0.6453	0.8135	−0.168	**	
Thr	**ACC**	1.8154	1.2705	+0.544	**	ACC
Thr	ACA	0.5641	0.7742	−0.210	**	
Thr	ACG	0.9379	1.1417	−0.203	**	
Tyr	TAT	0.5447	0.7627	−0.217	**	
Tyr	**TAC**	1.3839	1.2342	+0.149	**	TAC
Val	GTT	0.995	1.08	−0.085	*	
Val	**GTC**	1.2726	0.8775	+0.395	**	GTC
Val	GTA	0.4135	0.634	−0.220	**	
Val	GTG	1.2939	1.4084	−0.114	*	

Gene expression was measured using embryos. Putative optimal codons are in bold and underlined. The primary optimal codon per amino acid predicted by correspondence analysis is also shown. A total of 18 primary optimal codons were identified. * $P < 0.05$, ** $P < 0.001$ using *t*-tests. ΔRSCU, change in relative synonymous codon usage (rounded down to the nearest 1/1000); RSCU, relative synonymous codon usage (mean values); note that standard errors ranged between 0.010 and 0.050 and are not shown for presentation purposes.

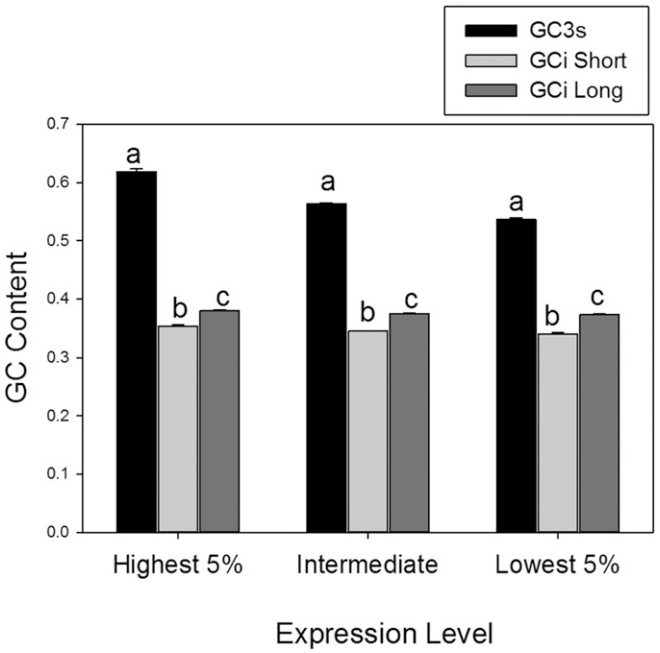

Figure 1 The average GC content at synonymous third codon positions (GC3s) and the GC content of introns (GCi). Results are shown for the set of coding sequences (CDS) with high (highest 5%), intermediate (between the 5th and 95th percentile), or low (lowest 5%) expression in *A. aegypti* embryos. GCi for short (≤130 bp) and long (>130 bp) introns (after removal of 50 bp at each end) per expression class are shown. Different letters (a, b, or c) above the bars showing mean values of GC3s, GCi (short) and GCi (long) within each expression class indicate a statistically significant difference ($P < 0.05$) using paired Mann-Whitney U (MWU) tests (the *P*-value was <0.001 in each contrast). Note that the GC3s values were also statistically significant different between the three expression classes using paired contrasts (MWU-test $P < 0.001$). Error bars are standard errors.

median) expression level in testes, and reduced expression in ovaries, for both short and long CDS (MWU-tests $P < 0.001$ for all contrasts between fold bias classes). In fact, CDS with ≥10-fold bias had the highest expression in testis (average = 771.6 ± 347.1 FPKM for short CDS and 368.2 ± 57.7 FPKM for long CDS), and the lowest expression in ovaries (6.9 ± 1.2 FPKM for short CDS and 3.3 ± 0.4 for long CDS) among all of the 3-fold bias categories (MWU-tests $P < 0.001$ for all contrasts, Figure 2, A and B). This trend concurs with findings in whole (early-stage) male/females in *Drosophila* and from sexual structures (gametophytes) in brown algae (*Ectocarpus*), where greater male bias resulted from both elevated expression in male tissues and decreased expression in female tissues (Lipinska *et al.* 2015; Perry *et al.* 2015).

For the ovary-biased CDS, a different trend was found. For short ovary-expressed genes, higher sex bias was linked to elevated expression in ovaries and no notable change in testis expression (Figure 2C). However, for long CDS, greater bias in ovary expression was linked to both a marked increase in ovary expression and a relatively mild reduction in testis expression in the ≥5- to 10-fold bias and ≥10-fold bias classes as compared to the ≥2- to 5-fold bias class (Figure 2D) (MWU-test $P < 0.001$ for each paired contrast). This finding differs from *Drosophila* and algae, wherein high bias in female tissues did not result from upregulation in females (which were largely unchanged across fold bias classes) but rather arose from downregulation in males (Lipinska *et al.* 2015; Perry *et al.* 2015). Thus, unlike those systems, in *A. aegypti*, ovary bias arises specifically from upregulation in the female gonad and is not determined by male expression levels.

It is notable that the expression of sex-biased gonad genes was higher for short than for long CDS for all expression classes (MWU-test $P < 0.001$ for all paired contrasts of each tissue type per fold biased category). This trend is consistent with observations of shorter CDS in highly expressed genes reported in various eukaryotic systems, which might involve selection to minimize synthesis costs of abundant proteins (Akashi 2001, 2003; Urrutia and Hurst 2003; Comeron 2004; Lemos *et al.* 2005; Williford and Demuth 2012; Whittle and Extavour 2016a). Our results extend this phenomenon to include genes with sex-biased gonad expression in *A. aegypti*.

High dN/dS and low optimal codon usage in ovary-biased genes

To study protein sequence divergence, we assessed dN/dS (Yang 2007) of *A. aegypti* and its sister species *A. albopictus*, an approach consistent with paired species sequence contrasts previously employed to reveal evolutionary patterns of sex-biased genes (Zhang *et al.* 2004; Proschel *et al.* 2006; see Ellegren and Parsch 2007 Assis *et al.* 2012). Values were determined using the Nei–Gojobori method (Nei and Gojobori 1986) in MEGA (Kumar *et al.* 2012) for all sex-biased and unbiased CDS [PAML was also utilized to estimate these values (Yang 2007); see below in this section, and Supplemental Note 2 in File S1]. These *Aedes* taxa have adequately diverged to accumulate effects on dN (average = 0.075 ± 0.001). All CDS had dN < 1 and nearly all CDS (97.0%) had dS < 1.5 (73.9% were <1.0), which comprises an effective range to study dN, dS, and dN/dS (*cf*. Castillo-Davis *et al.* 2004).

As shown in Figure 3, comparison of all testis-biased, ovary-biased, and unbiased CDS defined in *A. aegypti* revealed that dN/dS was statistically significantly higher for ovary-biased CDS than testis-biased CDS in this mosquito (MWU-tests $P < 0.001$). However, no differences were observed between ovary-biased and unbiased CDS (MWU-test $P > 0.05$). This represents an unusual pattern for metazoans, including other insects such as *Drosophila*, wherein male- (and/or testis-) biased genes typically evolve rapidly (Zhang *et al.* 2004; Ellegren and Parsch 2007; Haerty *et al.* 2007; Assis *et al.* 2012; Ellegren 2013; Harrison *et al.* 2015). Faster evolution in the female gonad genes might be related to the fact that ovary-biased expression results from active upregulation in the ovary in this taxon (Figure 2), rather than decreased testis expression (Lipinska *et al.* 2015; Perry *et al.*

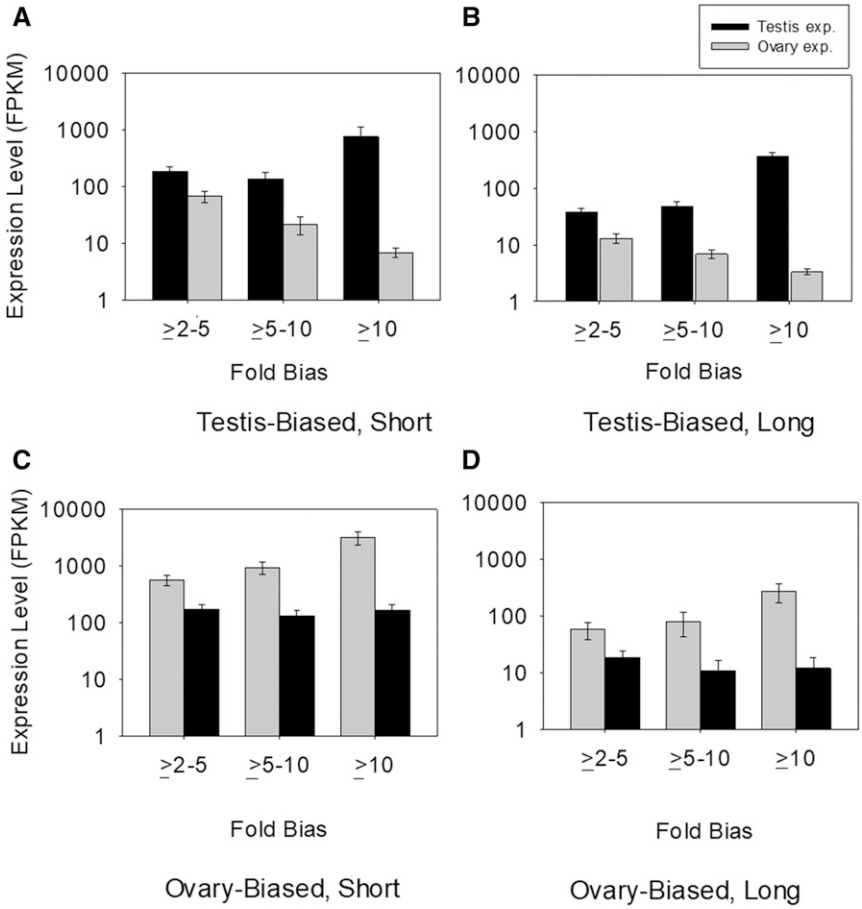

Figure 2 The average expression level in testes and ovaries of CDS with sex-biased expression in *A. aegypti*. The testis-biased and ovary-biased CDS were each subdivided with respect to fold bias, and the expression level in each tissue type for the biased genes is shown. (A and B) testis-biased short and long CDS. (C and D) ovary-biased short and long CDS. Within (A and B), all paired contrasts between fold class categories for testis-expressed and for ovary-expressed CDS were statistically significant ($P < 0.05$) with Mann-Whitney U tests $P < 0.001$ in each contrast. For (C), all paired contrasts between fold bias class were statistically significant for ovary expression ($P < 0.001$ for each contrast), but not for testis expression (all $P > 0.86$). For (D), all paired contrasts between fold classes were statistically significant for ovary expression, and between the ≥ 2 to 5 *vs.* the ≥ 5 to 10 and ≥ 10 classes for testis expression (P was < 0.001). Note the *y*-axes are in log scale. CDS, coding sequences; FPKM, frequency per kilobase million.

2015), suggesting a distinct mechanism of sex-biased expression. Further, the higher dN/dS in unbiased genes, which were in a similar range as ovary-biased genes, might indicate that ovary expression accelerates the evolution of the unbiased gene set (as compared to testis-biased), even when the genes are coexpressed in testis (see below section: *Ovary and testis expression relative to embryos and dN/dS*).

In addition to the Nei–Gojobori method (Nei and Gojobori 1986) in MEGA (Kumar *et al.* 2012), we also used PAML to measure dN, dS, and dN/dS, based on a substitution model that specifically includes codon usage bias (Yang 2007). Using this method, we found nearly identical results for all figures and tables (see Supplemental Note 2 in File S1). Further, values of dN/dS across all 9389 CDS under study were highly correlated between the two estimation methods (Figure S2 in File S1), with Spearman's $R = 0.95$, $P < 2.0 \times 10^{-6}$, as were dN ($R = 0.84$, $P < 2.0 \times 10^{-7}$) and dS ($R = 1.0$, $P < 2.0 \times 10^{-7}$) values.

With respect to codon usage, Fop was statistically significantly higher for testis- than ovary-biased CDS and unbiased CDS (Figure 3B), trends that also contrast with prior findings in other insects, where male-biased genes typically exhibited lower optimal codon usage (Zhang *et al.* 2004; Hambuch and Parsch 2005). While Fop has been proposed to be lower in longer CDS due to reduced selective constraint or genetic interference (Duret and Mouchiroud 1999; Hambuch and Parsch 2005), a gene length effect *per se* cannot explain the female effect herein because, as a group, ovary-biased CDS had shorter average lengths than testis-biased CDS (average = 416.6 ± 8.5 and 627.7 ± 6.7 codons, respectively; MWU-test $P < 0.001$). These trends in dN/dS and Fop in this mosquito resemble findings from a nonmetazoan, the fungus *Neurospora*, where genes with biased expression in female sex organs had higher dN/dS combined with lower Fop as compared to their male counterparts (Whittle and Johannesson 2013).

Fold bias, CDS length, and expression level and dN/dS

To evaluate in finer detail the relationship between sex-biased gonadal expression on molecular evolution in *A. aegypti*, we examined dN, dS, dN/dS, and Fop for each sex combination of the three classes of fold bias and the two classes of CDS lengths defined in our above section, *Fold sex bias in the gonads is linked to expression level*. The assessment is presented in detail in Figure S3, Supplemental Note 2, and Table S2 in File S1. In brief, the results showed that ovary-biased CDS exhibited higher dN in five of six categories of fold bias and CDS lengths, and higher dN/dS in four of six classes. Fop was lower for ovary-biased CDS than testis-biased genes for all six combinations of fold bias and CDS length (see also Figure S3 and Table S3 in File S1), indicating a persistently reduced Fop in the ovary-biased genes (compared

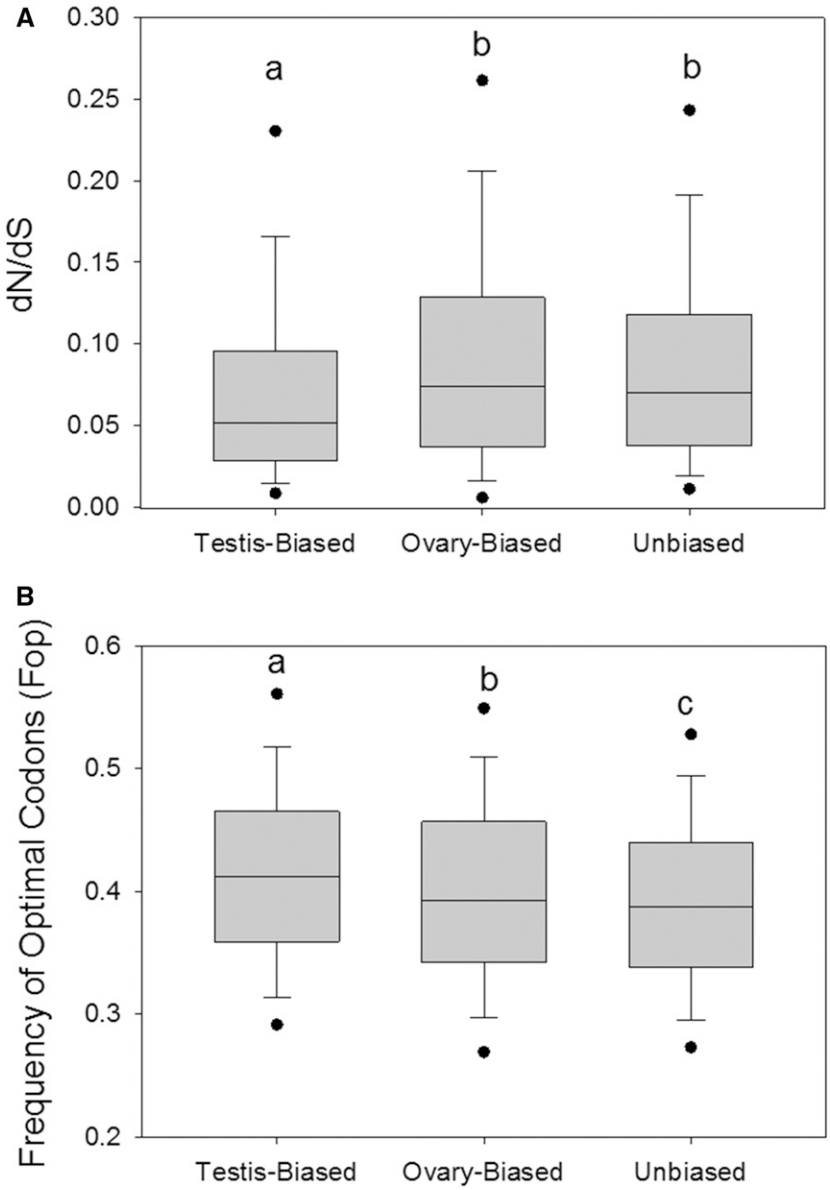

Figure 3 Box and whisker plots of dN/dS (A) and Fop (B) showing the distribution of all testis-biased, ovary-biased, and unbiased CDS in *Aedes*. Different letters above any two bars for the testis-biased, ovary-biased, and unbiased genes within each figure indicate a statistically significant ($P < 0.05$) difference in values using Mann-Whitney U tests (P was < 0.001 in all contrasts). $N_{\text{Testis-Biased}} = 2927$; $N_{\text{Ovary-Biased}} = 2013$, and $N_{\text{Unbiased}} = 4449$. Fop was measured using *A. aegypti*, and dN/dS measured using *A. albopictus* as a reference. CDS, coding sequences; dN/dS, nonsynonymous to synonymous substitution rates; Fop, frequency of optimal codons.

to testis-biased genes) irrespective of the level of sex bias and CDS length in *A. aegypti*. Nevertheless, we noted that dN/dS was higher for testis-biased CDS in short CDS with 5-fold or higher bias. Thus, while the primary signal is of high dN/dS in *A. aegypti*, a subset of short testis-biased genes appear to have evolved rapidly.

As a further analysis, we examined dN/dS and Fop in the most highly expressed testis-biased, ovary-biased, and unbiased genes (≥1000 FPKM). An extended description of the findings is provided in Supplemental Note 3 in File S1. In summary, we found that within the relatively small subset of sex-biased CDS that were extremely highly transcribed in this taxon ($N = 220$, or 2.34% of 9389 genes, Table 2), testis-biased and ovary-biased genes exhibited higher dN/dS and lower Fop as compared to unbiased genes (Table 2; note that dS varied among these gene sets and is discussed in Supplemental Note 4 in File S1). We conducted functional annotation targeted to the extremely highly expressed CDS using the gene ontology (GO) tool DAVID (database for annotation, visualization, and integrated discovery) (Huang da *et al.* 2009), which revealed that these testis- and ovary-biased CDS were highly represented by ribosomal protein genes (Table S4 in File S1). Accordingly, we propose that ribosomal protein genes, in addition to their core role as an essential component of the ribosome needed for translation, may also play a role in sexual differentiation in the sexual organs in *A. aegypti*. This notion is consistent with findings that ribosomal protein gene copies exhibit differential expression across tissues in plants, yeast, and certain animals, and suggestions that they are involved in tissue-specific gene regulation (Uechi *et al.* 2002; Komili *et al.* 2007; Whittle and Krochko 2009).

Taken together, the data show that fold bias, CDS length, and expression level are each relevant factors in the evolution

Table 2 Mean dN/dS, dN, dS, and Fop for the small subset of the most extremely high expressed (≥ 1000 FPKM) testis-biased and ovary-biased CDS in *Aedes*

Parameter	Testis-biased short	Ovary-biased short	Testis-biased long	Ovary-biased long	Unbiased short	Unbiased long
dN/dS	0.098 (0.023)a	0.061 (0.007)b	0.118 (0.014)a	0.083 (0.016)b	0.034 (0.016)[a]	0.083 (0.030)
dN	0.065 (0.017)a	0.040 (0.008)b	0.084 (0.013)a	0.053 (0.017)b	0.016 (0.009)[a]	0.021 (0.006)
dS	0.577 (0.116)a	0.566 (0.036)a	0.631 (0.049)a	0.503 (0.078)b	0.285 (0.034)[a]	0.279 (0.040)
Fop	0.499 (0.027)a	0.507 (0.011)a	0.448 (0.0169)a	0.507 (0.020)b	0.617 (0.014)[a]	0.621 (0.011)
N	21	107	49	17	23	3

Standard errors are in parenthesis. Different letters for each parameter within each length class (a versus b) indicate a statistically significant difference using randomization tests ($P < 0.05$). As unbiased long CDS had $N = 3$, the parameter values were included for completeness, but were not used in randomization tests. dN, nonsynonymous substitution rate; dS, synonymous substitution rate; Fop, frequency of optimal codons.

[a] The unbiased short CDS exhibit a statistically significant difference from testis- and ovary-biased CDS (short and long), $P < 0.05$.

of ovary-biased and testis-biased genes in *A. aegypti*, and that multiple approaches and analyses show a consistent and predominant effect of high dN/dS in ovary-biased genes.

Ovary and testis expression relative to embryos and dN/dS

To better understand the effects of sex-biased expression on molecular evolution, we sought to assess whether effects of testis or ovary expression on dN/dS and Fop may be influenced by coexpression (or lack thereof) in a nonsexual somatic structure, namely the embryos [noting that precursors to the germ cells are a minor part (<2%) of the population of cells therein] or by having expression specifically restricted to the ovaries or testes. Embryos exhibit a highly diverse transcriptome, expressing a majority of the genes in the genome across all embryonic stages, and are comprised of an array of cell types and tissues (*e.g.*, Diez-Roux *et al.* 2011; Mittmann and Wolff 2012; Combs and Eisen 2013; Schwager *et al.* 2015; Donoughe and Extavour 2016). Therefore, we considered that mixed-stage embryos (8–12 hr) would provide a suitable reference as a somatic nonsexual tissue for assessing genes with gonad-specific expression and gonad-somatic coexpression. We note that expression breadth is believed to influence molecular evolution, where genes expressed more broadly across tissues/structures are thought to be more pleiotrophic and constrained, and thus to evolve more slowly, than those that are tissue-specific (Mank and Ellegren 2009; Assis *et al.* 2012; Lipinska *et al.* 2015).

The Venn diagram in Figure 4A shows the number of the 9389 genes under study with specific or shared gene expression in the testis, ovaries, and embryos. We examined each of the seven nonoverlapping gene sets, including tissue-specific (testis-specific, ovary-specific, and embryo-specific), those expressed in two tissues (testis-ovaries, testis-embryos, and ovaries-embryos), and those expressed in all three tissues or none of the three tissues. For this, expression was defined as FPKM > 0 and nonexpression as FPKM $= 0$, or presence/absence of expression per tissue type. The results showed that, among all gene sets, dN/dS was highest for ovary-specific genes (Figure 4B). The fact that ovary-specific CDS had higher dN/dS than both testis-specific and embryo-specific CDS is consistent with accelerated evolution of genes from the female gonad (MWU-tests $P < 0.001$; Figure 4B), supporting our conclusions that ovary expression is typically connected to accelerated evolution in *Aedes*. The CDS lengths were shortest for ovary-specific CDS among all three categories (ovary-, testis-, and embryo-specific CDS lengths were 335.3 ± 32.3, 419.8 ± 32.4, and 449.9 ± 18.6 codons, respectively). As shorter CDS may be expected to be more conserved at the molecular evolutionary level (Akashi 2003; Lemos *et al.* 2005), CDS length cannot explain the faster evolution of these ovary-biased genes in *Aedes*. Thus, specific expression in ovaries *per se* appears to enhance the rate of protein evolution.

The results in Figure 4B suggest that the reason unbiased genes evolve faster than testis-biased genes in *Aedes* (Figure 3) is due to ovarian expression in the unbiased gene set. For instance, among genes expressed in two of the three tissue types, dN/dS was higher for testis–ovary and ovary–embryo genes than for testis–embryo genes. From this, we infer that coexpression in ovaries accelerates evolution, regardless of the other tissue in which the gene is expressed (*i.e.*, testis or embryos, Figure 4B). Moreover, testis–ovary coexpressed genes exhibited higher dN/dS than either testis-specific genes or testis–embryo coexpressed genes. These findings suggest that testis expression does not accelerate divergence, but rather that the high dN/dS values in the ovary–testis coexpressed gene set result from ovary expression, not testis expression. Together, it may be inferred that the elevated dN/dS observed for unbiased genes herein (Figure 3) likely results from gene expression in the ovaries. In other words, the reason why unbiased genes have more elevated dN/dS values than testis-biased genes (Figure 3) is likely due to ovary expression *per se*. Notably, this conclusion concurs with the data shown in Figure 2, A and B, which show that greater testis bias is linked to lower ovary expression, while unbiased genes by definition show no difference in expression with respect to testis. Therefore, ovary expression, assuming a link to higher dN/dS (Figure 4B), would be more apt to accelerate divergence of unbiased genes than testis expression. It can be concluded that unbiased expression between the ovaries and testes does not imply that the genes are not affected by sexual expression, but rather that their evolution may in fact be accelerated from ovarian transcription in *Aedes*.

Ovary-specific CDS also evolved faster than those CDS not expressed in any of the three tissue types (Figure 4B). We

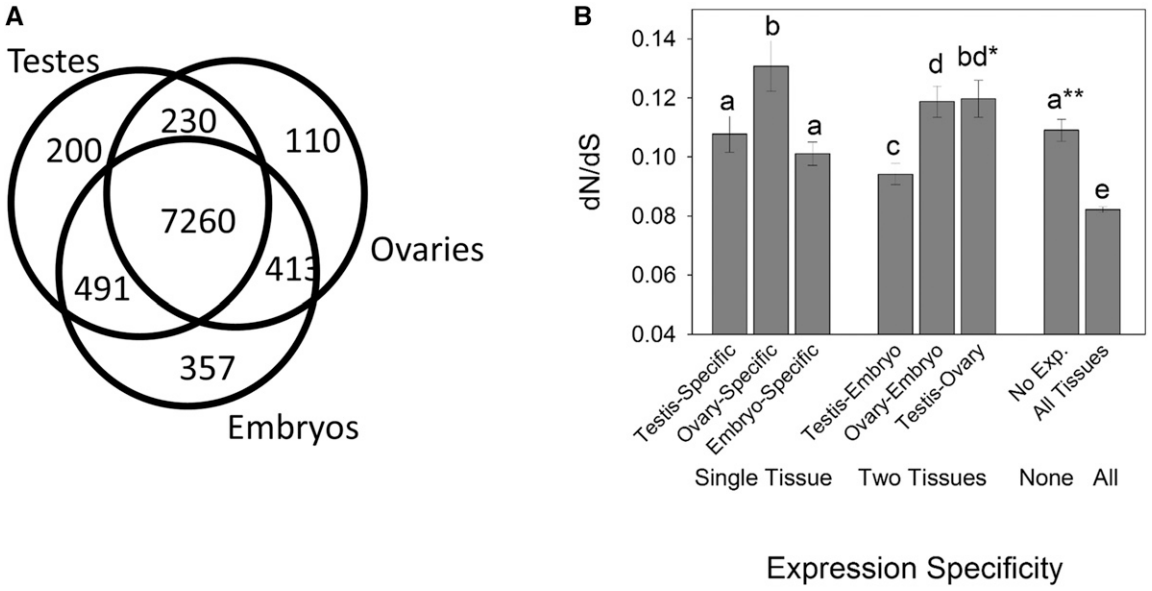

Figure 4 (A) The number of genes expressed in testes, ovaries, and embryos in *A. aegypti* among the 9389 genes under study (328 CDS had no expression). (B). The mean dN/dS of genes for tissue-specific (single tissue) and genes coexpressed (two tissue types or all three), and those not expressed in any tissues (none). Standard errors are shown in error bars. Different letters above any two bars indicate a statistically significant difference ($P < 0.05$) between those dN/dS values, using a paired Mann-Whitney U (MWU)-test (note that P was < 0.01 for all paired contrasts). *The ovary-specific and testis-ovary dN/dS did not differ across all genes per group (MWU-test $P > 0.05$), but the former had higher values using contrasts of values above the median ($P < 0.001$). No difference was observed between CDS coexpressed in ovary–embryo *vs.* testis–ovary as denoted by the same letter (d) in both categories. **No expression (No exp.) exhibited no difference in dN/dS as compared to testis-specific CDS, but was different from embryo-specific CDS. CDS, coding sequences; dN/dS, nonsynonymous to synonymous substitution rates.

would anticipate that genes not expressed in any tissues under study might be subject to reduced purifying selective forces (or relaxed selection) as compared to all of the other gene expression categories we consider here (Pal *et al.* 2001; Subramanian and Kumar 2004), particularly since embryos express genes from a large portion of the genome. Since ovary-specific genes in this mosquito evolve relatively rapidly, this finding suggests that the main mechanism of faster evolution of ovary-specific genes at least partly involves forces other than relaxed purifying selection (see below section: *Positive selection is more common in ovary- than testis- and embryo-specific genes*). Among all gene sets in Figure 4B, the slowest rates of evolution, or lowest dN/dS, were for those genes expressed in all three tissues (Figure 4B). Although the study of even more tissues is beyond the scope of our objectives here, and would be needed to fully conclude such a pleiotropic effect, we suggest that our results of embryonic gene expression analysis are consistent with enhanced pleiotropy and purifying selection in more broadly expressed genes (Mank and Ellegren 2009). For the findings on Fop for gene sets described in Figure 4, see Supplemental Note 5 in File S1.

Positive selection is more common in ovary- than testis- and embryo-specific genes

Given that positive selection is unlikely to be detected using whole CDS (dN/dS > 1) unless most sites in a CDS exhibit positive selection, we assessed whether sex-related genes exhibited signals of episodic positive selection at specific sites in *A. aegypti*. We assessed those genes with testis-, ovary-, and embryo-specific expression in Figure 4A, which are those genes most apt to indicate the effect of tissue type expression on events of positive selection. For this analysis, we studied trees of three for *A. aegypti*, *A. albopictus*, and their outgroup relative from the same family *C. quinquefasciatus* (Culicidae). This taxon was chosen due to availability of CDS data and its phylogenetic proximity to *Aedes* (Arensburger *et al.* 2010). We found that 63.6, 76.5, and 80.1% of the *Aedes* CDS list for ovary-specific, testis-specific, and embryo-specific, respectively, had putative orthologs in *C. quinquefasciatus*. The fact that we found fewer orthologs of genes belonging to the ovary-specific data set is consistent with the faster rates of evolution observed for these genes (Ellegren and Parsch 2007). Further, as *Culex* diverged from *Aedes* ~50 MYA (Arensburger *et al.* 2010), in those CDS with orthologs we observed substantial saturation for whole-branch dS (usually in the *Culex* branch) in some genes (*e.g.*, dS > 3, between 28.2 and 35.3% of each data set under study using a CODEML branch-model) (Yang 2007). Nevertheless, a majority of genes were not highly saturated for dS using an outgroup, and thus were suitable for analysis [note that, as described above, for the paired species within the *Aedes* genus, nearly all CDS (97%) had dS < 1.5 in our main analysis, but we permitted greater flexibility of dS < 3 when using the outgroup *Culex* in this present assessment].

For our assessment, we conducted branch site analysis using *A. aegypti*, our main target species under study that

was used in all sex-related expression analyses (Figure 4A), as the foreground branch. The results showed that the highest proportion of genes with statistically significant signals of positive selection [with $2 \times \Delta$ lnLikelihood χ^2 $P < 0.05$ (Yang 2007)] (see *Materials and Methods*) in the *A. aegypti* branch was for ovary-specific genes (23.7%), nearly double that of testis-specific (12.2%) and embryo-specific genes (11.9%). Nonetheless, we observed a nonnegligible level of positive selection in the latter two tissue types. A follow-up assessment using *A. albopictus* as the foreground branch also revealed greater incidences of positive selection in the ovary-specific gene set (28.9% of genes) than the testis- (19.1%) and embryo-specific (19.5%) gene sets (note that these gene sets showing signs of positive selection were largely nonoverlapping, and <6% per gene set had signs of positive selection in both taxa). While *A. albopictus* shows a greater tendency than *A. aegypti* for positive selection in all three tissue-specific data sets, as in *A. aegypti*, *A. albopictus* ovary-specific genes still exhibited more frequent instances of adaptive evolution than testis- or embryo-specific genes. Together, these results indicate a putative role of positive selection in the faster evolution of ovary-related genes in *Aedes* (Figure 3). Further, the results suggest that selective sweeps might contribute at least partly toward the inverse link between dN/dS and Fop (Figure 3).

Causes of fast evolution of ovary genes and GO

We used DAVID (Huang da *et al.* 2009) to conduct a targeted GO assessment of the ovary-specific and ovary–embryo coexpressed genes, as well as the testis-specific and testis–embryo coexpressed genes identified in Figure 4. These gene sets were chosen because we thought it was highly likely that they would be involved in gonad-specific functions, and would reflect the effect of gonadal expression on molecular evolution, such as that observed in Figure 4B. The results are provided in Table 3 and have been clustered by functionality, with the top three clusters shown per gene set. We found that the ovary-specific genes were strongly preferentially involved in olfactory functions, including odorant binding and odorant receptor activity (cluster 1 for that gene set, Table 3). Similarly, olfactory signaling genes were prevalent in the ovary–embryo coexpressed gene set (see cluster number 2 for that gene set), but were not a major functional cluster found for the testis-specific and testis–embryo coexpressed genes (Table 3). In *A. aegypti*, olfactory signals have been shown to be involved in male swarming and attraction of females, and thus are directly linked to mate recognition (Fawaz *et al.* 2014). Additionally, olfactory molecules have also been suggested to facilitate chemotaxis between sperm and oocytes within female reproductive structures to ensure fertilization in various organisms (Parmentier *et al.* 1992; Goto *et al.* 2001), including vector mosquitoes such as *A. aegypti* (Pitts *et al.* 2014). Further, after insemination, chemotaxis between sperm and female structures may direct the sperm into the spermathecae for their storage until fertilization (Degner and Harrington 2016). In fact, some authors have hypothesized that olfactory signaling may have evolutionarily originated from the reproductive system (Pitts *et al.* 2014) and later been co-opted for use in the antennae and other sensing systems. Our data suggest that such olfactory molecules are prevalent and rapidly evolving in ovary genes, raising the possibility that their rapid rates of evolution may be related to mating and fertilization processes. We speculate that such olfactory genes are absent as a top functional class in the testis-specific or testis–embryo coexpressed set because olfactory signals in female ovaries are continuously emitted, perhaps to maximize mating or fertilization opportunities, but may be restricted in male testis or sperm to brief periods directly linked to mating, and thus not observed for male gonads herein.

We propose that a hypothesis potentially explaining our findings is that sexual selection drives the rapid evolution of ovary-biased and ovary-specific genes. For instance, positive selection mediated by female–female competition acting to attract swarming males, or resulting from male mate choice among females (Oliva *et al.* 2014) [assuming some coexpression between sensilli and ovaries, as suggested in Foret and Maleszka (2006) and Matthews *et al.* (2016)], could cause fast evolution of ovary genes. Alternatively, it is possible that chemotaxis between ovaries and sperm is involved in attracting sperm movement into the reproductive tract after mating, which may promote the observed rapid evolution. For example, while the chemical activation underlying sperm movement into the spermathecae in mosquitoes (and other insects) is largely unknown, it has been suggested that secretions from one reproductive tissue might regulate sperm motility in another, such as the spermathecae (Degner and Harrington 2016). Thus, sperm movement could be driven, at least in part, by ovarian signals, and thus individuals containing ovaries with stronger olfactory reception or signaling may be more successful in compelling sperm to move into the spermathecae for storage (after mating), or triggering a high number of sperm to be released from the spermathecae for fertilization [a phenomenon that would be adaptive when sperm are plentiful (Degner and Harrington 2016)], thereby exerting an adaptive selective pressure on ovary-biased genes. In fact, evidence suggests that up to one-third of available sperm do not reach the spermatheceae after mating in *A. aegypti* [reviewed by Oliva *et al.* (2014)]. Putative signaling from ovary to sperm may be crucial for ensuring sperm storage, and thus supply, over a female's life span and reproductive success. Other speculative, yet conceivable, mechanisms include competition among eggs to attract incoming sperm, or male (sperm) choice among eggs [as numerous sperm may be released from the spermathecae for fertilization at a time; see Degner and Harrington 2016)], based on the strength or favorability of olfactory signaling. To determine the feasibility of these latter possibilities, it will be important for further studies to assess whether olfactory binders and receptors are observed not only in ovaries containing previtellogenic eggs (Raikhel and Dhadialla 1992), but also in the reproductive tract of mated females where fertilization

Table 3 Functional annotation of ovary-specific, testis-specific, ovary–embryo coexpressed, and testis–embryo coexpressed genes for *A. aegypti*

Gene type	Role	*P* value
Ovary-specific		
Cluster 1: enrichment score: 4.41	Odorant binding	6.50E−10
	Olfactory receptor activity	4.10E−05
	Plasma membrane	5.10E−05
	Olfaction	5.90E−05
	Olfactory receptor	6.20E−05
	Sensory transduction	1.00E−04
	Transducer	1.40E−03
	Receptor	7.60E−03
Cluster 2: enrichment score: 2.08	Integral component of membrane	5.00E−04
	Transmembrane helix	2.00E−02
	Transmembrane	2.00E−02
	Membrane	2.50E−02
Cluster 3: enrichment score: 1.24	Sensory perception of taste	1.90E−02
	7TM chemoreceptor	6.30E−02
	Cell membrane	1.60E−01
Testis-specific		
Cluster 1: enrichment score: 2.36	Leucine-rich repeat	3.60E−04
	LRR_TYP (Leucine-rich repeats, typical)	9.50E−03
	Leucine-rich repeat, typical subtype	2.40E−02
Cluster 2: enrichment score: 1.49	Ves allergen	1.60E−02
	SCP (SCP domain proteins)	3.10E−02
	Allergen V5/Tpx-1-related	4.60E−02
	CAP domain	4.60E−02
Cluster 3: enrichment score: 1.24	FBG (Fibrinogen-related domains)	4.40E−02
	Fibrinogen, α/β/γ chain, C-terminal globular domain	6.60E−02
	Fibrinogen, α/β/γ chain, C-terminal globular, subdomain 1	6.60E−02
Ovary–embryo coexpressed (absent testis)		
Cluster 1: enrichment score: 3.96	Integral component of membrane	2.90E−05
	Membrane	1.60E−04
	Transmembrane helix	1.70E−04
	Transmembrane	1.80E−04
Cluster 2: enrichment score: 2.37	Plasma membrane	5.10E−05
	Odorant binding	7.10E−05
	Olfactory receptor activity	1.10E−02
	Olfactory receptor	1.10E−02
	Olfaction	1.30E−02
	Transducer	1.60E−02
	Sensory transduction	2.10E−02
	Receptor	5.80E−02
Cluster 3: enrichment score: 1.96	Gustatory receptor protein	3.90E−03
	Gustatory receptor	1.80E−02
	Taste receptor activity	1.80E−02
Testis–embryo coexpressed (absent ovary)		
Cluster 1: enrichment score: 3.45	Secreted	7.90E−07
	Lipid metabolic process	1.30E−05
	Dol/Ves 1 allergen	5.50E−04
	Phosphatidylcholine 1-acylhydrolase activity	6.30E−04
	Lipase, N-terminal	7.30E−04
	Lipase	7.30E−04
	Carboxylic ester hydrolase activity	4.00E−01
Cluster 2: enrichment score: 2.69		
	Homeobox	9.00E−05
	Sequence-specific DNA binding	9.40E−05
	Homeodomain	1.70E−04
	HOX (Homeodomain and Homeodomain-like)	2.60E−04
	Homeobox, conserved site	3.30E−04
	Homeodomain, metazoa	1.20E−03
	DNA-binding	1.80E−03
	Homeodomain-like	3.60E−03
	Helix-turn-helix motif	5.30E−03

(continued)

Table 3, *continued*

Gene type	Role	*P* value
	Regulation of transcription, DNA-templated	4.10E−02
	Nucleus	4.60E−02
Cluster 3: enrichment score: 1.92	CAP domain	1.00E−02
	Allergen V5/Tpx-1-related	1.00E−02
	SCP (SCP domain proteins)	1.60E−02

The gene ontology was determined using the DAVID (database for annotation, visualization, and integrated discovery) system (Huang da *et al.* 2009), and the three clusters with the greatest enrichment score are shown per category. *P*-values are from a modified Fisher's test, wherein lower values indicate greater enrichment. The number of genes per category is provided in Figure 4A.

ultimately occurs, including the mature eggs and transferred sperm (Degner and Harrington 2016). Together, to distinguish between these possible scenarios, extensive further study will be needed to assess the causes of rapid evolution of ovary-expressed genes, including olfactory proteins and receptors, in *A. aegypti*.

Our finding that *A. aegypti* exhibits fast evolution of ovary-biased genes differs from most other models studied to date, including its Dipteran insect relative *Drosophila melanogaster*, which has been commonly shown to exhibit faster evolution of whole male-biased than female-biased genes, and testis-biased than ovary-biased genes (*e.g.*, Zhang *et al.* 2004, 2007; Proschel *et al.* 2006; Ellegren and Parsch 2007; Haerty *et al.* 2007; Meisel 2011; Assis *et al.* 2012; Parsch and Ellegren 2013; Perry *et al.* 2015). There are several factors that could underlie the observed differences. We note that, unlike *Drosophila*, ovary-biased expression in *A. aegypti* was caused by upregulation of genes in ovaries (Figure 2), whereas in *D. melanogaster*, greater ovary-biased expression (or preovary and pretestis depending on developmental stage) has been reported to result from downregulation in testes (Perry *et al.* 2015), suggesting that different mechanisms of gene regulation account for ovary-biased expression in the two insects. Further, the ovary gene sets in *A. aegypti* (Figure 4) include a high representation of olfactory signaling genes (Table 3), a finding not, to our knowledge, reported to be prevalent in *D. melanogaster*, and which might reflect a key difference in the biology of sperm attraction and storage between these two insect systems. We also note that unusually long sperm lengths and formation of sperm bundles in the female tract (which are thought to improve velocity) have been reported in *D. melanogaster*, features not believed to be inherent to *A. aegypti* [reviewed in Degner and Harrington (2016)]. Such sperm features might potentially reduce the need or effectiveness of ovarian or egg-based signaling in *D. melanogaster* to attract sperm to the spermatheceae, or the release of sperm from the spermatheceae for fertilization, and perhaps make *Drosophila* ovary-biased genes less likely to be subjected to adaptive evolutionary pressure than in *A. aegypti*. Based on these observations from our own work and that of others, we speculate that ovary-biased genes in *A. aegypti*, partly including olfactory signaling genes, might play an essential role in sperm attraction, storage, and fertilization, thus potentially exerting adaptive evolutionary pressures on mutations on these genes in the mosquito.

We propose that a secondary mechanism that may contribute toward the faster evolution of ovary-biased genes than testis-biased genes in *A. aegypti* is putatively reduced male–male sperm competition. In this mosquito, males ensure monogamy of females by transferring sufficient sperm to fertilize all eggs, and the accessory glands produce physical (and chemical) barriers in females that are normally formed and functional within 40 min after copulation (Oliva *et al.* 2014). This rapid postcopulation barrier to further mating could greatly limit sexual selection via male–male sperm competition (albeit not perfectly, given barriers are not established immediately Oliva *et al.* 2014), a feature differing from insects such as *Drosophila*, which exhibit strong intermale sperm competition after mating (Ellegren and Parsch 2007). In *D. melanogaster*, females mate with multiple males, and sperm from several males may become mixed in the female storage organs, resulting in strong sperm competition (Price *et al.* 1999). While various types of mating plugs form in *D. melanogaster*, they primarily ensure sperm are stored (and not lost from the female) in the storage organs, but do not prevent mating and sperm transfer from competing males (Avila *et al.* 2015). This differs from postmating barriers (initially physical barriers, followed by chemical barriers) that form after a first mating in *Aedes* females, which greatly limit or impede subsequent reinsemination by other males, and act to ensure female monogamy [reviewed by Oliva *et al.* (2014)]. As sperm competition between males is believed to contribute toward adaptive evolution of genes (Ellegren and Parsch 2007), this form of sexual selection might be highly limited or absent in *A. aegypti*, possibly decelerating the rate of evolution of testis-biased genes as compared to the ovaries in this insect.

Importantly, a prior report has shown that seminal fluid proteins with orthologs between *A. aegypti* and *A. albopictus* did not exhibit elevated dN/dS as compared to control housekeeping genes (Boes *et al.* 2014). In addition, no evidence of enhanced positive selection was observed across whole CDS or at specific codon sites in these *Aedes* species (Boes *et al.* 2014), findings in striking contrast to the typically fast and adaptive evolution of such genes observed in other organisms studied to date (Swanson and Vacquier 2002; Clark *et al.* 2006; Haerty *et al.* 2007). Together with our data (wherein testes samples included accessory glands; Akbari *et al.* 2013), this suggests that at least two male reproductive gene sets, namely testes and seminal fluid proteins, which typically

exhibit fast sequence evolution in studied metazoan systems, evolve slowly in species of *Aedes*.

We speculate that an additional factor that might contribute to fast evolution of ovary genes is infection. For instance, a bacterial infection of the germ lines (by *Wolbachia*), which has been shown to enhance female germ line fertility, has been linked to accelerated gene divergence in *D. melanogaster* (Flores *et al.* 2015). Thus, similar types of infections specifically affecting the ovaries, and fertility, is another potential avenue contributing to fast evolution of ovary-biased genes in *A. aegypti*. We note that the specific infection of *Wolbachia* might not naturally occur in or have this effect in *A. aegypti* (Segoli *et al.* 2014), but a similar symbiotic effect is plausible from other bacterial endosymbionts. Finally, we cannot exclude the possibility that ovary-biased CDS might be dispensable for reproductive success or fitness (Mank and Ellegren 2009), and thus some genes may exhibit relaxed selection, accelerating protein sequence evolution and reducing Fop. However, our finding of positive selection in ovary-biased genes suggests that this possibility cannot explain the findings. Therefore, we suggest that our findings are better explained by sexual selection, as described above. Further studies aimed toward addressing these possible causes may provide insights into why ovary-biased genes evolve fast in this mosquito.

Sex biases in whole males vs. females

As a final part of our assessment, we were interested in the connection between the gonads and somatic male and female sex biases in expression (Ellegren and Parsch 2007), and thus compared these two scales of sex-biased expression in *A. aegypti*. We assessed genes with ≥2-fold sex-biased expression from adult male and female carcasses, which excluded the sex organs (denoted hereafter as somatic-, or SOM-male and SOM-female, respectively) (Akbari *et al.* 2013), in *A. aegypti* (Table S1 in File S1). We found that SOM-male-biased genes ($N = 3743$) were more common than SOM-female-biased genes ($N = 774$), consistent with prior reports of sex-biased expression in the somatic tissues of this taxon (Akbari *et al.* 2013). Further, SOM-male-biased CDS had lower dN/dS than SOM-female-biased CDS and unbiased CDS ($N = 4872$; MWU-test $P < 0.001$, Figure S4A in File S1), and Fop was higher for the SOM-male- (than female-) biased genes (MWU-test $P < 0.001$; Figure S4B in File S1). Thus, these trends suggest that somatic female-biased genes exhibit faster evolution than somatic male-biased genes.

A total of 55.4% of the testis-biased gene set defined for our main analysis (Figure 3) were also SOM-male-biased ($N = 1621$ of 2927), while only 13.1% of ovary-biased genes were SOM-female-biased ($N = 264$ of 2013), indicating disparity in the gene sets exhibiting sex biases in the gonads as compared to the somatic tissues. Those genes that were both SOM-female- and ovary-biased had markedly higher median dN/dS as compared to those that were both SOM-male- and testis-biased (medians of 0.088 and 0.050, respectively, MWU-test $P < 0.007$, Figure S4C in File S1). However, importantly, after excluding genes with gonad-biased expression (including those only sex-biased in the soma), no difference in dN/dS profiles was observed between the SOM-female- ($N = 510$) and SOM-male-biased genes ($N = 2122$) (median = 0.069 and 0.068, respectively, MWU-test $P = 0.400$, Figure S4D in File S1). Collectively, the results suggest that the faster evolution of SOM-female- compared to SOM-male-biased genes (Figure S4, A and C in File S1) likely results from gene coexpression in the gonads (and gonad-biased expression, Figure S4D in File S1), rather than from sex-biased expression in the soma. Data from specific non-sexual organs and tissues in species of *Aedes* will help further ascertain whether or how the individual somatic structures contribute to whole-sex differences in expression and sequence divergence in *Aedes* (Matthews *et al.* 2016)

Together, these findings demonstrate that: (1) as ovary-biased genes were not necessarily SOM-female-biased ($N = 2013$ and 775, respectively), the sex-limited organs alone are linked to expression and sequence divergence in *Aedes*, in a manner that cannot be explained by biases in whole males *vs.* females; and (2) faster evolution of somatic female-biased genes than somatic male-biased genes (Figure S4A in File S1) may be explained by the presence of gonad-biased genes in each data set (Figure S4, C and D in File S1).

It is worth noting that a recent study in preprint for four species of *Anopheles*, a related mosquito from the same family as *Aedes* (Culicidae), has indicated that female-biased genes identified after blood feeding evolve faster than male-biased genes in that genus (Papa *et al.* 2016) [see also a recent study by of testis *vs.* male carcass genes (Cassone *et al.* 2017)]. Both female carcass- (excluding the gonads) and ovary-biased genes showed elevated dN/dS as compared to their male counterparts (Papa *et al.* 2016). Papa *et al.* (2016) hypothesized that blood feeding contributes to rapid evolution of female (carcass)-biased genes, as high dN/dS genes of genes upregulated in somatic carcasses of blood-fed females were preferentially involved in functions of salivary glands, digestion, and immunity. We do not consider blood feeding to be a primary candidate to explain our findings on expression (Figure 2), dN/dS, (Figure 3 and Figure 4), or GO functions (Table 3) in ovary genes in *A. aegypti*, as first, the females and thus ovaries were not blood fed herein, and second, ovaries are not directly involved in the somatic physical mechanisms of blood feeding. Nonetheless, as blood feeding is perhaps the most obvious biological novelty of vectors such as mosquitoes, an indirect role is possible, for instance, via coexpression of some genes in non-blood-fed and blood-fed ovaries. As described above, our findings point toward a primary role of selection during mating and fertilization, including a possible role of olfactory genes, in the fast evolution of ovary genes in *A. aegypti* (Figure 4 and Table 3), and are not consistent with a major role of blood feeding.

The findings from our present study, and those of Papa *et al.* (2016), suggest that rapid evolution of ovary-biased genes as compared to testis-biased genes might be a common feature

of the mosquitoes, spanning both blood-fed and nonblood-fed ovaries. Mating plugs have historically been thought to limit sperm competition in many metazoans, particularly mammals and insects (Parker 1970). As described above (see section: *Causes of fast evolution of ovary genes and GO*), the employment of mating plugs in *A. aegypti* promotes female monogamy and may greatly limit sperm competition in this mosquito (Oliva *et al.* 2014), a possibility that has also been noted for at least some species of *Anopheles* (Rogers *et al.* 2009; Oliva *et al.* 2014; Papa *et al.* 2016), and might contribute to slowed evolution of testis-biased genes in both genera. Additional research across more genera of mosquitoes will be essential to assess whether fast evolution of ovary-biased genes is a ubiquitous feature of this taxonomic group.

Conclusions

Here, we have provided an uncommon example in metazoans showing that ovary-biased genes evolve rapidly as compared to testis-biased genes in *A. aegypti*. We propose that the fast evolution of ovary-biased and ovary-specific genes, including those involved in olfactory functions, may partly result from sexual selection (positive selection) during courtship/mating and/or fertilization. In addition, we propose that higher dN/dS in ovary- than testis-biased genes may partly arise from the formation of post-mating barriers in the female reproductive tract, resulting in low male-male sperm competition. Further behavioral research in mating in this taxon, including male and female choice, and gene expression analysis in post-mating sperm and eggs in the female reproductive tract, including olfactory genes, will help further discern the factors contributing to the evolution of sex-biased gonad genes in this mosquito.

Materials and Methods

Expression in *A. aegypti* testes, ovaries, embryos (8–12 hr), and whole male and female carcasses was determined by mapping all reads to the list of 14,678 CDS under study from *A. aegypti* using Geneious 9 (www.Geneious.com: Kearse *et al.* 2012). Expression was determined as frequency per kilobase million (FPKM, or RPKM since all were single reads; Supplemental Material, Table S1 in File S1). These expression values were utilized for subsequent expression analyses.

Optimal codon usage

To determine optimal codon usage, we used embryo tissues and relative synonymous codon usage (ΔRSCU) values between the 5% least and most transcribed in the CDS list for *A. aegypti* across the full CDS list described in the *Results and Discussion*. For the correspondence analysis of codon usage, we used CodonW (Peden 1999) and the complete CDS list for *A. aegypti*. The correspondence analysis separates the codons into discrete classes on the principle axis based on the effective number of codons (ENC, Wright 1990) and predicts the list of putative optimal codons for a taxon (those linked to high expression), which should be followed-up with expression data analysis for confirmation, as conducted herein using ΔRSCU (Table 1) (Peden 1999). We note that for *A. aegypti*, a partial list of seven optimal codons had been suggested previously, where the list included codons ending in G, C, A, or T (Behura and Severson 2011). However, this comprises an atypical and small optimal codon list (Sharp *et al.* 1986; Stenico *et al.* 1994; Duret and Mouchiroud 1999; Cutter *et al.* 2006; Ingvarsson 2008; Whittle *et al.* 2011), and was obtained for different goals than herein. Thus, we conducted an assessment to identify optimal codons herein using gene expression data and correspondence analysis, yielding the list of 18 optimal codons in Table 1. Further details are provided Supplemental Note 1 in File S1.

Orthology and expression analysis

In our analysis of sex-biased genes, *A. aegypti* and *A. albopictus* orthologs were determined using reciprocal BLASTX (https://blast.ncbi.nlm.nih.gov). Only those CDS having a top match (with $e < 10^{-6}$) in both reciprocal searches between the two species were retained for further sex bias analysis for a total of 9489 complete CDS. Sex-biased CDS were identified as those with at least twofold difference in expression, with $P < 0.05$, and FPKM ≥ 1 in at least one of the compared tissues. *P*-values were determined using expression values normalized to the median in Geneious 9, which is based on the same method used in DeSeq (Anders and Huber 2010), and its two-sample expression contrast method employing the Binomial distribution (http://www.geneious.com/ Kearse *et al.* 2012). We presented results for all genes with $P < 0.05$ rather than corrected values as we had a large cutoff for definition as sex-biased [≥2-fold and $P < 0.05$; rather than 1.25- or 1.5-fold with 10% FDR used in other studies (Mank *et al.* 2007)], and we aimed to study all available genes exhibiting signals of sex-biased expression. Nonetheless, we repeated all our molecular evolutionary analysis of testis- and ovary-biased genes using only the gene sets identified after Bonferroni correction, which classified an additional 11.0% of the 9389 genes as unbiased (N = 5484 *vs.* N = 4449), resulting mostly from the weakest (<10 FPKM) expressed genes. This approach yielded nearly identical results for all tables and figures (data not shown). In the calculation of expression level of unbiased CDS (Table 2), we averaged the expression in testis and ovaries.

Alignments and dN/dS

To measure dN, dS, and dN/dS, orthologs for *A. aegypti* and *A. albopictus* were aligned by codons using MUSCLE (Edgar 2004) as implemented in MEGA (Kumar *et al.* 2012) and gaps removed. The dN and dS (and dN/dS) values were calculated using the Nei–Gojobori method (Jukes–Cantor) in MEGA (Kumar *et al.* 2012) with γ variation among sites. Values were also calculated using maximum likelihood and yn00 in PAML (Yang 2007), which includes codon bias as a parameter. Results in all tables and figures are for Nei–Gojobori (both methods yielded highly similar results), and PAML

findings are cited in the *Results and Discussion* and shown in Figure S2 in File S1.

Pairwise contrasts of dN, dS, dN/dS, or Fop using randomization tests (Ludbrook and Dudley 1998) were conducted as follows: for each comparison, genes from the two compared categories were pooled. The pooled data set was resampled with replacement 1000 times. Each sample was randomly divided into two bins of size N_1 and N_2, corresponding to the gene sample sizes of category one and two, respectively. For each of the 1000 samples, we determined ΔX = mean $X_{\text{Category 1}}$- mean $X_{\text{Category2}}$, where X = dN/dS, dN, dS, or Fop depending on the parameter under study. This gives the sampling distribution of differences in X under the null hypothesis. *P*-values were calculated as the fraction of samples in the distribution wherein ΔX exceeded the observed ΔX.

Intron analysis

Introns were extracted using the annotation files in Table S1 in File S1. Evidence suggests that short introns are nearly entirely selectively neutral while longer introns may be subjected to some selection, particularly in regulatory flanking regions (Farlow *et al.* 2012). Thus, we divided introns into short and long classes approximately divided by the median (130 bp), and removed the 50-bp ends from the long introns prior to analysis of GC content (Williford and Demuth 2012). All introns <20 bp were excluded from analysis.

Positive selection

For assessing episodic positive selection in *A. aegypti* for the tissue-specific genes in Figure 4, we used trees of three closely related species, *A. aegypti*, *A. albopictus*, and the outgroup species *Culex quinquefasciatus*, a species that represents a reasonably close mosquito relative (from the same subfamily as *Aedes*) with whole genomic sequence available for study (available at http://metazoa.ensembl.org/). Orthologous CDS in all three species were identified using reciprocal BLASTX (https://blast.ncbi.nlm.nih.gov) with CDS sequences of our main study organism *A. aegypti* as the query and *A. albopictus* (as described above) or *C. quinquefasciatus* CDS as the target CDS with a cutoff *e*-value of $e < 10^{-6}$. Alignments were conducted for CDS of the three species in MEGA as described above (in *Materials and Methods*) and gaps removed (aligned by codons). Prior to branch-site analysis, branch analysis was conducted using CODEML in PAML (Yang 2007) for the three taxa using the free-ratios model, and poor or very saturated alignments (due to distance of *Culex* to *Aedes*) measured by dS > 3 (*cf*. Lloyd *et al.* 2015) in the (typically longest) *Culex* branch were discarded. The proportion of CDS without orthologs or with high saturation is cited in the *Results and Discussion*. Subsequently, branch-site analysis was conducted using CODEML (Yang and Swanson 2002; Yang 2007), which allows dN/dS to vary among branches and sites. *A. aegypti* or *A. albopictus* was used as the foreground branch in an unrooted tree of the three species. Positive selection was inferred for all CDS with a statistically significant value for $2 \times \Delta$ln Likelihood using a χ^2-test ($P < 0.05$) (Yang and Swanson 2002; Yang 2007). The number of CDS under study for ovary-specific, testis-specific, and embryo-specific data sets is provided in Figure 4A.

Data availability

Sources for all data used for analysis are provided in Table S1 in File S1 and were downloaded between August and November 2015 (*A. aegypti*) and in March 2016 (*A. albopictus*). No custom scripts or tools were created to perform the present analysis.

Acknowledgments

We are grateful to the anonymous reviewers for valuable comments on the manuscript. This work was supported by funds from Harvard University and National Institutes of Health grant 5R01H D0-73499 to C.G.E.

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Communicating editor: B. P. Lazzaro