OXFORD GENETICS

GENETICS, 2023, 224(1), iyad034
https://doi.org/10.1093/genetics/iyad034
Advance Access Publication Date: 3 March 2023
Investigation

Selection and geography shape male reproductive tract transcriptomes in *Drosophila melanogaster*

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Abstract

Transcriptome analysis of several animal clades suggests that male reproductive tract gene expression evolves quickly. However, the factors influencing the abundance and distribution of within-species variation, the ultimate source of interspecific divergence, are poorly known. *Drosophila melanogaster*, an ancestrally African species that has recently spread throughout the world and colonized the Americas in the last roughly 100 years, exhibits phenotypic and genetic latitudinal clines on multiple continents, consistent with a role for spatially varying selection in shaping its biology. Nevertheless, geographic expression variation in the Americas is poorly described, as is its relationship to African expression variation. Here, we investigate these issues through the analysis of two male reproductive tissue transcriptomes [testis and accessory gland (AG)] in samples from Maine (USA), Panama, and Zambia. We find dramatic differences between these tissues in differential expression between Maine and Panama, with the accessory glands exhibiting abundant expression differentiation and the testis exhibiting very little. Latitudinal expression differentiation appears to be influenced by the selection of Panama expression phenotypes. While the testis shows little latitudinal expression differentiation, it exhibits much greater differentiation than the accessory gland in Zambia vs American population comparisons. Expression differentiation for both tissues is non-randomly distributed across the genome on a chromosome arm scale. Interspecific expression divergence between *D. melanogaster* and *D. simulans* is discordant with rates of differentiation between *D. melanogaster* populations. Strongly heterogeneous expression differentiation across tissues and timescales suggests a complex evolutionary process involving major temporal changes in the way selection influences expression evolution in these organs.

Keywords: testis, accessory gland, expression, evolution, population, cline, Africa

Introduction

Inferring the importance of various genetic and population phenomena responsible for the maintenance of intraspecific genetic and phenotypic variation is the foundational problem of population genetics. Of the several possible selective mechanisms for the preservation of such variation, spatially varying selection has probably received the most attention, likely because consistent geographical patterns of phenotypic and genetic variation, such as their correlations with latitude or altitude, have been observed in a wide variety of organisms (e.g. Haldane 1948; Endler 1977). The genus *Drosophila* has been a central model system in this context, as several species and traits exhibit clines (e.g. Dobzhansky 1944; Mettler *et al.* 1977; Singh *et al.* 1992; Gilchrist *et al.* 2004; Levitan and Etges 2005; Arthur *et al.* 2008; Allen *et al.* 2017). Moreover, the evidence that selection is a dominant force patterning genomic variation in *Drosophila* generally (Begun and Aquadro 1992; Charlesworth *et al.* 1993; Andolfatto 2005; Halligan and Keightley 2006; Begun, Holloway, *et al.* 2007; Begun, Lindfors, *et al.* 2007; Sella *et al.* 2009; Langley *et al.* 2012) suggests that spatially varying selection likely contributes substantially to clines (e.g. Kolaczkowski *et al.* 2011; Fabian *et al.* 2012; Reinhardt *et al.* 2014). *D. melanogaster* latitudinal clines are among the most comprehensively described in the genus. This ancestrally African species has recently spread as a human commensal across Eurasia, the Americas, and Australia (David and Capy 1988): latitudinal clines have been observed in each region (Imasheva *et al.* 1994; Calboli *et al.* 2003; Hoffmann and Weeks 2007; Adrion *et al.* 2015; Fabian *et al.* 2015). While the investigation of continental clines in the Americas and Australia has received much attention, the possible role of selection in shaping phenotypic traits during the initial intercontinental colonization of non-African regions has received less.

The possible role of geographic gene expression differentiation in local adaptation has recently been investigated in several animal and plant taxa (e.g. Fraser 2013; Morris *et al.* 2014; Svetec *et al.* 2015; Juneja *et al.* 2016; Allen *et al.* 2017; Mack *et al.* 2018; Rivas *et al.* 2018; Huang *et al.* 2019; Ravindran *et al.* 2019; Jacobs *et al.* 2020; Blanc *et al.* 2021). In previous work (Zhao *et al.* 2015), we investigated latitudinal gene expression differentiation in North American populations of *D. melanogaster* and its sibling species, *D. simulans*, using whole-male transcriptome data from populations sampled from Panama City (Panama) and Fairfield (Maine, USA). That study revealed extensive latitudinal expression differentiation for both species and significant interspecific parallelism, which is difficult to explain by a mechanism other

Received: January 25, 2023. **Accepted:** February 20, 2023

than natural selection. However, although *Drosophila* reproductive tissues are often fast-evolving (e.g. Meiklejohn *et al.* 2003; Whittle and Extavour 2019), we observed no enrichment of testis-biased or testis-specific genes among the differentially expressed genes. Because many genes expressed in a male-biased or male-specific manner are testis-biased or testis-specific (Parisi *et al.* 2003), we interpreted this finding as supporting the idea that much of the observed whole-male expression differentiation was driven primarily by expression variation in somatic tissues. Nevertheless, that inference was indirect and not particularly biologically informative in that it provided no information on the tissues responsible for whole-male expression differentiation. Moreover, whole animal transcriptome data almost certainly lead to underestimates of the number of differentially expressed genes at the tissue or organ level (Chintapalli *et al.* 2007) and provide little insight into possible differences of the relative importance of selection acting on expression phenotypes in different tissues. Finally, most work on patterns of "Out-of-Africa (OOA)" phenotypic variation has focused on transcriptome comparisons of African and European populations (Hutter *et al.* 2008; Müller *et al.* 2011; Catalan *et al.* 2012; Huylmans and Parsch 2014), leaving the relationship of African and American gene expression variation unclear.

Here, we begin to address some of these gaps in our understanding through an analysis of transcriptome differentiation in two male reproductive tissues, one primarily germline, the testis, and one somatic, the accessory gland (AG), which produces seminal fluid proteins (Sfps), which are transferred to females along with sperm during mating and are essential for fertilization, similar to the seminal fluid of the mammalian prostate (reviewed in Poiani 2006; Wilson *et al.* 2017). We use populations from Maine, USA and Panama City (Panama) from the well-studied American cline, and a Zambia (Siavonga) population of *D. melanogaster*, which among sampled African populations seems to be to the closest approximation to an ancestral-like African population of this species (Pool *et al.* 2012). Finally, we contrast patterns of geographic expression differentiation in these two tissues to longer-term patterns of expression divergence between *D. melanogaster* and its sibling species, *D. simulans*, to extend our investigation of rate heterogeneity of transcriptome divergence on different timescales.

Methods

Flies, tissues, and sequencing

D. melanogaster males were sampled from isofemale lines established from inseminated females collected from Fairfield, Maine (2011 September), Panama City, Panama (2012 January) (Zhao *et al.* 2015), and Siavonga, Zambia (2010 July) (Pool *et al.* 2012). Stocks were maintained on standard yeast-cornmeal-agar food at 25°C on a 12-h light:dark schedule. For Maine and Panama, we used 12 isofemale lines for each population. Prior to dissection, males were collected within 4 h of eclosion, placed in vials with other males, and aged for 3–5 days. For each of these two populations a replicate consisted of tissue isolated from two males from each of the 12 lines. We generated three replicates for each tissue and population. Thus, there were 2 tissues × 2 populations × 3 replicates for a total of 12 libraries. Testis and accessory gland + anterior ejaculatory duct (henceforth referred to as AG) were dissected in 1× PBS buffer, transferred directly into Trizol on ice, then stored at −80°C until RNA extraction. For the Zambia population sample, we generated data from six isofemale lines, with three replicates per line. For RNA extraction, tissues were homogenized in 200 µl Trizol, then the volume of Trizol was adjusted to 1 ml, followed by the addition of 200 µl chloroform. Samples were shaken for 20 s then incubated at room temperature for 5 min. After centrifugation (13,000 rpm at 4°C) the upper phase was collected; 1 µl glycogen was added, followed by 500 uµl isopropanol. Samples were placed at −20°C overnight and centrifuged again for 15 min. Pellets were washed with 70% EtOH, the supernatant was removed, the pellet was dried for 10 min, then dissolved in 50 µl nuclease-free water. DNase digestion was performed with the TURBO DNA-free kit (Ambion) using the manufacturer's protocol; samples were cleaned up with HighPrep RNA Elite beads (MagBio Genomics). After fragmentation, first-strand synthesis was carried out via random hexamer priming, followed by second-strand synthesis. After end repair and A-tailing, adaptors were ligated, followed by size selection, amplification, and purification; 150 bp paired-end reads were generated on an Illumina NovaSeq 6000 machine.

Differential expression analysis

We created three Zambia pseudo-pools by contributing one replicate from the six individually sequenced Zambia strains to each pool. This allowed us to generate an African data set that was more similar to that of the Maine and Panama pooled population samples. The number of reads obtained from each library from each of the three populations is in Supplementary Table 1.

To generate tables of read counts for each population sample, we ran featureCounts using -p–countReadPairs (Liao *et al.* 2014, 2019). Differential gene expression was then estimated using *limma* with $\mathrm{lfc} = \log_2(1)$ (Ritchie *et al.* 2015; Phipson *et al.* 2016). Because the number of reads for the Maine and Panama populations was similar for both tissues, we used the *limma-trend* procedure for normalization, however, because the Zambia data contained many more reads than the Maine and Panama data we used the *voom* function (Law *et al.* 2014). To ensure that differences in normalization procedures did not influence our results, we also used *limma-voom* to normalize the Maine/Panama AG comparison. While *limma-trend* finds more DE genes, there is substantial overlap between the two methods, with 97% of the genes found by *limma-voom* also found by *limma-trend*; 78% of genes found by *limma-trend* were found by *limma-voom*. Further filtering of candidate DE genes was done by restricting candidates to genes expressed at TPM ≥ 1 in at least one of the two populations in a comparison.

For some analyses, we restrict our attention to differential expression of *D. melanogaster*/*D. simulans* one-to-one orthologs. Orthologs were identified from a table of *D. melanogaster* orthologs identified in other *Drosophila* (FlyBase, downloaded 2021 May 12). We generated a featureCounts table for both AG and testis to compare all three *D. melanogaster* populations to *D. simulans*. We then ran *limma* to identify differentially expressed genes as above.

Genes were considered to show latitudinal DE for a tissue if we observed differential expression between the Maine and Panama populations. Genes that showed differential expression between both Maine and Zambia and Panama and Zambia, but not between Maine and Panama were considered to show "Out-of-Africa" DE. A third category includes genes that show DE for all three pairwise population comparisons.

Sequence alignment, expression estimates, and population comparisons

Reads were then aligned to version 6.41 of the *D. melanogaster* genome (FlyBase, downloaded 2021 August 9) using *Hisat2* v.2.1 (Kim *et al.* 2015) with default parameters. We used StringTie v2.1.4

(Pertea et al. 2016) to calculate abundance for use in some downstream analyses and as a way to summarize relative expression levels. We used a custom gtf file for our abundance calculations that added de novo gene annotations from Cridland et al. (2022) to the gtf file from *D. melanogaster* 6.41 from FlyBase.

We additionally aligned a w^{501} *D. simulans* AG library (Majane et al. 2022) and four wXD1 *D. simulans* testis libraries (SRR9025055 and SRR9025060 from Chakraborty et al. (2021), and SRR7410596 and SRR7410597 from Lin et al. (2018); downloaded from NCBI, 2022 January 19) to version 3 of the *D. simulans* genome (GCF_016746395.1; downloaded from NCBI, 2021 February 1) and measured transcript abundance and interspecific DE as described above.

PCA analysis

To summarize population affinities based on gene expression we encoded each gene as either expressed (TPM ≥ 1) or not expressed (TPM < 1) in each population sample and tissue, followed by PCA analyses on these values in R (v4.1.2, R Core Team 2021) using the prcomp function; AG and testis were analyzed separately. Additionally, we used bcftools (Danecek et al. 2021) to identify SNPs in the transcriptome data from each biological replicate from Maine, Panama, and our Zambia pseudo-pools as well as our *D. simulans* w^{501} sample. We required a coverage of ≥ 20 in a sample to call a genotype at a site as well as a coverage of ≥ 3 for the minor allele to identify heterozygous sites. Further screening was then done at the site level where we only kept sites that had missing data in no more than one individual sample. We performed PCA analyses on the SNPs identified from the transcriptomes of the three populations. We used *plink* to generate the input files (Purcell et al. 2007) and the package SNPRelate to perform the SNP based PCA (Zheng et al. 2012).

Four taxa expression branch analysis

We examined lineage-specific expression differences by calculating a population branch statistic (PBS) for each expressed gene in the Maine and Panama populations using a four population tree as described in Jiang and Assis (2020). The four populations were Zambian *D. melanogaster*, Panama *D. melanogaster*, Maine *D. melanogaster*, and *D. simulans*. We used TPM estimates to calculate PBS values separately for the AG and testis.

Tissue-biased genes

We downloaded the FlyAtlas2 fastq files (Leader et al. 2018) from SRA (2020 December 1). We downloaded all male data and calculated TPMs for each using *Hisat2* and StringTie as above. Median TPM values were then calculated for each tissue. We calculated τ for each gene as described in Yanai et al. (2005) to measure expression bias. Tissue-biased genes were defined as genes with TPM ≥ 1 in the focal tissue where the gene was most highly expressed and having τ value ≥ 0.9.

GO analysis

We performed gene ontology (GO) analyses on the sets of genes showing latitudinal and Out-of-Africa DE in the AG and testis. We used GOrilla (Eden et al. 2007, 2009) to identify processes, components, and functional categories that were elevated in these lists compared to a background of all genes expressed in the tissue in question. We used the implementation of GOrilla on (http://cbl-gorilla.cs.technion.ac.il/) and the default *P*-value threshold.

Population genetic analysis

We estimated F_{ST} on genomic pooled population samples from Fairfield, ME, Homestead, FL, and Panama City, Panama as previously described (Svetec et al. 2016) following corrections for pooled sequencing data (Kolaczkowski et al. 2011). We also calculated mean F_{ST} per gene (i.e. "genic F_{ST}") with gene defined as the transcription start-to-end, with a minimum of five SNPs per gene for inclusion.

Inversion genotyping

We used the inversion breakpoints identified in Corbett-Detig and Hartl (2012) to identify read-pairs indicating the presence of *In(1)Be* in populations from the American cline (Svetec et al. 2015, 2019) and in two *D. melanogaster* sequencing libraries from Australia (Bergland et al. 2016). Read-pairs that supported the presence of the inversion were defined as those where both members of the pair mapped to the same strand of the reference sequence and the read-pairs straddled a breakpoint. The frequency of the inversion was estimated based on fragment coverage of inversion vs standard arrangements. Additionally, we used published primers (Corbett-Detig and Hartl 2012) to PCR-amplify breakpoints for both the *Standard* and *In(1)Be* X chromosome arrangements in 17 lines from the Panama population. We used the published inversion breakpoints (Corbett-Detig and Hartl 2012) to identify the genes spanned by the inversion.

Results

General patterns

The total number of genes expressed in the *D. melanogaster* accessory gland + anterior ejaculatory duct (AG) and testis (defined as mean TPM ≥ 1 in at least one of the three focal populations) were 9,496 and 13,442, respectively (Supplementary Table 2). There are 35 genes called as expressed in Panama AG (mean TPM ≥ 1) but not Maine AG (mean TPM < 0.1). Such genes would be the population level analog of the recently reported neomorphic or amorphic AG-expressed genes (Cridland et al. 2020). While for many of these genes the Panama expression level is low, three (Supplementary Table 3) express at a relatively high level in Panama (TPM ≥ 5). Similarly, 27 genes exhibit the converse pattern, TPM ≥ 1 in Maine and TPM < 0.1 in Panama, of which two express at TPM ≥ 5 in Maine. In total, the Panama AG expresses about 500 more genes than the Maine transcriptome at the TPM ≥ 1 cutoff, which supports the idea that the Panama AG transcriptome is slightly more complex than the Maine AG transcriptome. We observed 56 genes that were expressed in Zambia AG (TPM ≥ 1) but in neither Panama nor Maine AG (TPM < 0.1 for both). As was true of the Panama-but-not-Maine expression, most ($n = 46$), but not all of these Zambia-specific AG-expressed genes exhibit TPMs < 5 in Zambia. We observed 211 genes expressed in Zambia testis but neither Panama nor Maine testis, seven of which were putative de novo genes (Zhao et al. 2014; Cridland et al. 2022) and fourteen of which exhibited Zambia TPM ≥ 5 (Supplementary Table 4). Only a handful of testis-expressed genes were expressed only in Maine (6) or Panama (12).

To ask more generally about the potential relevance of natural transcriptomes for studying the basic biology of *D. melanogaster*, we identified genes that were expressed at TPM ≥ 1 in the testis for at least one of our population samples but were expressed at TPM < 0.01 in a commonly used community resource, FlyAtlas2; we found 513 such genes. While many of these genes were expressed

in only one population, 65 exhibited TPM ≥ 1 in all three populations; 22 are annotated as non-coding genes (Supplementary Table 5). The three-population mean TPM for these 65 genes was 4.2. Such observations raise interesting questions about the possible biological relevance of substantial tissue expression that is not observed in community gene expression resources.

Overall patterns of transcriptome similarity for Maine, Panama, Zambia, and *D. simulans* are summarized in Fig. 1. A PCA analysis of encoded expression for the AG shows that as expected, *D. melanogaster* populations are well separated from *D. simulans*, and that the Zambia *D. melanogaster* population is more similar to *D. simulans* than are the American populations. It also shows that the Maine population is more similar to the Zambia population than is the Panama population (Fig. 1a). For the testis we see the expected separation of *D. melanogaster* and *D. simulans*, and the Zambia population is more similar to *D. simulans* than are the American populations. However, there is no obvious difference between Maine and Panama with respect to similarity to Zambia. These patterns support the idea that for both organs the Zambia *D. melanogaster* population has retained more ancestral-like transcriptomes, as might be expected based on population genomics inferences (Pool *et al.* 2012), but that the relative divergence of the American populations is heterogeneous across tissues, with recent expression divergence between Maine and Panama in the AG, but not the testis. Thus, we see clear evidence of population-by-tissue interaction effects for recent expression differentiation.

Overall patterns of expression differentiation

Table 1 shows for AG and testis, the number and proportion of expressed genes that show DE between Maine and Panama (hereafter, latitudinal DE), and the number and proportion of genes that show DE for both Zambia vs Panama and Zambia vs Maine, but are not differentially expressed between Maine and Panama. For convenience, we refer to this latter case as OOA DE. The premise of this model is that expression differentiation of the American populations from Africa (but not from each other) reflects the shared evolution that occurred in the initial establishment of all non-African populations. Accordingly, we assume that expression divergence between Maine and Panama has occurred since the colonization of the Americas. We acknowledge that more complex scenarios cannot be ruled out in the absence of additional sampling, especially from Africa and Europe.

The most striking patterns are (1) the finding of latitudinal DE in the AG, but not the testis and (2) the presence of much greater OOA DE for the testis than the AG. Consistent with the expression PCA, the geographic/temporal patterns of transcriptome divergence are dramatically different for these two organs, with the testis exhibiting much more differential expression on the longer OOA timescale and the AG exhibiting much more differential expression on the shorter latitudinal timescale. Tissue-biased genes ($\tau > 0.9$) are significantly more likely to exhibit latitudinal DE and OOA DE for the AG (Fig. 2a) and more likely to exhibit OOA DE in the testis, with enrichments ranging from roughly 2- to 4-fold. Similarly, latitudinal DE genes had substantially higher mean τ values, 0.9 in DE genes vs 0.75 in other genes (Wilcox test;

Table 1. Differentially expressed genes.

Category	AG	% AG	Testis	% Testis
Latitudinal DE	411	4.3	0	0.0
Out-of-Africa DE	802	8.4	1,831	13.6
Three-way DE	139	1.5	0	0.0
Tissue-biased	531	5.6	3,006	22.4

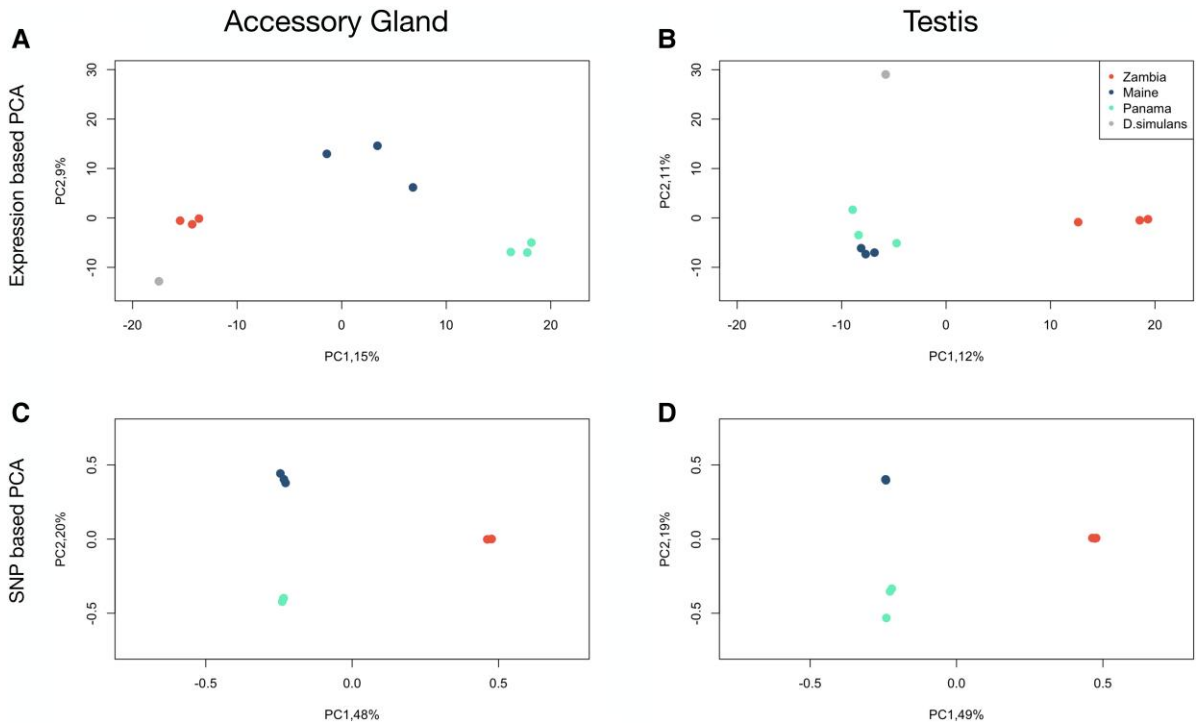

Fig. 1. Separation of three *D. melanogaster* populations by gene expression and genetic variation. a) PCA based on gene expression in the accessory gland. b) PCA based on gene expression in the testis. c) PCA based on SNPs identified in accessory gland transcripts. d) PCA based on SNPs identified in testis transcripts.

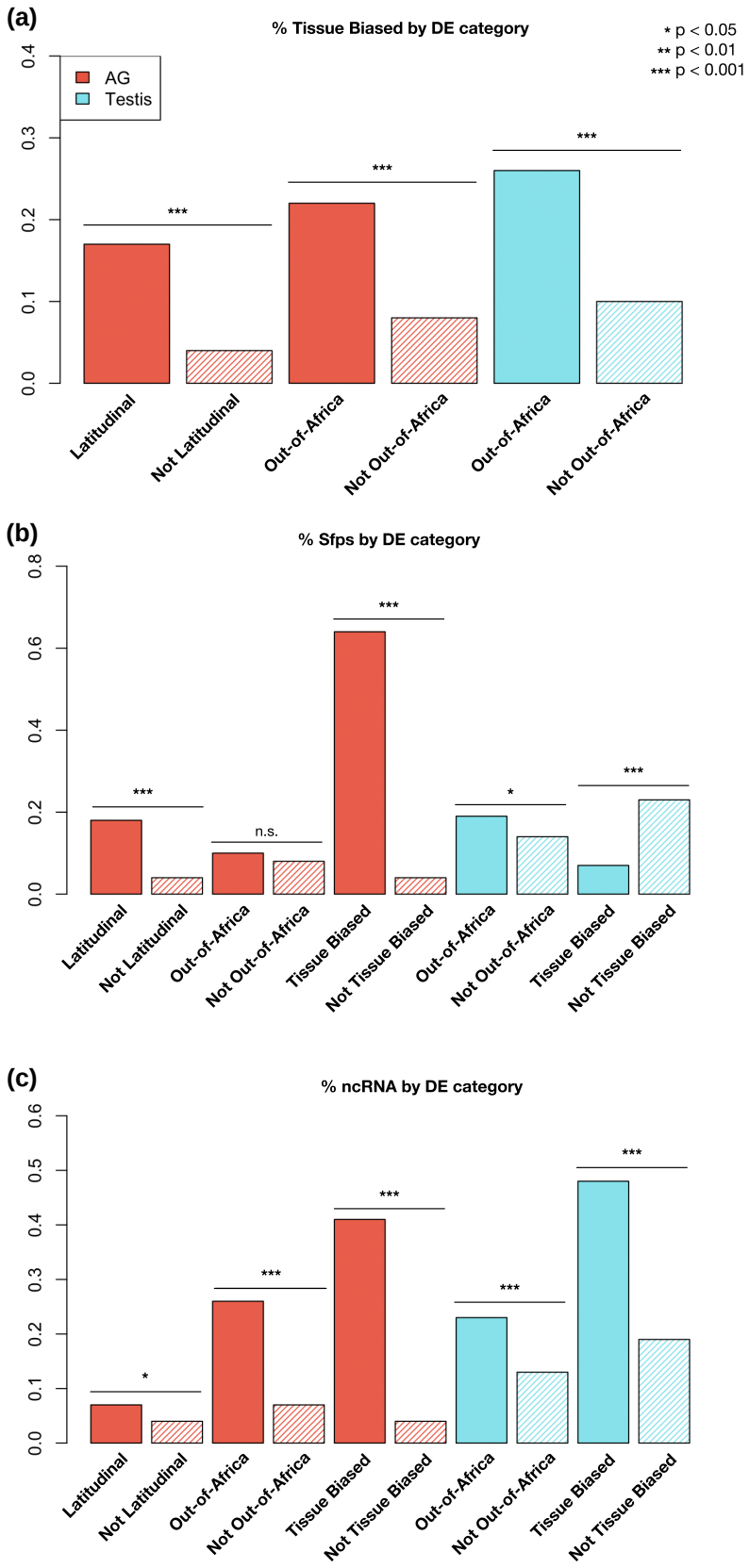

Fig. 2. Contrasts in gene numbers between differential expression categories. a) The proportion of genes exhibiting tissue bias. b) The proportion of genes identified as seminal fluid proteins (Wigby *et al.* 2020). c) The proportion of genes that are ncRNA.

$P = 2.9 \times 10^{-69}$) as calculated using the FlyAtlas2 data set (Leader et al. 2018). Furthermore, in both the testis and the AG, OOA DE genes show much higher mean τ values (0.87 OOA DE vs 0.74 not OOA DE in the AG, $P = 3.9 \times 10^{-84}$; 0.88 OOA DE vs 0.79 not OOA DE in the testis, $P = 4.8 \times 10^{-106}$). This supports the idea that the geographic differentiation is likely at least partially the result of functional effects of expression in these tissues rather than the consequence of pleiotropic effects of expression variation in other tissues.

Expression differentiation

Latitudinal expression differentiation in the accessory gland

General patterns

For the 411 latitudinally DE AG-expressed genes, 173 (42%) exhibited a positive $\log_2$FC, indicating greater expression in Panama than Maine, which is a much smaller proportion than was observed in non-DE genes expressed in at least one of these two populations (4305/8262 or 52%, $P = 7.74 \times 10^{-5}$). Thus, AG latitudinal DE is associated with lower expression in Panama/higher expression in Maine. We calculated a four PBS (Jiang and Assis 2020) using expression estimates (TPM) from Maine/Panama/Zambia/*D. simulans* to partition Panama vs Maine transcriptome differentiation to either the Panama or Maine branch. Latitudinally DE genes were significantly enriched for longer branches to Panama relative to non-latitudinally DE genes (Table 2). Thus, much of the latitudinal AG DE appears to derive from evolution in the Panama population. A quantitative comparison of mean branch lengths (Table 2) supports the notion that latitudinal DE genes show substantially greater divergence on the Panama branch than other gene categories.

One possible explanation for the observation that latitudinal DE genes in the AG exhibit greater divergence in Panama is that the Panama population is more genetically diverged from an ancestral-like African population, and transcriptome divergence is a correlated effect of greater overall genetic divergence. However, greater genetic differentiation between Panama and Zambia than between Maine and Zambia would be unexpected given population genetic results from the literature suggesting that relative to high-latitude American populations, low-latitude American populations should be more genetically similar to African populations (e.g. Bergland et al. 2016; Kao et al. 2015). To investigate this matter, we used previously published SNP frequencies from Maine, Panama, and Zambia (Pool et al. 2012; Svetec et al. 2015) to summarize genetic differentiation between the three populations. We found that genome wide, differentiation as estimated by genic F_{ST} was lower between Panama and Zambia (0.079) than between Maine and Zambia (0.088, Wilcox test P-value $= 8.12 \times 10^{-39}$), which supports the general pattern from the literature (Fig. 1, c and d).

To investigate whether strongly latitudinally differentiated SNPs in the Americas also exhibit this pattern we focused on the 1% tail of high-F_{ST} Maine vs Panama SNPs and determined whether for these SNPs, the Panama vs Zambia F_{ST} was less than the Maine vs Zambia F_{ST}. This analysis revealed that similar to the general genomic pattern, the most latitudinally differentiated American SNPs (mean $F_{ST} = 0.34$) exhibit much lower F_{ST} between Panama and Zambia (mean $F_{ST} = 0.09$, t-test P-value $= \sim 0$) than between Maine and Zambia (mean $F_{ST} = 0.28$, t-test P-value $= 3.38 \times 10^{-149}$). Thus, there is strong evidence that genetically, Panama is more similar to Zambia than Maine is to Zambia. Against this background, the greater AG transcriptome divergence in Panama is discordant, and implies strongly heterogeneous processes driving geographic differentiation of AG expression and the genetic differentiation of the corresponding populations. To check that the discordant transcriptome vs genomic divergence cannot be explained by an unknown laboratory error (e.g. labeling error) we identified SNPs from the AG and testis transcriptomes from the same three populations and used these SNPs to estimate F_{ST}. We observed the same general patterns in these analyses—Panama vs Zambia genetic differentiation was lower than Maine vs Zambia genetic differentiation (Table 3). Given that for these analyses the same animals and RNA-seq reads were used to estimate the patterns of transcriptome and genetic differentiation, we conclude the phenotypic vs genetic discordance is genuine.

To further investigate the possible relative importance of drift and directional selection in the elevated divergence of the Panama AG transcriptome, we summarized nucleotide heterozygosity in Maine and Panama, with the premise that if the Panama population divergence was due to increased drift relative to Maine, then Panama would have lower heterozygosity. All four autosomal arms show consistently higher heterozygosity in Panama than Maine (Supplementary Fig. 1), consistent with the literature (Kolaczkowski *et al.* 2011; Fabian *et al.* 2012; Reinhardt *et al.* 2014), suggesting that lower latitude populations from the Americas and Australia are more heterozygous than higher latitude populations. These results support the idea that the elevated divergence of the Panama AG transcriptome cannot be explained

Table 3. F_{ST} between population pairs.

Type	Tissue	Maine vs Panama F_{ST}	Maine vs Zambia F_{ST}	Panama vs Zambia F_{ST}
Genomic	—	3.63×10^{-2}	8.76×10^{-2}	7.84×10^{-2}
Transcriptomic	Testis	5.69×10^{-2}	8.51×10^{-2}	7.96×10^{-2}
Transcriptomic	AG	5.71×10^{-2}	8.53×10^{-2}	7.02×10^{-2}

Table 2. Lineage-specific expression divergence in the accessory gland.

Category	% LED longer in Maine	Fisher's test vs all genes	Mean LED to Maine	P (binomial) in Maine	Mean LED to Panama	P (binomial) in Panama
Latitudinal DE	10.8	2.95×10^{-30}	0.27	2.53×10^{-2}	1.52	1.59×10^{-76}
Not latitudinal DE	42.9		0.20		0.32	
Out-of-Africa DE	33.6	7.80×10^{-4}	0.17	9.58×10^{-3}	0.52	1.30×10^{-10}
Not Out-of-Africa DE	42.1		0.20		0.35	
Tissue-biased	27.0	1.93×10^{-5}	0.14	7.05×10^{-6}	0.58	2.10×10^{-6}
Not Tissue-biased	42.0		0.20		0.36	
All genes	41.6	—	0.20	—	0.36	—

entirely as a result of recent drift, and likely is at least partially the result of selection in the Panama population.

Biology of latitudinal DE

The relatively large number of latitudinal DE genes in the AG permits a general investigation of their properties. We find that seminal fluid protein genes (Sfps) are dramatically over-represented (roughly 5-fold) amongst DE genes (Fig. 2, Supplementary Table 6), consistent with previously observed rapid expression divergence of this class of genes (Majane *et al.* 2022). AG-biased genes and non-coding genes are also substantially enriched among the DE genes (Fig. 2b, Supplementary Table 6). To address the possibility that transcript abundance may influence our ability to detect DE and that this may be confounded with tissue-bias or Sfp status (i.e. Sfp or not-Sfp), we performed logistic regressions in R (DE ~ Expression of Population 1 + Expression of Population 2 + Sfp + Tissue-biased) for each population comparison and calculated McFadden's pseudo-R. We found no evidence that greater transcript abundance of Sfps/AG-biased genes explains their enrichment among DE genes (McFadden's pseudo-R ≤ 0.102 for all comparisons). We additionally found no evidence of multicollinearity between DE and Sfp or tissue-biased status (variance inflation factors < 4 for all comparisons). GO enrichment analyses of DE gene lists support over-representation of functional categories associated with Sfps, accessory gland protein genes (*Acps*), and sperm leucine aminopeptidase genes (*S-Laps*), and further indicate functional differences separated by population (Supplementary Table 7).

Seminal fluid protein genes

Among the differentiated Sfps, only four, *Acp26Aa* (*ovulin*), *Anp*, *Spn77Bc*, and *Dup99B*, are annotated with functional information related to reproduction (Wigby *et al.* 2020, Supplementary Table 3). The absolute value of the $\log_2$FC was marginally greater for latitudinal DE Sfps (2.1) than latitudinal DE non-Sfps (1.87) (MWU, $P = 0.085$). Much more striking than the quantitative magnitudes of DE for Sfps vs non-Sfps is their directionality. Of the 49 Sfps exhibiting latitudinal DE, 48 have a negative $\log_2$FC indicating lower expression in Panama. In contrast, for the 362 DE genes that are not Sfps, 190 exhibit a negative $\log_2$FC while 172 exhibit a positive $\log_2$FC. Thus, there is very strong enrichment of directional DE for Sfps, which is much more likely than other DE genes to show greater expression in Maine ($P = 1.9 \times 10^{-14}$). When considered in light of the results from the previous section, a plausible interpretation is that selection has tended to reduce Sfp expression in Panama.

Many possible factors that could generate locally variable selection on Sfp expression, among them, temperature-related fitness variation, population density, or mating system variation, none of them can be distinguished with existing data. However, a possible genetic cause of mating system variation is sex-ratio X chromosome drive, which results in excess production of female offspring (Morgan *et al.* 1925; Jaenike 1996). While strong sex-ratio X chromosomes have not been identified in *D. melanogaster*, a weak sex-ratio *X* chromosome has been reported (Reed *et al.* 2005) and appears to be correlated with inversion *In(1)Be* (Corbet-Detig and Hartl 2012). We used existing population genomic data to genotype this inversion following Corbett-Detig and Hartl (2012) and found that in stark contrast to previous work characterizing this inversion as a low-frequency African endemic found only in South Africa and west Africa (Aulard *et al.* 2002; Corbett-Detig and Hartl 2012), the frequency in Panama (Svetec *et al.* 2014) is estimated as 0.15. However, we see no evidence from population genomic data that it segregates in Maine, Connecticut, Pennsylvania, Georgia, Florida, or Australia (Svetec *et al.* 2015; Bergland *et al.* 2016; Svetec *et al.* 2019); a recent report (Coughlan *et al.* 2022) estimated its frequency in Raleigh, North Carolina as 3.6% (3/84), and its overall frequency in Out-of-Africa and African populations as 2% (5/241) and 1.7% (9/516), respectively. We obtained an independent estimate of the frequency of the inversion in Panama using PCR (Corbet-Detig and Hartl 2012) on a sample of 17 independently sampled flies, which yielded an estimated frequency of 0.18, very similar to that obtained through our bioinformatic analysis. Given the very young age of the inversion, 60 years (95% CI, 6–373 years) as estimated by sequence divergence near its breakpoints (Corbett-Detig and Hartl 2012), we hypothesize that this inversion has recently spread under selection in Panama, which could generate a slight female bias in the Panama population. Whether such a phenomenon could be associated with selection on Sfp expression is a matter for speculation.

To further investigate the possible connection of the X-linked inversion to population genetic patterns we summarized X chromosome geographic differentiation between Maine and Panama. Compared to normally recombining regions of the X chromosome, we observed a slight elevation of F_{ST} in the region spanned by the inversion (Fig. 3), which is not surprising given our estimated frequency of the inversion in Panama. More striking, however, is the much greater *X*-linked F_{ST} over the tip and base of the chromosome in the Maine vs Panama comparison relative to the Maine vs Florida comparison. Previous surveys of variation in these populations revealed that all autosomal arms were more differentiated than the *X* chromosome (Fabian *et al.* 2012; Reinhardt *et al.* 2014), ostensibly as a consequence of frequency variation of autosomal inversion polymorphisms, and that the rank order of autosomal arm F_{ST} was $3R > 2L > 3L > 2R$ (Reinhardt *et al.* 2014). Using the analysis pipeline employed here, we recapitulated that observation for the Maine vs Florida comparison. In contrast, in the Maine vs Panama comparison we observe *X*-linked F_{ST} to be only slightly less than *3R*, and greater than *3L*, *2L*, and *2R*, though the rank order is preserved among those arms. Thus, the Panama *X* chromosome exhibits elevated differentiation, partly as a result of the *In(1)Be* region and partly as a result of increased differentiation at the tip and base of the chromosome. Whether these phenomena are functionally related is unknown.

Genes

While the number of AG latitudinal DE genes is too great for comprehensive discussion, here we note a few interesting cases that are not Sfps but for which some functional annotation is available. The DE gene with the greatest absolute $\log_2$ fold-change ($\log_2$FC) is *dpr16* (mean TPMs in Panama and Maine are 8 and 0.07, respectively). This gene carries an Immunoglobulin-like domain, is not annotated as being expressed in the AG in any public database, and belongs to a gene family thought to function in the nervous system. Interestingly, a second gene from this family, *dpr17*, also exhibits latitudinal DE in the AG.

Among the DE genes are other families. For example, all seven *S-Lap* (Sperm leucine aminopeptidase) genes annotated in the *D. melanogaster* genome exhibit DE and all exhibit greater expression in Panama than in Maine. They all show extremely high levels of testis-biased expression (FlyAtlas2), leaving their function in the AG a matter for speculation. However, Hurtado *et al.* (2022), in contrast to Wigby *et al.* (2020), hypothesize that *S-Lap7* is a seminal fluid protein. This observation (and those noted below) prompted

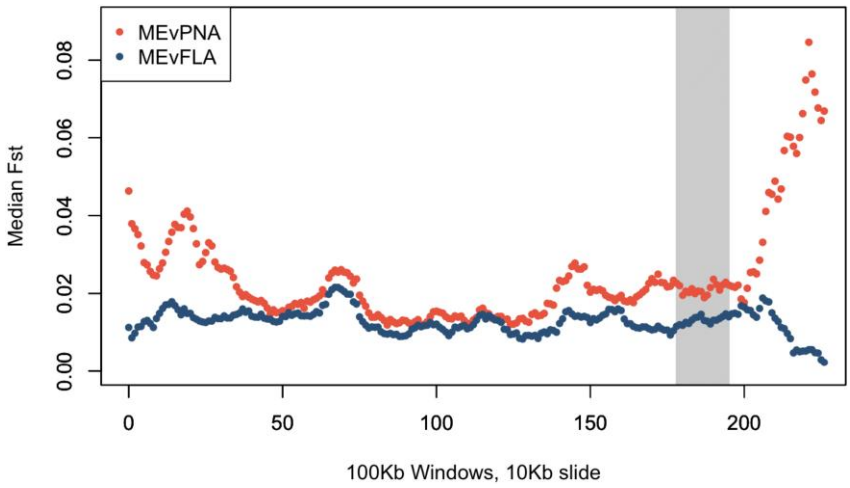

Fig. 3. Median F_{ST} in 100 kb windows with a 10 kb slide on Ch X. Variation in F_{ST} along the X chromosome. The shaded region indicates the location of *In(1)Be*.

us to investigate the general patterns of testis expression for genes exhibiting AG latitudinal DE. Using FlyAtlas2 data (Leader *et al.* 2018) we found that genes showing latitudinal DE in the AG were roughly twice as likely as non-DE genes (38 vs 20%) to have their greatest expression level in the testis ($P = 3 \times 10^{-17}$). This is consistent with the idea that genes that are more strongly testis-biased are more likely to experience divergent expression in the AG, though the fact that there is so little latitudinal DE in the testis suggests that the geographic patterns of differentiation are largely separable from the shared patterns of tissue expression more generally.

Given their very strong testis-biased expression in public databases (modENCODE, FlyAtlas2) we investigated whether the general *S-Lap* DE pattern could be explained by testis contamination of our AG dissection that affected one population more than the other. We identified the testis-specific genes ($\tau = 1$) from FlyAtlas2 and found only three expressed in our AG data (mean TPM < 1 in all three populations) with no evidence that Panama AG expressed more of these putatively testis-specific genes than Maine AG. These observations suggest that if there is testis contamination of our AG RNA, it is low and of similar magnitude in both populations.

Early work in the melanogaster subgroup suggested the possibility that odorant-binding proteins (*Obp*) could be a component of seminal fluid (Begun *et al.* 2006; Findlay *et al.* 2008). We observed three *Obp* genes (*Obp22a*, *Obp44a*, *Obp51a*) as DE in the AG. Two of these genes, *Obp22a* and *Obp51a*, have been categorized as Sfps (Wigby *et al.* 2020), while the third has no reproductive function in any annotation.

The gene *timeless* (*tim*), a canonical clock gene (Sehgal *et al.* 1994) that interacts with *period* to regulate circadian rhythms (reviewed in Cai and Chiu 2021) exhibits DE with roughly 2.4-fold greater expression in Maine than Panama. Two *tim* isoforms generating different behavioral phenotypes were previously shown to exhibit a latitudinal cline in the United States (Pegoraro *et al.* 2017). Peripheral clocks have been observed in multiple *D. melanogaster* tissues (reviewed in Ito and Tomioka 2016), though have never been investigated in the AG. Given that mating in *D. melanogaster* exhibits circadian patterns (Sakai and Ishida 2001), it seems plausible that selection could act on *tim* in the AG via circadian effects on seminal fluid production (Giebultowicz *et al.* 2001; Beaver *et al.* 2002; Ito and Tomioka 2016). Alternatively, *tim* could plausibly influence AG function via effects on seasonality of male reproduction or reproductive dormancy (Kubrak *et al.* 2016; Abrieux *et al.* 2020) or could have non-circadian functions in peripheral tissues (reviewed in Cai and Chiu 2021).

Genomic distribution

Given previous results on the non-random chromosomal distribution of AG-expressed genes and their expression attributes (Cridland *et al.* 2022), we investigated the chromosome arm distributions of latitudinally DE genes. For the most part, deviations from expected arm distributions (relative to all AG-expressed genes) were modest, though significant, with the exception of arms *X*, and *4* (Supplementary Table 8). There was a nearly 3-fold under-representation of *X*-linked genes that were DE. More striking, however, is the exceptional (nearly 10-fold) over-representation of latitudinally DE genes on chromosome 4; 27 of the 70 annotated AG-expressed fourth-chromosome genes, none of which is a Sfp (Wigby *et al.* 2020), exhibit latitudinal DE. None of the common cosmopolitan inversions known to exhibit clines in North America (Stalker 1976; Mettler *et al.* 1977; Knibb *et al.* 1981) are enriched for DE genes (Supplementary Table 9).

We investigated larger-scale patterning of DE genes across chromosomal arms more generally by visualizing mean $\log_2$FC between Maine and Panama in 51-gene sliding windows. This revealed that mean $\log_2$FC fluctuates on a substantial physical scale, with the most prominent feature being a major trend of negative $\log_2$FC (lower gene expression Panama) in the centromere proximal region of *2R* (Fig. 4a, other arms in Supplementary Fig. 2). Because there are relatively few annotated fourth-chromosome genes and because of the expected high levels of linkage disequilibrium (Bridges 1935; Wang *et al.* 2002; Arguello *et al.* 2010), we consider the entire chromosome rather than windows. All 27 *4th* chromosome latitudinal DE genes showed negative lfc. Thus, the highly heterochromatic *4th* chromosome exhibits patterns of AG expression variation similar to those observed in the pericentric regions of chromosome arm *2R*. Chromosome arm *3L* also exhibits a suggestive trend near the centromere (Supplementary Fig. 2), though its physical scale is reduced compared to *2R*.

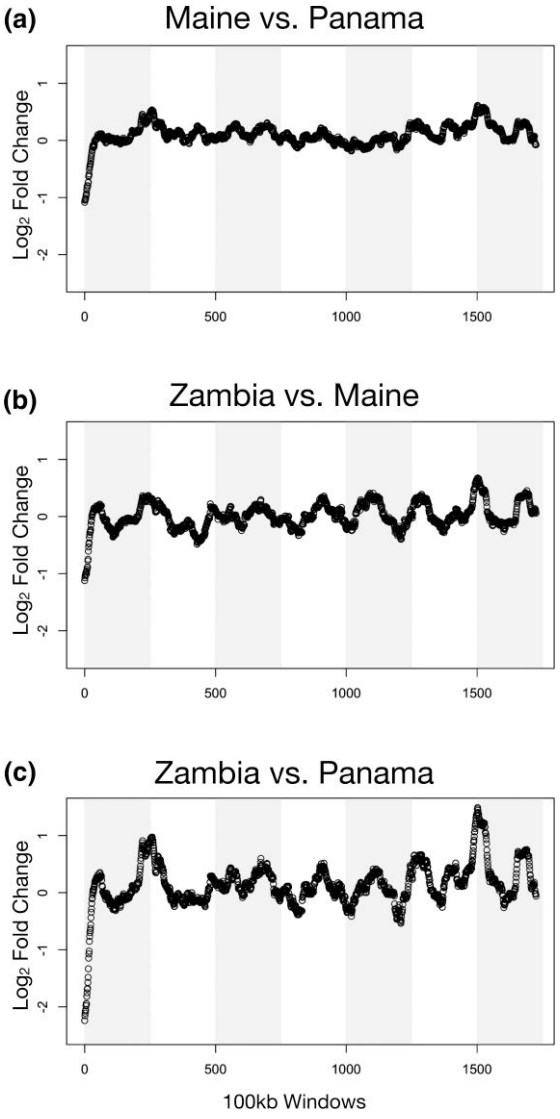

Fig. 4. $\log_2$FC on chromosome 2R for AG-expressed genes in 51-gene windows. a) Maine vs Panama. b) Zambia vs Maine. c) Zambia vs Panama.

Overall these patterns support the idea that some fraction of geographic expression differentiation in the AG may result from population differences in the magnitude of centromere proximal heterochromatic effects. More specifically, we hypothesize that the *4th* chromosome and the centromeric region of *2R* (and perhaps *3L*) carry more heterochromatin in Panama, resulting in greater *cis*-effect suppression of expression (reviewed in Talbert and Henikoff 2006) in Panama in these genomic regions. While *trans*-mediated effects of heterochromatin from other regions, including the Y-chromosome, are also plausible and completely consistent with our observations, Y-chromosome influences on expression appear to be genomically widespread with no evidence of strongly regional effects as observed here (Lemos *et al.* 2008). Thus, it seems unlikely that Y-linked *trans* effects explain all aspects of our data.

Association with genetic differentiation

Unsurprisingly, the regulatory genetics of AG expression variation appear to be complex (Cridland *et al.* 2022). Nevertheless, if *cis*-effects were a major contributor to geographic expression differentiation, then given the scale of linkage disequilibrium in *D. melanogaster* (e.g. Langley et al. 2012), latitudinal DE could be associated with allele frequency differentiation of *cis*-acting regulatory non-coding SNPs in or near DE genes. Alternatively, frequency differences at *trans*-acting regulatory SNPs could contribute to geographic DE. To investigate these questions we used a collection of whole-male eQTLs (associated with genes based on physical distance) identified in a large sample of *D. melanogaster* inbred strains originating in Raleigh, NC (Everett *et al.* 2020) to determine whether latitudinal DE genes were more likely to be associated with higher F_{ST} eQTL compared to non-DE genes.

In our sample of 95 eQTL associated with 62 DE genes and 2491 eQTL associated with 1709 non-DE genes we observed no evidence that mean F_{ST} of DE eQTL were greater than mean F_{ST} of non-DE eQTL (Wilcoxon test, $P = 0.52$). Nevertheless, we did observe examples of high F_{ST} (90% tail, Maine vs Panama) eQTL associated with DE genes. The most suggestive is eQTL SNP *2R:* 9820476, predicted to act in *cis* with the latitudinal DE gene, *CG34033*, a Sfp; it exhibits high F_{ST}, 0.11, between Maine and Panama, and between Panama and Zambia (0.12), but no differentiation between Zambia and Maine, supporting recent evolution in Panama. The frequency of the derived allele (polarized using *D. simulans*) is 0.88 in Panama, in contrast to 0.49 in Zambia, which supports the idea of a rapid spread of the the derived allele in Panama correlated with the derived expression phenotype. A second example is eQTL SNP *2R:*4624258, which is associated in *trans* with *CG17486*. This SNP has very high F_{ST} between Maine and Zambia (0.34) as well as high F_{ST} between Maine and Panama (0.13) and consequently a derived allele frequency of 45% in Maine as opposed to a derived allele frequency of 1% in Zambia.

Under a polygenic model of spatially varying selection on regulatory SNPs, selection on small-effect SNPs could generate consistent small directional geographic allele frequency difference without generating many F_{ST} outliers (e.g. Reinhardt *et al.* 2014; Erickson *et al.* 2020). To investigate this possibility we identified the derived allele for each eQTL SNP and then asked whether geographic differentiation of eQTL for DE genes was more consistent that observed for non-DE genes. More specifically, we tested the hypothesis that the frequency of the derived allele of the eQTL SNP (polarized using *D. simulans*) occurred at higher frequency in Panama more often for latitudinal DE genes than for non-DE genes. The premise of this hypothesis is that since the Panama expression phenotype is more derived than the Maine phenotype, functionally relevant eQTL might have higher derived allele frequencies in Panama than in Maine. However, we observed no significant correlation between derived allele frequencies of eQTL and DE. Note that the Everett *et al.* eQTL were identified based on whole-male expression data, while our data are from the AG, which may substantially weaken our power to detect correlations between eQTL frequencies and AG expression.

Finally, given the possible role of *trans*-acting variants in geographic expression variation in the AG, we extended the search for candidate *trans*-acting factors driving latitudinal DE in two ways. First, we conditioned specifically on nsSNPs in transcription factors expressed in Maine and/or Panama AG and asked if there were F_{ST} outliers ($\geq$ 90% relative to nsSNPs in other AG-expressed genes); we found 98 such SNPs distributed across 73 genes (Supplementary Table 10). Of these, 13 outlier nsSNPs distributed across 10 genes corresponded to genes attached to Everett eQTLs —6 were classified as potential *trans*-eQTLs, three as *cis*-eQTL, and one was associated with both *cis*- and *trans*-eQTL. These are potentially attractive candidates for clinically varying protein polymorphisms that drive some of the latitudinal DE in the AG.

Second, we identified the annotated transcription factors that exhibited latitudinal DE in the AG and found seven such cases (Supplementary Table 3), though none corresponded to Everett eQTLs. This amino acid and expression variation is an interesting material for further investigation.

Out-of-Africa expression differentiation

Accessory gland

We observed 802 OOA DE genes, roughly twice the number observed as latitudinally DE. Among these, only 28 (3.5%) are Sfps, which does not represent an enrichment relative to AG-expressed genes (Fig. 2, Supplementary Table 6). Thus, compared to their representation in OOA DE, Sfps are about three times more likely to show latitudinal DE. Nevertheless, AG-biased genes more generally are still roughly 3-fold enriched for OOA DE. Also similar to the latitudinal DE, non-coding genes are enriched among the OOA DE genes, however, the enrichment is much more pronounced in OOA DE, roughly 4-fold, as opposed to the less than 2-fold enrichment for non-coding genes in latitudinal DE (Fig. 2, Supplementary Table 6). The different enrichment patterns for latitudinal vs OOA DE genes support the notion that heterogeneous selective regimes operate on the AG transcriptome across these two geographic/temporal dimensions. OOA DE genes were enriched for signal transduction, drug metabolic processes, and microtubule-related processes (Supplementary Table 7).

Of the AG OOA DE genes, 133 were associated with 204 Everett eQTL. A further 1,638 non-OOA genes were associated with 2,382 Everett eQTL. Eighteen Everett eQTL SNPs were Zambian vs American ≥ 90% F_{ST} outliers (Supplementary Table 11). However, as was the case for latitudinal DE, we saw no evidence that OOA DE gene eQTL had higher mean F_{ST} than non-DE eQTL (Wilcoxon test, $P = 0.15$). AG OOA genes generally showed similar patterns of chromosome arm heterogeneity as the latitudinal DE genes, with an excess of DE on *2L* and *4*, and a deficit on the *X*, though both the *X*-linked under-representation and the *4th* chromosome over-representation are less extreme for OOA genes (Supplementary Table 7). The OOA genes additionally showed a moderate deficit on *3R*, which was not observed among latitudinal DE genes.

We then investigated whether the chromosomal patterning observed for latitudinal expression differences in the AG was also present in the OOA comparison. To do so, we plotted $\log_2$FC in 51-gene sliding windows for Maine vs Zambia and Panama vs Zambia. This analysis revealed similar patterns in the two different continental comparisons. As we observed in the Panama vs Maine AG analysis, the centromere proximal region of *2R* shows a sharp decline in $\log_2$FC values indicating highest expression in Zambia, followed by lower expression in Maine and then Panama (Fig. 4, b and c). Thus, over the first six megabases of chromosome *2R*, 30 of 53 AG-expressed genes exhibit expression differentiation; 21 latitudinal DE genes and 9 OOA DE genes. This further motivates *2R* as a target for analysis of the regulatory and population processes driving this chromosomal patterning. We see similar patterns in the centromere proximal region of *3L* and again on chromosome 4, consistent with our observations between Maine and Panama (Supplementary Figs. 3 and 4).

Testis

In striking contrast to the absence of latitudinal DE in the testis, OOA DE was abundant, with 1,831 genes (13.6% of expressed genes) exhibiting DE at our cut-offs (Table 1). Of these, 774 (42%) were strongly testis-biased ($\tau > 0.9$) in their expression, representing a more than 2-fold enrichment relative to other testis-expressed genes in our data. Similar to our observation of the AG, genes annotated as non-coding were roughly 2-fold more likely to exhibit OOA DE compared to genes that were not DE (Fig. 2c, Supplementary Table 6). We observed 113 genes with absolute $\log_2$FC > 5 and of these, 80 were annotated as non-coding. One hundred nine of these 113 genes were expressed at a higher level in Zambia. Indeed, for these 113 genes the median Zambia, Maine, and Panama TPMs were 182.6, 11.7, and 14.7, respectively; several Zambia-expressed genes would be classified as unexpressed in American populations. More generally, OOA genes tend to be expressed at a higher level in Zambia, with 1287/1831 (70%) exhibiting a negative mean $\log_2$FC between both the Zambia vs Maine and Zambia vs Panama comparisons, indicating higher expression in Zambia. Alternatively, for non-OOA genes only 4,935 of 10,526 (47%) had a negative mean $\log_2$FC ($P = 1 \times 10^{-91}$).

Among the top 20 Out-of-Africa DE genes by $\log_2$FC, only four, *Che53b* (perception of chemical stimulus), *CG14245* (chitin binding), *Gr92a* (sensory perception of taste), and *Cpr72Ea* (chitin-based cuticle development), had any functional annotation. In addition to *Gr92a*, three other gustatory receptors (*Gr22c*, *Gr22f*, and *Gr47a*) are testis OOA; all four *Grs* exhibit greater expression in Zambia, with mean $\log_2$FC = 2.4. Ten odorant-binding proteins are differentially expressed, of which, only one, *Obp56i*, is a Sfp. The possible role of *Obps* and *Ors* in testis is poorly understood, though testis cyst cell RNAi knockdown of *Obp44a* leads to defects in spermatid nuclear bundling (Bouska and Bai 2021), and testis knockdown of odorant receptor co-receptor (*orco*) also affects testis cyst cell function (Dubey *et al.* 2016). *Odorant receptor 71a* is expressed in Zambia testis (mean TPM = 2.3) but shows no evidence of expression in the Americas, while OOA gene *Or67b* shows roughly 10-fold greater expression in Zambian than in American testis. While the possible functions of most of these genes in *Drosophila* reproduction are unclear, evidence that sperm-associated chemical perception genes may regulate sperm chemotaxis in the female reproductive tract in many animal taxa (Miller 1985; Rihani *et al.* 2021) suggests the possibility that differential expression of this class of genes plays a role in the divergence of male-female communication in the reproductive tract of African and non-African flies.

Of the 244 Wigby *et al.* Sfps expressed in testis, 47 (19%) exhibit testis OOA; 43 of these (91%) show a positive mean $\log_2$FC indicating greater expression in the Americas (mean $\log_2$FC = 3). Thus, there is substantially more OOA Sfp DE in the testis than in the AG (3.5% of expressed genes), supporting the possibility that the functions of Sfps in the two organs may not be completely redundant. Many of the most significantly enriched GO terms (Supplementary Table 12) for OOA testis DE are driven by ribosomal protein genes. Of the 35 DE ribosomal protein genes, 33 are OOA with higher expression in the Americas. Ribosomal protein genes tend to be expressed in the early stage of spermatogenesis (Witt *et al.* 2019; Mahadevaraju *et al.* 2021) and are also abundant in mature sperm (Fischer *et al.* 2012). We undertook a more general investigation using markers for cell type/developmental stage of spermatogenesis identified by Mahadevaraju *et al.* (2021) and found for the two earliest stages (germline and early spermatocytes) there was a significant deficit of OOA genes, whereas for the middle and late primary spermatocyte cell types, there was an enrichment of OOA genes (Supplementary Table 13). In particular, the late primary spermatocytes (L1) cell type exhibited a substantial excess of OOA genes. Within this set were 31 genes that had much higher expression in Zambia than North

America, with $\log_2$FC ≥ 5. Of these, 15 were markers only in the L1 cell type; none has any functional annotation. Thus, late primary spermatocyte transcript abundance may be a hotspot of geographic differentiation. However, given the importance of translational regulation during spermatogenesis (Schafer *et al.* 1990; Giorgini *et al.* 2002; Hempel *et al.* 2006), the functions of the encoded proteins could be realized substantially later during sperm differentiation.

Of the seven annotated *Dhc* (dynein heavy chain) genes, which function in axoneme assembly and function, six exhibit OOA DE; all have greater transcript abundance in the Americas. The gene *Dnah3* (dynein axonemal heavy chain 3) and *PpR-Y* (orthologous to human *DRC3*, the dynein regulatory complex 3 gene) also show significantly greater expression in the Americas. Moreover, of the eight annotated *Sdic* (sperm-specific intermediate dynein chain) genes, four exhibit OOA DE and they, too, have greater transcript abundance in the Americas. These patterns are suggestive of potential geographic variation in sperm size, perhaps associated with larger sperm in American populations.

Two other Y-linked protein phosphatase genes in addition to *PpR-Y*, *Pp1-Y2*, and *PpY-55A*, show significantly higher expression in Zambia (consistent with other Y-linked DE genes—see below). Whether the observed primary expression of *PpY-55A* in the testis cyst cell (Armstrong *et al.* 1995) and the cyst cell knockdown phenotype of OOA DE gene *Obp44a* point to somatic cyst cell functional variation across populations is an interesting question. In support of this possibility, an additional 168 of the 1,341 testis cyst cell markers identified in (Mahadevaraju *et al.* 2021) also showed OOA DE in the testis, although OOA DE genes are not enriched in this cell type ($P = 0.13$). Three of these markers (*mt:CoIII*, *RNASEK*, and *Gprk1*) were very highly expressed in Zambia relative to North America ($\log_2$FC ≥ 5).

Testis OOA DE genes were distributed heterogeneously across chromosome arms, in general exhibiting similar patterns as the AG, supporting previous work on correlated gene expression in these two tissues (Cridland *et al.* 2020, 2022). For example, similar to patterns in the AG, testis OOA genes were over-represented on chromosome 4, exhibiting a roughly 4-fold enrichment (Supplementary Table 8). Also, similar to the AG, the testis exhibited a moderate enrichment of OOA DE on arm 2*L* and an underrepresentation of DE genes on 3*R*. In general, neither testis nor AG exhibited significant enrichment of OOA DE with chromosomal regions spanned by cosmopolitan inversions, the exceptions being *In(3L)P* for the testis and *In(2R)NS* for the AG, though only the *In(2R)NS* enrichment is significant after multiple test correction (Supplementary Table 9). There were two major differences between tissues in chromosomal distribution of OOA DE genes. First, the *X* chromosome showed a moderate underrepresentation for the AG but no evidence of such a phenomenon for the testis. Second, of 42 Y-linked testis-expressed genes in our data, 16 (38%) exhibited an OOA pattern, representing a 3-fold enrichment. In contrast, the AG expresses only three Y-linked genes, none of which showed evidence of geographic expression differentiation. For all 16 Y-linked testis OOA genes the Zambia population showed substantially greater expression than the American populations (mean $\log_2$FC = 1.86).

We examined $\log_2$FC in 51-gene windows for all three-population pairs (Supplementary Figs. 5–7). Both the Zambia vs Maine and Zambia vs Panama comparisons were very similar to each other, with highly similar patterns of $\log_2$FC observed over the length of each chromosome. As we saw in the AG comparisons, there is a sharp negative trend in $\log_2$FC in the centromere proximal region of 2*R* and a more pronounced trend of negative $\log_2$FC for the centromere proximal region of 3*L* for testis than for AG (Supplementary Figs. 6 and 7). Like the AG, the testis also showed consistent negative $\log_2$FC for the 4*th* chromosome.

Latitudinal and Out-of-Africa DE in the accessory gland

We observed 139 genes that were differentially expressed in both the Maine vs Panama comparison and were also differentially expressed Out-of-Africa, i.e. were differentially expressed in all three pairwise population comparisons. From a geographic perspective, these genes exhibit the most rapidly evolving AG gene expression. Of these, the absolute value of the Zambia vs Panama $\log_2$FC was greater than the absolute value of the Zambia vs Maine $\log_2$FC for 129 genes, consistent with the latitudinal DE pattern, showing greater divergence in the Panama vs Zambia contrast than in the Maine vs Zambia contrast. Fifteen of the 139 genes are Sfps. These genes have a similar pattern of enrichment as other latitudinal DE genes for tissue-bias, Sfps, and ncRNA.

Geographic variation for novelties

Recent work on the origin and/or spread of de novo genes and novel organ expression phenotypes in *D. melanogaster* has focused on AG and testis expression in inbred lines from North Carolina (Zhao *et al.* 2015; Cridland *et al.* 2020, 2022). While geographic patterns of expression for such genes could potentially provide clues about the factors influencing their abundance in populations, there is no work directly addressing this issue.

Of the 133 putative AG-expressed de novo genes previously identified in a Raleigh sample of six inbred lines (Cridland *et al.* 2022), 34 were called as expressed (mean TPM > 1) in at least one of our three focal populations. The median TPMs across all 34 genes, including those that were unexpressed in some populations, were 2.1, 1.3, 0.9 for Zambia, Maine, and Panama, respectively. Twenty-six of the genes were expressed in Zambia, while the corresponding numbers for Maine and Panama were 21 and 16, respectively. Ten putative de novo genes had TPM > 1 in all three populations. Of the 34 AG-expressed putative de novo genes, 6 (18%) exhibited latitudinal DE in the AG, while only 4% of all AG-expressed genes exhibited latitudinal DE (P-value = 3.4×10^{-4}); eight putative de novo genes (24%) exhibited OOA DE in the AG (P-value = 1.6×10^{-3}). These results support the idea that recent selection on novel AG-expressed genes has operated since the Out-of-Africa event in this species. Four of the 139 genes showing DE in all three pairwise population AG contrasts were candidate de novo genes. Cridland *et al.* (2020) identified 31 neomorphic AG-expressed genes in a Raleigh sample. None of these genes were latitudinally DE while six (20%) (*snky*, *Marf1*, *CG8641*, *CG32816*, *Cp15*, *CG13084*) exhibited OOA DE.

Among a conservative set of 168 testis-expressed putative de novo genes (53 fixed and 115 polymorphic, Zhao *et al.* 2014; Cridland *et al.* 2022), 131 were categorized as expressed in at least one focal population. Of these, 38 (29%) were OOA, significantly more than the proportion of all testis-expressed genes that were OOA (1,831/13,442, 14%) ($P = 1.2 \times 10^{-6}$). Interestingly, of the 38 putative de novo genes exhibiting OOA expression, 36 exhibited higher expression Zambia, suggesting the possibility that previous work in North American flies has substantially underestimated the number of testis-expressed de novo genes in the species. Of the 17 testis neomorphs identified by Cridland *et al.* (2020), 16 were identified as testis-expressed in these data; four (*phyl*, *CG8960*, *Osi23*, *Muc4B*) were categorized as testis OOA. Further investigation of novel genes and expression phenotypes in African

samples would provide a clearer picture of the possible association between recent colonization and their spread.

Comparing patterns of within and between species divergence

The heterogeneous *D. melanogaster* AG and testis transcriptome population differentiation raise the question of how population level transcriptome differentiation relates to longer timescale patterns of interspecific divergence. The time since the *D. melanogaster* vs *D. simulans* split, roughly 2–3 million years (Obbard *et al.* 2012), is thought to be at least 100-fold greater than the *D. melanogaster* OOA event, roughly 10–15 K years ago (David and Capy 1988; Lack *et al.* 2015). To put geographic patterns of *D. melanogaster* variation into a longer timescale context, we compared them to patterns of interspecific DE between Zambian *D. melanogaster* and *D. simulans* (Supplementary Tables 14–16), using the same methods that were used to characterize within-species variation.

We found that 3,399 of 11,174 testis-expressed genes (30.4%) showed interspecific testis DE. Of the 9,527 AG-expressed genes, 1,937 (20%) exhibited interspecific DE. Thus, the rate of interspecific testis DE accumulation is roughly fifty percent higher than that of the AG. Overall, testis exhibits more DE genes than the AG on the two longer timescales (interspecific and OOA), while the AG exhibits dramatically more DE than the testis in the Americas. The likely explanation for these patterns is that the AG transcriptome has experienced an astonishing rate of divergence relative to the testis since colonization of the Americas.

The proportion of AG expressed genes in the OOA DE category (8.4%) and latitudinal DE category (4.3%) is substantially greater on a per year basis (8.4% of genes/∼15,000 years≈0.056% of genes/year, and 4.3% of genes/∼100 years≈0.043% of genes/year) than the estimated accumulation of DE genes/year in the interspecific comparison (20% of genes/$\sim 2 \times 10^6$ years$\approx 10^{-5}$% genes/year). At least four factors may contribute to this discordance. First, the intensity of directional selection on expression could have increased in the recent past in the *D. melanogaster* lineage. Second, the pairwise Zambian *D. melanogaster* vs *D. simulans* comparison leaves open the question of the rate of DE accumulation specifically in the *D. melanogaster* branch, though it seems highly unlikely that lineage heterogeneity could be so great as to generate the observed discordance. Third, it is possible that a fraction of the estimated interspecific DE is attributable to intraspecific *D. simulans* variation. Finally, it is possible that rapid expression evolution has led to an underestimate of the underlying rate of divergence.

To investigate this last possibility we determined the degree of concordance between the AG-expressed DE genes on different timescales by comparing latitudinal DE genes to the interspecific DE genes, an appropriate comparison given the absence of shared data for the two comparisons. Of 7,783 AG-expressed orthologs we found that 285 exhibited latitudinal DE and 1,962 exhibited interspecific DE. Assuming independence, the number of genes expected to show DE in both comparisons is 62, while the observed number is 109, representing a highly significant enrichment ($P = 2.744 \times 10^{-6}$) (Supplementary Table 15). Thus, some AG-expressed genes have the propensity to exhibit expression divergence over multiple timescales. This is consistent with the idea that for rapidly evolving expression phenotypes our estimates of transcriptome divergence are downwardly biased due to parallel/convergent expression evolution. Interspecific DE genes in AG have higher average τ values than non-DE genes ($P = 5.7 \times 10^{-28}$), however, unlike latitudinal DE genes, they are less likely to be most highly expressed in the testis ($P = 0.039$), illustrating further differences in the expression properties of genes that are DE at different time scales.

The consistently greater DE for the testis than for the AG on the interspecific and OOA timescales raises the question of the relative rates of testis DE on these two timescales. The interspecific timescale is roughly 100-fold greater than the Out-of-Africa colonization, yet the former exhibits only a roughly 2-fold greater number of testis DE genes relative to the latter (3,399 vs 1,831). We investigated the overlap between genes identified as OOA DE and interspecific DE in the testis, but found no enrichment of shared genes ($P = 0.13$), despite the correlated nature of the analyses (both comparisons share the Zambian *D. melanogaster* data), further supporting heterogeneous processes generating transcriptome differentiation across these dimensions.

We find the chromosome arm distribution of interspecific DE genes to exhibit less heterogeneity compared to the distributions observed in OOA and latitudinal DE genes (Supplementary Table 17). There is neither over- nor under-representation of AG DE genes on any of the autosomes, and only a modest under-representation of interspecific DE genes for the testis on chromosome 3L. A smaller under-representation (∼15%) was observed in X-linked interspecific DE genes for both tissues than was observed in either the OOA or latitudinal DE sets in the AG.

In contrast to observed GO enrichments for geographically differentiated *D. melanogaster* AG expression, these genes showed no enrichment for any functional categories in a GO analysis (Supplementary Table 18). Alternatively, interspecific testis DE genes were associated with multiple GO enrichments, including DNA-binding transcription factor activity, serine-type peptidase activity, and genes annotated as having extracellular regions.

Further investigation of the composition of genes in the interspecific DE sets reveals that both AG and testis exhibit significant enrichment of Sfps (Wigby *et al.* 2020) and tissue-biased genes (Leader et al. 2018) (Supplementary Table 6). We compared testis interspecific DE genes to marker genes in spermatogenesis (Mahadevaraju *et al.* 2021). For every developmental stage examined, there was a significant deficit of interspecific DE genes (Supplementary Table 19). In contrast, in the AG we find an enrichment of interspecific DE genes (Table 4) amongst both the main cell and ejaculatory duct cell markers (Majane *et al.* 2022). Indeed, a very large proportion of ejaculatory duct markers included for the interspecific comparison, 44%, show DE ($P = 2.55 \times 10^{-4}$). This observation supports inferences from Majane *et al.* (2022) that the ejaculatory duct transcriptome appears to evolve more quickly than the accessory gland transcriptome per se.

Discussion

Our investigation of transcriptome differentiation between three populations—Zambia, Maine, and Panama—in two male reproductive organs in *D. melanogaster* revealed evidence of extreme heterogeneity across tissues and populations as well as population-by-tissue interactions. While our interpretations of the observed geographic patterns are parsimonious in light of current understanding of continental-scale heterogeneity in the species, data from several additional African, European, and American populations would be required to strengthen support for these interpretations.

The *Drosophila* literature points to the testis as exhibiting one of the most rapidly evolving transcriptomes between species (e.g. Meiklejohn *et al.* 2003; Whittle and Extavour 2019), consistent with our finding that there are 1.5-fold more interspecific DE genes

Table 4. Accessory gland cell markers and differential expression.

Cell cluster	# Expressed cells in cluster	Category	DE genes	Total expressed genes	Cell type & DE category	Ratio cluster in DE/Cluster in all expressed genes	*P* (binomial)
Main	212	Latitudinal DE	411	9,496	43	4.69	2.43×10^{-17}
Main	212	Out-of-Africa DE	802	9,496	44	2.46	3.45×10^{-8}
Main	212	Three-way DE	139	9,496	21	6.77	8.18×10^{-13}
Main	139	Interspecific DE	1,937	9,527	39	1.38	1.10×10^{-2}
Secondary	80	Latitudinal DE	411	9,496	1	0.29	8.61×10^{-1}
Secondary	80	Out-of-Africa DE	802	9,496	10	1.48	8.13×10^{-2}
Secondary	80	Three-way DE	139	9,496	1	0.85	3.27×10^{-1}
Secondary	70	Interspecific DE	1,937	9,527	10	0.70	8.68×10^{-1}
Ejaculatory duct	50	Latitudinal DE	411	9,496	10	4.62	1.56×10^{-5}
Ejaculatory duct	50	Out-of-Africa DE	802	9,496	6	1.42	1.35×10^{-1}
Ejaculatory duct	50	Three-way DE	139	9,496	0	0.00	5.20×10^{-1}
Ejaculatory duct	39	Interspecific DE	1,937	9,527	17	2.14	2.55×10^{-4}

between *D. melanogaster* and *D. simulans* for testis than for AG (Supplementary Table 20). Despite this longer timescale pattern, however, we observed many latitudinally DE genes in the AG, yet none in the testis. The lack of substantial testis DE between the Maine and Panama populations is consistent with our previous speculation from whole-male transcriptomes that DE between these populations is primarily the result of expression divergence of somatic rather than germline tissues (Zhao *et al.* 2015). Whether the AG exhibits more latitudinal DE than other male somatic organs remains unknown.

Patterns of AG expression differentiation between Maine and Panama suggest that much of the population difference can be explained by evolution in the Panama population. Moreover, three pieces of evidence support a role for natural selection in this recent divergence. First, the Panama population is genetically more similar to the Zambia population than the Maine population, but is more phenotypically different from the Zambia population. The greater genetic similarity of Panama to Zambia is consistent with at least two hypotheses. First, selection tends to favor the same "low-latitude" alleles in Panama and Zambia. Second, American populations exhibit an "ancestry" cline resulting from dual introduction of flies to the Americas from two different sources in close temporal proximity, one high-latitude European population that ostensibly experienced no African admixture, and an African population, followed by secondary contact and genetic drift (Bergland *et al.* 2016). Regardless, the robust discordance between genetic and phenotypic similarity observed here suggests the action of natural selection. Second, the Panama population is more heterozygous than Maine, making it unlikely that increased rates of drift are the sole explanation for its more rapid evolutionary rate. Finally, seminal fluid proteins are not only over-represented among AG-expressed latitudinal DE genes, but also exhibit greatly exaggerated asymmetry, with the vast majority of differentially expressed Sfps showing lower expression in Panama. It seems unlikely that such strong enrichments and directional trends could be explained by drift. While the agents of selection driving AG transcriptome divergence between Maine and Panama cannot be addressed directly with our data, these findings strongly motivate investigations of potential male fertility phenotypes and the genetics and biology of female post-mating phenotypes in these populations.

While the correlates of DE on AG function remain unknown, a few broad patterns in addition to Sfp DE deserve mention. First, genes annotated as non-coding are major contributors to both latitudinal and OOA DE. These results are consistent with our recent work (Cridland *et al.* 2022) supporting an important but poorly known role of non-coding genes in AG function (Maeda *et al.* 2018; Immarigeon *et al.* 2021) and adaptation. Second, several of the DE genes observed here were either not known to be expressed in the AG at all and/or have no known function in male reproduction. To the extent that at least some of the DE documented here can be taken as indirect evidence of evolutionary or biological importance, these results suggest that a full understanding of AG function cannot be achieved without accounting for natural variation, both within and between populations. Third, among the latitudinally DE genes, a few ancient gene families are represented—some, for example, the Sperm Leucine Aminopeptidase family, are very strongly testis-biased in expression. This supports previous work suggesting a correlation between AG and testis expression in *D. melanogaster* (Cridland *et al.* 2020, 2022). This correlation is also apparent at the protein level (Garlovsky *et al.* 2022; McCullough *et al.* 2022).

Our AG data capture three cell types: main, secondary, and ejaculatory duct, with main cells the large majority (about 90%) and therefore represented/conveyed well in bulk tissue expression data (Majane *et al.* 2022). Of the 78 secondary cell markers reported in Majane *et al.* (2022) and expressed in our data set, only one, *CG43185* (not a high-confidence Sfp; Wigby *et al.* 2020), is latitudinally differentially expressed, though given the small percentage of secondary cells in a bulk tissue dissection (about 4%), this small number is not surprising. However, a larger proportion (20%) of 50 reported ejaculatory duct markers expressed in our data exhibit latitudinal DE ($P = 1.56 \times 10^{-5}$) and another 12% exhibit Out-of-Africa DE ($P = 0.13$) (Table 4). While formal inferences on cell type DE from bulk data are challenging, given that only about 4% of AG-expressed genes are DE in bulk transcriptomes and given that like secondary cells, ejaculatory duct cells constitute a minority of the cells in bulk tissue (about 5%, Majane *et al.* 2022), we speculate that we have likely underestimated the degree of enrichment of DE for the ejaculatory duct, though population differences in the relative size of the ejaculatory duct would also be consistent with our observations.

While we observed no evidence that latitudinal DE in the AG is influenced by the well-known latitudinally varying cosmopolitan paracentric inversions in *D. melanogaster*, our analysis revealed three chromosomal patterns. First, latitudinal DE in the AG is enriched in the pericentric regions of chromosome arm 2*R* and on the highly heterochromatic chromosome 4. Second, enrichment in these chromosome regions is directional toward lower expression in Panama. The mechanisms underlying these chromosomal patterns are an interesting topic for future investigation, with geographically differentiated *cis*- or *trans*-effects of heterochromatin

being one possibility. Overall chromosomal trends in the Out-of-Africa DE genes in the AG for 2*R* and 4 generally follow patterns similar to those observed for latitudinal DE genes, but to a lesser extent.

In contrast to the much greater transcriptome evolution of the AG (compared to the testis) in the Americas, the Out-of-Africa testis transcriptome shows dramatically more differentiation than that of the AG. Testis OOA genes are enriched for non-coding genes, and GO enrichment patterns for DE genes are suggestive of increased levels of translational machinery (e.g. ribosomal proteins) in the testis of non-African flies. Several genes associated with axoneme assembly also show OOA DE and strong trends for greater transcript abundance in the Americas. Furthermore, we find a substantial enrichment in OOA DE genes for markers of middle and late primary spermatocytes (Mahadevaraju *et al.* 2021), with the late primary spermatocyte apparently being a particular hotspot of Out-of-Africa differential transcript abundance. Another clear trend in the data is the abundance of Sfps that exhibit OOA DE in the testis but not the AG. This finding supports recent emerging evidence that Sfps may have specific functions in spermatogenesis in addition to their role in production of seminal fluid (Galvin *et al.* 2021). Finally, testis OOA DE genes are heterogeneously distributed across the genome, with dramatic trends in the centromere proximal regions of 2*R* and 3*L* similar to those observed for the AG, with a very substantial enrichment on the *Y* as well. In general, it appears that large genomic regions with low recombination rates, including chromosome 4 and the *Y*, play a significant role in OOA transcriptome differentiation in the male reproductive tract.

The amount of testis transcriptome differentiation exhibits considerable discordance with current estimates of the species' demographic history. For example, we observed about twice as much DE in testis between Zambian *D. melanogaster* and *D. simulans* as we did between Zambian *D. melanogaster* and American *D. melanogaster*, even though the timescale of the former is many fold greater than the latter. Additional transcriptome data from several populations in the Americas, Europe, and Africa would likely clarify the geographical and temporal patterns of phenotypic differentiation for these two tissues.

Finally, most evolutionary analysis of *Drosophila* de novo genes and novel gene expression patterns has tended to focus on a comparative/phylogenetic approach (Dickinson 1980; Ross *et al.* 1994; Begun *et al.* 2006; Levine *et al.* 2006; Begun, Holloway, *et al.* 2007; Begun, Lindfors, *et al.* 2007; Zhou *et al.* 2008; Rebeiz *et al.* 2011; Heames *et al.* 2020) or on intraspecific variation in a single population (Zhao *et al.* 2014; Cridland *et al.* 2020, 2022). Thus, the possible role of recent processes associated with colonization or local adaptation in the spread of such novelties has received little attention. The results reported here suggest the possibility that a detectable fraction of novelties expressed in the *D. melanogaster* testis or AG have been influenced by these short timescale phenomena. This raises interesting questions about (1) how the factors driving evolutionary novelty may vary with geography and the demographic and selective processes associated with range expansion across heterogeneous environments, and (2) whether the relative contributions of de novo gene expression to male reproductive tissue transcriptomes differ between African and non-African populations.

Data availability

Sequence data are available at SRA under PRJNA890638. Supplementary Material is available online at figshare: https://doi.org/10.25386/genetics.22185853.

Supplemental material available at GENETICS online.

Acknowledgments

We thank Hayley Sheehy and Libby Polston for assistance with lab work and John Pool for the flies from Zambia. We thank Rachel Thayer and other members of our lab for comments. Ben Hopkins, and two anonymous reviewers also provided several useful comments. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Funding

This work was supported by the National Institute of Health, NIH R35GM134930 awarded to D.J.B.

Conflicts of interest

The authors declare no conflict of interest.

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Editor: M. Hahn