Correspondence

Female-biased gene expression in the malaria mosquito *Anopheles gambiae*

Matthew W. Hahn[1*] and Gregory C. Lanzaro[2]

Females of the malaria-carrying mosquito, *Anopheles gambiae*, must deal with a number of tasks never confronted by males, including blood-feeding and defense against *Plasmodium* infection. Here, we examine global gene expression differences between the sexes in *A. gambiae* via Affymetrix GeneChip® microarrays and find a dramatic over-representation of genes expressed more highly in females. Approximately 10% of the genome has greater than a fourfold difference in expression between males and females, with 71% of these differences being female-biased. In addition to measuring sex bias in gene expression, this is the first experiment to examine expression in the full *A. gambiae* genome. Through comparative genomic analysis with *Drosophila melanogaster*, we find that the functions of sex-biased genes are conserved across the Diptera, even if the same genes do not fill these roles. Unlike *D. melanogaster*, however, there is no evidence for a dearth of male-biased genes on the *A. gambiae* X chromosome.

Hybridization of cRNA from adult, unmated male and female mosquitoes revealed that 4,490 of 14,900 predicted genes had detectable levels of expression in either sex (see Supplemental Data published with this article online). In a comparison of male and female gene expression, we found 2,901 genes that were differentially expressed at $P < 0.05$. Bonferroni correction for the 4,490 tests carried out between males and females results in a set of 167 genes that are differentially expressed at a nominal $P < 0.05$ level (Figure 1). The false discovery rate in this set is extremely low (FDR < 0.00025), and thus we are highly confident that these genes are differentially expressed.

Recent work in *D. melanogaster* has shown that there are many differences in gene expression between the sexes [1–5], and that these dissimilarities are due to differences in both somatic and germline cells [5]. Of the transcripts detected, 17% were differentially expressed at a twofold level between the sexes in *D. melanogaster* [5]; at this level, we found 29% of the genes to differ between males and females (Figure 1). Parisi *et al.* [5] found that there was more male-biased expression among genes in *D. melanogaster*. We found the opposite to be the case in *A. gambiae* — in our high-confidence set of genes, 118 of 167 genes (71%) are expressed more highly in females. This bias appears to get stronger as the difference between the sexes gets larger: in the top 2,000 most-biased genes (by *p*-value), 55% are female-biased; in the top 1,000, 59%; and in the top 500, 63%. The larger difference in sex-biased gene expression in mosquitoes relative to *Drosophila* likely reflects larger differences between the sexes in behavioral (e.g., blood-feeding) and immune (e.g., response to *Plasmodium*) traits in *Anopheles.*

Only 22 of the genes in our high-confidence set were previously annotated. We were able to assign possible functions to 122 of the 167 high-confidence genes by similarity search and found *D. melanogaster* homologs for 96 of them. Four of the *A. gambiae* genes found to be sex-biased in their expression have homologs in *D. melanogaster* that are also sex-biased [5]. Three of the genes are female-biased in both species and one is male-biased in both species (see Supplemental Data).

Though it is hard to draw quantitative conclusions from such a small group of annotated proteins, there are a number of patterns that stand out. A substantial fraction of the female-biased genes (22 of 122) are involved in antigen-related defense, blood-feeding, or are expressed solely in salivary glands [6,7]. Furthermore, many of the biological processes assigned to genes found to be female-biased in *D. melanogaster* [5] are also found in *A. gambiae*: genes encoding proteins involved in ribosomal function, translation initiation, DNA replication and RNA binding, and genes expressed in the ovary. Many of these genes are probably expressed in order to produce eggs in female mosquitoes. While female-biased genes in *A. gambiae* seem to both complement and extend the transcriptionally active set found in female *D. melanogaster*, those genes that are the most male-biased fall into expected categories involved in germline functions [5]. We found that males expressed an abundance of mitochondrial genes, genes encoding protein transport components and heat-shock proteins, and sperm-specific genes, similar to male-biased genes in *D. melanogaster*.

Previous work revealed a significant paucity of genes with male-biased gene expression on the *Drosophila* X chromosome [3], suggesting a role for natural selection in the location of sex-biased genes. Unlike in *D. melanogaster*, however, we found no deficit of male-biased genes on the X chromosome (at any *p*-value cut-off), or indeed any non-random distribution of sex-biased genes on chromosomes; the excess of genes showing high relative expression in females is evenly distributed throughout the mosquito genome (see Supplemental Data).

There also does not appear to be any bias in the movement of genes onto or off of the X chromosome: 53% of all orthologs between *A. gambiae* and *D. melanogaster* remain on the X chromosome after approximately 250 million years [8]. In our dataset three of the five sex-biased genes on the X chromosome in *A. gambiae* that have *D. melanogaster* orthologs

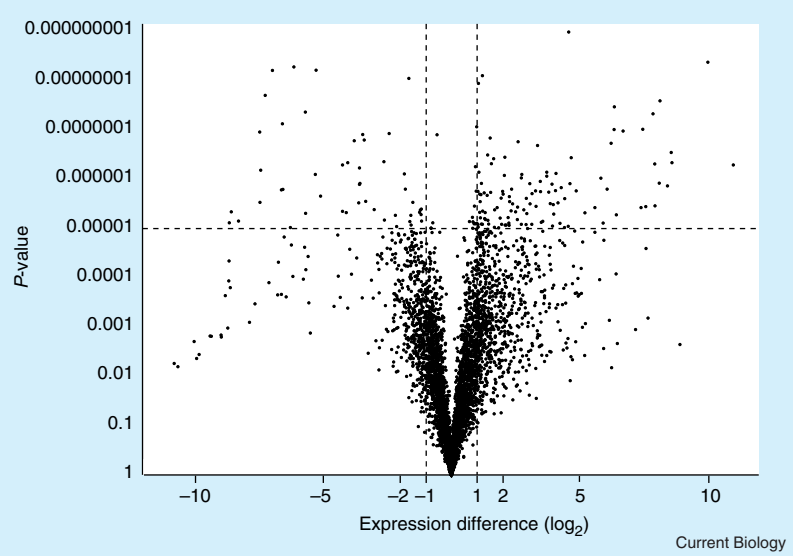

Figure 1. Volcano plots of significance against relative expression differences for the male vs. female comparison.

Each dot represents one of the 4,490 genes that had detectable expression in either sex. The *y* axis is the *P*-value associated with a t-test for differences between the sexes; the horizontal dashed line represents the Bonferroni-corrected threshold for significance, P=0.00001. The *x* axis shows the $\log_2$-transformed difference in expression between male and female mosquitoes; positive values are associated with those genes that are expressed at a higher relative level in females. The vertical dashed lines represent twofold differences in expression.

remain on the X chromosome in *D. melanogaster*. Neither do we find any deficit in apparent sequence conservation of sex-biased genes in *A. gambiae*, as was reported for *D. melanogaster* [3]. Approximately 60% of all mosquito genes have one-to-one orthologs in *D. melanogaster* [8], while we found orthologs for 57% (96/167) of the genes in our high-confidence set (chi-squared = 0.16, P = 0.69).

Although only four of the sex-biased genes in mosquitoes appear to have sex-biased orthologs in *Drosophila*, our comparison shows that the roles played by sex-biased genes — especially in germline function — appear to be conserved. Unlike in *Drosophila* [3], we did not find any evidence for the partitioning of sex-biased genes among chromosomes in *Anopheles*, such as avoidance of the X chromosome by male-biased genes. Various hypotheses have been put forth for both the appeal and the abhorrence of the X chromosome for male-biased gene expression [9,10], but it is unclear why any mechanism should predominate in one or the other species considered here. Examining sex-biased gene expression in another dipteran such as the mosquito *Aedes aegyptii* — where there are no heteromorphic sex chromosomes — may shed light on the fly- or mosquito-specific forces responsible for the observed disparities in sexually dimorphic transcription.

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Supplemental data

Supplemental data including additional discussion, a table of the differentially expressed genes, and experimental procedures are available at http://www.current-biology.com/cgi/content/full/15/6/R192/DC1/

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Supplemental Data: Female-biased gene expression in the malaria mosquito *Anopheles gambiae*

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All mosquitoes came from the F19 generation of Lanzaro Lab stock MA, a non-*Plasmodium* infected stock started from 16 wild-collected, gravid females from N'Gabacoro Droit, Mali. 80 virgin females and 60 virgin males were collected ~36 hr post-eclosion. Adults of both sexes were fed only sugar solution. Groups of 20 mosquitoes of each sex were separately frozen in liquid nitrogen, ground to a fine powder, and then 1 ml of TRIzol was added. After homogenization and wash, the RNA was extracted using the RNeasy Mini Kit (Qiagen). One cycle cDNA synthesis, cDNA cleanup, and biotin labeling was carried out with kits from Affymetrix, according to the manufacturer's instructions. Labeled cRNA from each of the seven samples (3 male, 4 female) was then hybridized separately to Affymetrix GeneChip® *Plasmodium*/*Anopheles* Genome Arrays and scanned at the UC Davis School of Medicine Microarray Core Facility.

Hybridization data were analyzed using the program ArrayAssist (Stratagene). Data were normalized using the GC-RMA method (Z. Wu, R. Irizarry, R. Gentleman, F. M. Murillo, and F. Spencer; http://www.bepress.com/jhubiostat/paper1), and all probesets with mean intensities of less than 50 across chips were removed. Of the remaining probesets, 4,490 were from the *A. gambiae* genome, while only 5 were from the *P. falciparum* genome (the *Plasmodium*/*Anopheles* array contains approximately 4,510 probesets from the *Plasmodium* genome, but none are expected to hybridize as the mosquitoes are not infected; the five probesets detected are likely to be false positives). Similar experiments in *D. melanogaster* using Affymetrix microarrays found 7,679 out of 13,996 genes with detectable expression [S1]. The difference between these studies in the number of genes expressed is likely due to the larger number of genotypes (10) used in the *D. melanogaster* study compared with the single line used here, and to the fact that the *A. gambiae* gene predictions are less well supported than in *D. melanogaster*, leading to many 'genes' on the array that do not have detectable expression. Using these 4,490 genes, we conducted *t*-tests with unequal variance for a comparison of male and female probesets. Bonferroni correction for 4,490 tests suggests using a *P*-value of 0.000011 for a nominal 0.05 false positive rate; we rounded this down to 0.000010 for convenience and found that 167 genes were significant at this level of significance (Table S1).

Surprisingly, given that the mosquitoes in our experiment were never challenged with *P. falciparum*, a number of genes implicated in defense against the parasite were present at detectable levels. *CTL4* and *CTLMA2*, C-type lectins involved in *Plasmodium* development in the mosquito midgut [S2], were both present in males and females and appeared to each be slightly female-biased (P=0.0098 and P=0.087, respectively). This slight bias toward females suggests that expression is not a generalized response to bacterial or fungal infection in both sexes. Because it was previously believed that genes of the mosquito innate immune system were activated only upon invasion by *Plasmodium* [S2,S3], and because mounting an immune response is believed to be costly [S4], further work into possible pleiotropic roles for these genes should be undertaken. Additionally, we found that very few of the odorant-binding proteins implicated in host-seeking [S4] were expressed in adult mosquitoes. Because female mosquitoes do not generally seek human hosts until after mating [S5], it may be that these proteins are not expressed until after mating. Future comparisons of mosquitoes that feed on human and non-human hosts must bear in mind this delayed expression, or extremely low level of expression, when looking for the differences that affect host-seeking behavior.

We used Ensembl (http://www.ensembl.org) to assign probable function to the 167 genes in our high-confidence set via Interpro or Pfam annotation, and to find homologs in *D. melanogaster* by best reciprocal blast hit or by synteny around a best reciprocal hit. We were able to assign function to 122 genes, and were able to find apparent orthologs for 96 genes. Of this small number of annotated genes, most are female-biased and female-mosquito-specific, including genes encoding proteins involved in blood-feeding (D7s, apyrase [S6,S7]) and the immune response (serine proteases [S8]), and genes found exclusively in the salivary glands (GSGs [S9]). Four *A. gambiae* genes with *D. melanogaster* orthologs were also among the sex-biased genes in the dataset of Parisi *et al.* [S10]. Three of the genes are female-biased in both species (*Anopheles*/*Drosophila* gene IDs: ENSANGG00000016924/*nop5*, ENSANGG00000009193/*CycA*, and ENSANGG00000015589/CG7338) and one is male-biased in both species (ENSANGG00000015789/CG8086). A caveat to this comparison is that there may have been other *A. gambiae* homologs in *D. melanogaster* that were sex-biased, but that were below the statistical threshold used by Parisi *et al*. [S10] and thus were not reported. Alternatively, the fruitfly homologs may be sex-biased in their expression, but not

Table 1. Highly significant sex-biased genes.

Affy Gene ID	F/M	Affy Gene ID	F/M	Affy Gene ID	F/M	Affy Gene ID	F/M
Ag.2L.1014.0	–5.686	Ag.2R.273.0	2.07	Ag.3L.904.0	–2.19	Ag.X.106.0	0.829
Ag.2L.1432.0	–7.444	Ag.2R.274.0	–0.723	Ag.3R.10.0	0.822	Ag.X.13.0	7.475
Ag.2L.151.2	1.33	Ag.2R.277.0	1.078	Ag.3R.1218.0	–1.579	Ag.X.179.0	2.92
Ag.2L.156.1	–2.635	Ag.2R.279.0	1.104	Ag.3R.1316.0	–4.253	Ag.X.257.0	1.177
Ag.2L.158.0	0.988	Ag.2R.284.2	–0.562	Ag.3R.1364.0	–2.141	Ag.X.341.1	–6.639
Ag.2L.163.0	1.054	Ag.2R.286.0	1.002	Ag.3R.15.0	10.01	Ag.X.69.0	1.04
Ag.2L.164.0	3.999	Ag.2R.31.0	4.633	Ag.3R.1518.0	–7.471	Ag.X.7.0	–1.209
Ag.2L.180.0	3.804	Ag.2R.318.0	2.509	Ag.3R.155.0	0.843	Ag.X.879.0	–4.105
Ag.2L.2.0	1.21	Ag.2R.3223.0	3.285	Ag.3R.165.0	0.886	Ag.UNKN.1934.0	–3.57
Ag.2L.21.0	4.874	Ag.2R.334.0	8.434	Ag.3R.172.0	1.133	Ag.UNKN.212.0	3.073
Ag.2L.229.0	3.364	Ag.2R.346.1	3.304	Ag.3R.177.0	0.895	Ag.UNKN.89.0	3.566
Ag.2L.241.0	5.925	Ag.2R.348.0	–0.796	Ag.3R.1834.0	4.236		
Ag.2L.250.0	1.9	Ag.2R.36.0	7.404	Ag.3R.189.0	–1.796		
Ag.2L.260.0	2.277	Ag.2R.3635.0	1.175	Ag.3R.19.0	11.011		
Ag.2L.2621.1	–5.696	Ag.2R.3716.0	–3.792	Ag.3R.191.0	2.925		
Ag.2L.2856.0	–1.179	Ag.2R.3744.0	–1.159	Ag.3R.194.0	1.155		
Ag.2L.297.0	1.452	Ag.2R.3772.0	–7.27	Ag.3R.1947.0	–3.585		
Ag.2L.299.0	3.72	Ag.2R.381.0	1.558	Ag.3R.198.0	7.938		
Ag.2L.304.0	4.682	Ag.2R.462.0	2.089	Ag.3R.20.0	4.561		
Ag.2L.307.0	5.21	Ag.2R.472.0	–1.576	Ag.3R.2018.0	0.953		
Ag.2L.3169.0	–6.284	Ag.2R.477.0	2.589	Ag.3R.204.0	–1.169		
Ag.2L.35.0	7.588	Ag.2R.556.0	3.864	Ag.3R.220.0	8.616		
Ag.2L.357.0	–3.586	Ag.2R.567.0	1.401	Ag.3R.238.0	4.089		
Ag.2L.36.0	8.144	Ag.2R.568.0	0.702	Ag.3R.2444.1	–6.577		
Ag.2L.364.0	2.264	Ag.2R.659.0	–1.835	Ag.3R.2548.0	3.064		
Ag.2L.4.0	0.939	Ag.2R.69.0	–2.431	Ag.3R.2624.0	8.587		
Ag.2L.406.0	1.687	Ag.2R.719.1	3.932	Ag.3R.292.0	2.754		
Ag.2L.586.1	3.479	Ag.2R.737.2	3.975	Ag.3R.307.0	1.034		
Ag.2L.627.0	2.735	Ag.2R.859.0	1.843	Ag.3R.326.0	3.951		
Ag.2L.630.0	2.571	Ag.2R.86.0	–3.349	Ag.3R.336.0	3.154		
Ag.2L.641.1	1.791	Ag.2R.888.0	2.327	Ag.3R.347.0	–3.466		
Ag.2L.664.0	–1.659	Ag.3L.1.0	1.044	Ag.3R.351.0	3.508		
Ag.2L.689.0	8.12	Ag.3L.112.0	2.022	Ag.3R.3557.0	–0.794		
Ag.2L.723.0	–5.282	Ag.3L.114.0	1.514	Ag.3R.360.0	1.212		
Ag.2L.806.0	6.233	Ag.3L.117.2	1.102	Ag.3R.371.0	1.958		
Ag.2L.835.2	2.136	Ag.3L.121.0	1.652	Ag.3R.38.0	7.874		
Ag.2L.885.0	–8.308	Ag.3L.133.0	4.423	Ag.3R.388.0	2.0		
Ag.2L.9.0	0.897	Ag.3L.1336.0	–3.014	Ag.3R.438.0	2.948		
Ag.2R.1148.1	3.728	Ag.3L.134.0	1.303	Ag.3R.474.1	2.24		
Ag.2R.1272.0	1.942	Ag.3L.137.0	6.365	Ag.3R.491.0	2.828		
Ag.2R.13.0	6.44	Ag.3L.17.0	5.604	Ag.3R.5.0	6.706		
Ag.2R.1757.0	–6.146	Ag.3L.173.0	1.428	Ag.3R.50.0	6.35		
Ag.2R.1859.0	–6.594	Ag.3L.2127.0	–1.503	Ag.3R.622.0	–1.621		
Ag.2R.1883.0	–2.612	Ag.3L.213.0	1.218	Ag.3R.641.0	–8.672		
Ag.2R.2137.0	–5.316	Ag.3L.218.0	2.733	Ag.3R.652.0	3.141		
Ag.2R.2234.0	–4.049	Ag.3L.2212.0	2.259	Ag.3R.705.0	–5.105		
Ag.2R.263.0	2.218	Ag.3L.240.0	2.387	Ag.3R.715.0	–7.483		
Ag.2R.264.106	1.281	Ag.3L.251.0	2.35	Ag.3R.825.0	–8.589		
Ag.2R.265.0	1.318	Ag.3L.329.1	2.103	Ag.3R.84.0	7.952		
Ag.2R.266.0	1.42	Ag.3L.638.0	4.291	Ag.3R.855.0	4.164		
Ag.2R.267.0	1.125	Ag.3L.720.0	–6.984	Ag.3R.992.2	–1.245		
Ag.2R.271.12	3.476	Ag.3L.786.0	1.53	Ag.X.10.0	5.836		

Gene names according to Affymetrix IDs are shown in order of their chromosomal locations. Female/Male expression is given in $\log_2$ (male-biased genes are therefore negative). Only the top 167 most biased genes ($P < 1E–5$) are shown.

represented on the array used by these authors.

To test for under-representation of male-biased genes on the X chromosome [S11], we compared the number of male-biased genes on this chromosome to the number of male-biased genes across the genome. Because 6.8% of all expressed genes in our experiment are found on the X chromosome, we expect this percentage of all male-biased genes on the X chromosome given no under-representation. Considering all the male-biased genes in our dataset, we found 132 on the X chromosome (143 expected; chi-squared = 0.85, $P = 0.36$). Considering only male-biased genes different from

females at $P < 0.05$, we found 78 on the X chromosome (89 expected; chi-squared = 1.36, $P = 0.24$). And considering only male-biased genes in our high-confidence set ($P < 1E–5$), we found 3 on the X chromosome (3 expected; chi-squared = 0, $P = 1$). There is therefore no evidence of a non-random distribution of sex-biased genes on any chromosome (Table S1).

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