Species often produce sterile hybrids early in their evolutionary divergence, and some evidence suggests that hybrid sterility may be associated with deviations or disruptions in gene expression. In support of this idea, many studies have shown that a high proportion of male-biased genes are underexpressed, compared with non–sex-biased genes, in sterile F1 male hybrids of *Drosophila* species. In this study, we examined and compared patterns of misexpression in sterile F1 male hybrids of *Drosophila simulans* and 2 of its sibling species, *Drosophila mauritiana* and *Drosophila sechellia*, at both the larval and adult life stages. We analyzed hybrids using both commercial *Drosophila melanogaster* microarrays and arrays we developed from reverse transcriptase–polymerase chain reactions of spermatogenesis and reproduction-related transcripts from these species (sperm array). Although the majority of misexpressed transcripts were underexpressed, a disproportionate number of the overexpressed transcripts were located on the X chromosome. We detected a high overlap in the genes misexpressed between the 2 species pairs, and our sperm array was better at detecting such misexpression than the *D. melanogaster* array, suggesting possible weaknesses in the use of an array designed from another species. We found only minimal misexpression in the larval samples with the sperm array, suggesting that disruptions in spermatogenesis occur after this life stage. Further study of these misexpressed loci may allow us to identify precisely where disruptions in the spermatogenesis pathway occur.

Introduction

Disruption of gene networks in hybrids provides a unique view of how those networks evolve in the first place. (Clark 2006)

Many empirical studies have established that natural populations harbor considerable variation in gene expression (see reviews in White 2001; Wray et al. 2003; Ranz and Machado 2006; Whitehead and Crawford 2006). Because of this high intraspecific variation, differences in gene expression are also often observed between closely related species, resulting from sequence divergence in pathways at trans- and/or cis-acting sites. One unexpected finding from the last decade is that component parts of regulatory pathways sometimes diverge through time between species or isolated populations with no accompanying change in the final phenotype, a process termed “developmental systems drift” (True and Haag 2001). Hence, although 2 species may bear a consistent phenotype, the underlying regulatory architecture may be distinct.

One consequence of developmental systems drift would be the disruption of pathways in species hybrids (see review in Ortiz-Barrientos et al. 2006). If 2 divergent regulatory architectures are brought together in a single individual, the resultant phenotype could be anomalous. Species often produce sterile hybrids early in their evolutionary divergence (see review in Coyne and Orr 2004), and there is some evidence that hybrid sterility may be associated with divergence or disruptions in “normal” gene expression. For example, the only cloned hybrid male sterility gene, *Odysseus*, bears a homeodomain region (Ting et al. 1998), and its transcript is differentially localized between sterile and fertile individuals (Sun et al. 2004). Theoretical models have also shown how the evolution of binding strength of regulatory proteins with promoter regions can provide biologically plausible and powerful postmating reproductive isolation, such as hybrid sterility (Johnson and Porter 2000; Porter and Johnson 2002). Disruptions in gene expression in species hybrids have been studied extensively in the *Drosophila melanogaster* group (Ranz et al. 2004) and particularly between *Drosophila simulans* and *Drosophila mauritiana* (Michalak and Noor 2003, 2004; Haerty and Singh 2006). Using microarrays derived from *D. melanogaster*, Michalak and Noor (2003) examined patterns of expression in *D. simulans*, *D. mauritiana*, and their sterile male F1 hybrids. They found many genes severely underexpressed in the hybrids relative to the pure species, and these were disproportionately associated with spermatogenesis or other male-specific phenotypes. This finding was confirmed using testes of male hybrids of *D. simulans* and both *D. melanogaster* and *Drosophila sechellia* (Haerty and Singh 2006). In a subsequent study, Michalak and Noor (2004) found that sterility and misexpression of 5 transcripts were strongly correlated even in fifth generation backcross hybrids. Thus, it is possible (though far from certain) that misexpression of essential genes involved in spermatogenesis caused sterility in these hybrids.

Phylogenetic studies suggest that *D. simulans* may be slightly more closely related to *D. mauritiana* than either is to *D. sechellia* (Kliman et al. 2000; Ting et al. 2000), but more loci appear to contribute to hybrid sterility between the more closely related pair (Palopoli et al. 1996). Male F1 hybrids of *D. simulans* to its sister species do not produce motile sperm, yet their testes appear normal, spermatogenesis is initiated, and meiosis is completed (Lachaise et al. 1986; Wu et al. 1992; Maside et al. 1998). Additionally, 2D gel electrophoresis performed on testes in one study found that sterile males had remarkably similar protein profiles to fertile males, with only a few specific changes, showing that there is no general breakdown in protein synthesis (Zeng and Singh 1993). Sterility in these hybrids is...
therefore believed to be associated with spermiogenic (post-meiotic) failures rather than large-scale disruptions in reproductive tissue or organ development (Wu et al. 1992).

Our study builds on earlier work in 2 significant directions. First, we use a novel microarray platform wherein we printed reverse transcriptase-polymerase chain reactions (RT–PCRs) of 48 genes known or thought to be involved in spermatogenesis or reproduction (hereafter, “sperm array”). These RT–PCRs were performed on the strains of D. simulans, D. mauritiana, and D. sechellia studied here, so sequence divergence between the array and the RNA samples does not confound the analysis. We also use the D. melanogaster Drosophila Genomics Resource Center (DGRC) array-1 (hereafter “genome array”) for comparison. Second, we examine expression both at the adult stage and at the third instar larval stage, hence potentially localizing when the disruptions occur during the development.

Materials and Methods

Drosophila Strains

Drosophila simulans Florida city, D. mauritiana Synthetic, and D. sechellia Robertson stocks were maintained on standard sugar medium at 20 °C, 12-h light–dark cycle. Virgin D. simulans females were crossed to either D. mauritiana or D. sechellia males. Virgin pure species and F1 males were collected, aged 4 days, then immediately frozen at −80 °C between 1 and 2 hours after “lights on” on the fourth day. Third instar larvae were sexed based on the presence of visible testes in males. Three trial collections were made of 4–16 male larvae per vial and were allowed to eclose to check the accuracy of the larvae-sexing procedure. After we determined that the sexing was accurate, larvae were collected at the third instar stage and frozen immediately at −80 °C.

Creation of Custom Sperm Array

All 3 alleles (D. simulans, D. mauritiana, and D. sechellia) of 48 genes known or suspected to be involved in spermatogenesis or reproduction (White-Cooper et al. 2000; Michalak and Noor 2003; Peregazga et al. 2004; White-Cooper 2004; Grumbling et al. 2006), 2 “housekeeping” genes (18SrRNA and Actin5C), and 2 genes of interest for another study (CG9775 and SHT2) were included on the custom sperm array. Note that, although our goal was to have every gene known or suspected to be involved in spermatogenesis on the array, several genes were not able to be included due to either the lack of available sequence or difficulty in amplification in our samples. The procedure for the creation of the array is described in the Supplementary Material online. The primers and amplified sequence for the array are listed in supplementary table 1, Supplementary Material online.

RNA Extraction and Hybridization to Custom and Genome-Wide Arrays

RNA extractions were performed for D. simulans, D. mauritiana, D. sechellia, and the 2 hybrids, D. simulans–D. mauritiana (sim–mau) and D. simulans–D. sechellia (sim–sec). RNA was extracted from 35 to 50 flies or 4 to 30 larvae using the Qiagen (Valencia, CA) RNeasy Mini Kit, yielding a total of, approximately, 10 μg of RNA for hybridization to the arrays.

Two array platforms were used: the custom sperm array described above and a genome-wide D. melanogaster chip (DGRC-1) purchased from the DGRC (Bloomington, Indiana), hereafter called “genome array.” The genome array contains long amplicons (100–600 bp) of 88% of the genome (12,012 genes) on Corning Gaps II printed slides. The long amplicons increased the likelihood of the slightly divergent sequence of D. melanogaster’s sibling species hybridizing. Hybridizations were performed at the Duke University Microarray Core Facility (Durham, NC) using Cy5 and Cy3 fluorescent labels. Six to 10 samples of each type (parental or F1) were hybridized in the scheme shown in figure 1. Larval male RNA was hybridized to the sperm array; adult male RNA was hybridized to either the sperm array or the genome array.

Data Analysis

Background-subtracted expression values were log2 transformed and normalized on the median for the genome array. Expression values were normalized based on Actin5C expression on the custom sperm array because we expect a shift in the median expression value for hybrids in spermatogenesis genes, which are the primary constituent of this array. For comparison, the values from the genome array were analyzed for a second time using only the genes also present on the sperm array and normalizing by Actin5C. The data were analyzed with a mixed-model analysis of variance (ANOVA) (Wolfgang et al. 2001; Cui and Churchill 2003) using PROC MIXED with SAS 9.1 software (SAS Institute, Cary, NC). The mixed-model ANOVA takes into account both fixed and random effects of variance; in this experimental model, dye and species are fixed effects, whereas array and spot are random effects. The mixed model provides a single statistical framework that includes the expression levels of a set of clones over all arrays and an analysis of whether these clones differ significantly between treatments.

A locus was considered significantly misexpressed in the hybrids if its P value was below 0.05 except where specified otherwise. Although this significance threshold may
allow for some false positives, we nonetheless wanted to fully explore the range of genes highlighted, particularly, in comparisons between the 2 hybrid classes. Misexpression was considered significant when the hybrid expression was different from both parents—it would simply be a demonstration of dominance (not misexpression) if the hybrid expression value is only significantly different from one parent. Additionally, the locus must be significantly over- or underexpressed relative to both parents because if the hybrid is in between the 2 parental values, it demonstrates additivity, not misexpression. PANTHER software (Mi et al. 2005) was used to evaluate the number of loci in each functional category for both the entire genome and the subset of misexpressed genes. Significant loci for both array platforms (genome and sperm) and both life stages (adult and larvae), as well as the representative functional classes are presented in supplementary table 2A–C, Supplementary Material online.

To examine general patterns in the microarray data and to compare different hybridizations, the expression levels of the hybrids were compared with the mean expression levels of the parental species. The log10 of the ratio of the normalized gene expression level of the hybrids to the mean expression level of the 2 parental species was calculated. This value provided a measure of the deviation in expression level of the hybrid from both parents. Regressions (α = 0.05) of this hybrid–midparent value were performed in Microsoft Excel to compare different hybridizations. Comparisons of 1) hybridizations to the D. melanogaster probes on the genome array versus D. simulans probes on the sperm array and 2) hybridizations to heterospecific (D. mauritiana or D. sechellia) probes versus conspecific (D. simulans) probes on the sperm array were done to identify effects of sequence divergence on array hybridization between these closely related species. Comparisons were also done for hybridizations of larval RNA versus hybridizations of adult RNA. We excluded both the control genes (Actin5C and 18S rRNA), which were used for normalization, and gonadal, which was only the gene to give contradictory results between the platforms and therefore sampled the 47 remaining genes with both platforms.

Results

Misexpressed Loci in Hybrids

We identified 30 genes as overexpressed and 190 genes underexpressed, based on hybridizations to the D. melanogaster genome array in D. simulans–D. sechellia (sim–sec) hybrids and 63 genes overexpressed and 505 genes underexpressed in D. simulans–D. mauritiana (sim–mau) hybrids (supplementary table 2C, Supplementary Material online). Of these loci, 128 were misexpressed in both sets of hybrids, and all but 4 of these transcripts were those that were underexpressed compared with the parental species.

There was an overall trend of genes that are misexpressed to be expressed at lower levels in hybrids than in the parental species (see also Michalak and Noor 2003; Haerty and Singh 2006). Additionally, 18.9% of the genome showed strong dominance in their expression, with an equal amount showing D. simulans–like expression (1,127 loci) as D. sechellia–like expression (1,140). In the sim–mau hybrids, 2852 transcripts appeared to show dominance, with a greater amount showing D. simulans–like expression (1,771) than D. mauritiana–like expression (1,081).

Although 16.7% of the genes on the genome array are located on the X chromosome, only 9.1% of the sim–sec and 6.2% of the sim–mau misexpressed genes were on the X chromosome (table 1). There is a disproportionately small number of misexpressed loci on the X, even when we make the conservative comparison to genes that have testis-specific expression, of which 10.6% are located on the X in D. melanogaster (Parisi et al. 2004). Of greater interest, however, is that a disproportionate number of genes exhibited misexpression via overexpression on the X. For those genes located on the X chromosome, 55% of the genes that were significantly misexpressed in sim–sec and 34.3% of those in sim–mau were overexpressed, whereas only 9.5% (sim–sec) and 9.3% (sim–mau) of misexpressed genes on the autosomes were overexpressed.

Of the genes that were misexpressed in hybrids, genes involved in transcription were underrepresented (6.9% genome vs. 2.2% sim–sec, 1.6% sim–mau), as were genes involved in DNA binding (6.4% vs. 1.4%, 2.4%) (supplementary table 2C, Supplementary Material online). Functional categories that were more prevalent than expected include chaperones (4.3× more for sim–mau misexpressed genes) and protein folding (4.5×, sim–mau), mitochondrial transport (5.2×, sim–sec; 5.1×, sim–mau), calcium binders (3.1×, sim–sec; 3.1×, sim–mau), microtubule-associated proteins (3.6×, sim–sec; 3.8×, sim–mau), epimerase/racemase (3.1×, sim–sec), and apoptosis (3×, sim–sec).

When we compare functional categories of misexpressed loci with those prevalent for testis-specific loci in D. melanogaster (Parisi et al. 2004), the same trends generally hold, with 2 exceptions: there is no longer an underrepresentation of loci involved in transcription, as this category is less prevalent for testis-specific genes, and loci for DNA binding are overrepresented as misexpressed in hybrids. However, this comparison to D. melanogaster is somewhat contrived, as some misexpression may be unrelated to the testes or male fertility or both.

We also detected misexpression in adult hybrid males using the D. simulans probe of our sperm array (supplementary table 2B, Supplementary Material online). Based on results from these probes, we found 1 gene (tra2) to be significantly overexpressed and 7 genes significantly underexpressed in sim–mau hybrids. In sim–sec hybrids,
we found 2 genes to be significantly overexpressed and 11 genes underexpressed. Assignments of misexpression were relatively robust to significance threshold: even at the conservative Bonferroni adjusted $P < 0.000005$, 4 genes were still significantly misexpressed in sim–mau hybrids and 4 genes in sim–sec hybrids (dj was still significantly underexpressed in both), as assayed with these probes on the sperm array.

Commonalities in Misexpression between Species Hybridizations

We sought to determine whether there was disproportionate overlap in the sets of misexpressed genes between the 2 species hybridizations. Such overlap would indicate commonalities in the regulatory networks disrupted in species hybrids (but see Discussion). As our sets of misexpressed genes from the sperm array were very small, we performed this analysis on the results from the genome array. We found a large and statistically significant overlap in the genes disrupted in hybrids from the 2 crosses. This overlap was still significant when we excluded all genes expressed in one or more of the pure species at a level more than twofold below the mean hybridization intensity across the array (see table 2), hence accounting for possible artifacts resulting from genes not truly expressed in adult males. The overlap was particularly large for the sets of underexpressed genes: 25–65% of genes significantly undervexpressed in 1 hybridization were also undervexpressed in the other (the percentage depends on which direction the comparison is made). Furthermore, among the 65 genes significantly undervexpressed in only the sim–sec hybridization, 43 were expressed at levels below both parental species in the sim–mau hybridization (but not significantly so). Similarly, among the 366 genes significantly undervexpressed only in the sim–mau hybridization, 282 were expressed at levels below both parental species in the sim–sec hybridization (but not significantly so). Therefore, our analyses may greatly underestimate the extent of overlap in misexpression between these 2 hybridizations.

Table 2
Overlap in Adult Hybrid Male Misexpressed Transcripts between Drosophila simulans–Drosophila mauritiana and D. simulans–Drosophila sechellia Hybridizations, after Eliminating Genes Expressed in One of the Pure Species at a Level More than Twofold below the Mean Hybridization Intensity across the Array

<table>
<thead>
<tr>
<th>Underexpressed transcripts</th>
<th>D. simulans–D. sechellia</th>
<th>D. simulans–D. mauritiana</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>9214</td>
<td>366</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Overexpressed transcripts</td>
<td>D. simulans–D. sechellia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>9693</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>$P = 0.0073$</td>
<td></td>
</tr>
</tbody>
</table>

Effects of Developmental Stage

Using our sperm array, we detected much less misexpression in hybrid male third instar larvae than in the adult hybrid males. Based on hybridization to the D. simulans probe, the only gene significantly misexpressed in hybrids relative to pure species was Mst84Dc, which was underexpressed in sim–mau hybrids. A few other genes were misexpressed, when all probes on the sperm array were considered: Odsh in sim–mau, roxl and Mst84Dc in sim–sec.

Given the small number of significantly misexpressed genes above, we also examined the overall general trend in hybrid expression in larvae of transcripts shown to be underexpressed in adult hybrids. In the D. simulans–D. mauritiana hybridization, 3 of the 7 transcripts significantly underexpressed in adult hybrids were underexpressed (non-significantly) in larvae (see supplementary table 2A, Supplementary Material online). This representation is greater than expected by chance ($P = 0.01$). However, in the D. simulans–D. sechellia hybridization, only 2 of 11 significantly underexpressed transcripts were nonsignificantly underexpressed in larvae, which was the proportion expected by chance.

We also compared in adults and larvae the overall estimated levels of expression in hybrids relative to the midparent of the 2 parental species using the D. simulans probes for each gene. This was done to assess whether there is a correlation between larval and adult relative expression values in hybrids. In the D. simulans–D. mauritiana hybridization, deviation from midparent expression in adult hybrids was significantly correlated with that in larval hybrids ($r = 0.615; P < 0.0001$; see supplementary fig. 1, Supplementary Material online). However, in the D. simulans–D. sechellia hybridization, deviation from midparent expression was not significantly correlated between adult and larval hybrids ($r = 0.214; P = 0.128$; see supplementary fig. 1, Supplementary Material online).

Technical Comparison of Microarray Platforms and Probes

We compared the success of the D. simulans probe in detecting misexpression with that of the D. melanogaster probe on the genome array to identify effects of sequence divergence on array hybridization. We acknowledge that this comparison is imperfect because of the use of different platforms. In both hybridizations, more genes were suggested to be misexpressed based on the sperm array, than based on the D. melanogaster genome array (table 3). This difference was statistically significant in the D. simulans–D. sechellia hybridization ($P = 0.0005$) but not in the D. simulans–D. mauritiana hybridization. Further, in both cases, no single transcript was found to be significantly misexpressed by the D. melanogaster genome array that was also not found to be misexpressed using the sperm array. Overall expression levels in hybrids relative to the midparent (mean expression of each of the parent species) was strongly correlated in the D. simulans–D. mauritiana hybridization ($r = 0.595, P < 0.0001$) but not in the D. simulans–D. sechellia hybridization ($r = 0.181, P = 0.23$) between the 2 platforms, with the genome array detecting
Table 3
Number of Genes Showing Statistically Significant Misexpression via a Mixed-Model ANOVA in F1 Adult Male Hybrids Compared with Parents for the Different Microarray Platforms and Probes

<table>
<thead>
<tr>
<th>Hybridization</th>
<th>D. simulans</th>
<th>D. mauritiana</th>
<th>D. sechellia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genome array versus sperm array</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No misexpression detected</td>
<td>38</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>D. melanogaster probe</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>D. simulans probe misexpression only</td>
<td>4</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Both probes misexpression</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Probes within sperm array</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No misexpression detected</td>
<td>34</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>“Foreign” D. simulans group species probe misexpression only</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>D. simulans probe misexpression only</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Both probes misexpression</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

Numbers Indicate Genes for Which the Higher Intensity Was Noted for the Heterospecific Spot, and the Second Indicate Genes for Which a Higher Hybridization Intensity 

Table 4
Relative Hybridization Intensity in Heterospecific: Conspecific Spots on Sperm Array. The First Numbers Indicate Genes for Which a Higher Hybridization Intensity Was Noted for the Heterospecific Spot, and the Second Numbers Indicate Genes for Which the Higher Intensity Was Noted for the Conspecific Spot

<table>
<thead>
<tr>
<th>RNA Source</th>
<th>D. simulans</th>
<th>D. mauritiana</th>
<th>D. sechellia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foreign probe</td>
<td>22:28</td>
<td>19:31</td>
<td></td>
</tr>
<tr>
<td>D. simulans</td>
<td>16:34</td>
<td>—</td>
<td>21:29</td>
</tr>
<tr>
<td>D. sechellia</td>
<td>23:27</td>
<td>29:21</td>
<td>—</td>
</tr>
</tbody>
</table>

(i.e., over- or underexpressed) in these cases, even though it was not statistically significant.

Discussion

We have approached the genetic basis of hybrid dysfunction by identifying genes and pathways that are misexpressed in hybrids relative to pure species, using the D. simulans species complex as a model system. Consistent with the findings of previous studies in this species group (Michalak and Noor 2003; Haerty and Singh 2006), we observed that genes that were misexpressed in hybrids of these Drosophila species tended to be underexpressed relative to the parental species. If F1 hybrids bore shrunken testes relative to parental species, then finding a preponderance of underexpressed loci could reflect the smaller proportion of mRNA derived from testes relative to the rest of the body. However, based on our dissections and multiple published studies (e.g., Coyne 1984; Thomas and Singh 1992), testes do not differ in size or shape between these pure species and their F1 hybrids. Further, some testes-specific transcripts were expressed in hybrids at levels virtually identical to one or both pure species in our whole body assays (e.g., Crtp and CG7813; Boutanaev et al. 2002).

We also observed that, although a smaller number of X chromosomal loci than expected were misexpressed, a disproportionate number of overexpressed loci were located on this chromosome. Although we have no compelling explanations for the cause of this pattern, we note that the one characterized hybrid male sterility gene, OdysseusH, is an X chromosome locus believed to cause disruptions in spermatogenesis via a gain of expression in the testes (Sun et al. 2004). To elucidate at which precise stage of spermatogenesis disruptions occur, we have overlaid the misexpression results onto part of the known spermatogenesis pathway for D. melanogaster (fig. 2). Although this is a preliminary attempt at identifying points within the developmental network at which misexpression occurs, we note the large number of late-stage downstream loci exhibiting misexpression, whereas relatively few early-stage loci are misexpressed. This is an exciting first step toward identifying the causal stage of disruptions in spermatogenesis and it also supports our suggestion that the disruptions in spermatogenesis occur postmeiotically and are not due to an overarching failure of spermatogenesis or organ development.

Few loci involved in transcription or DNA binding were misexpressed in hybrids, suggesting either a constraint...
on the evolution of these loci or a robustness of the expression of these loci in spite of perturbations. Conversely, a larger number of misexpressed loci than expected were involved in protein folding, chaperone function, mitochondrial transport, calcium binding, microtubule function, epimerase/racemase function, and apoptosis. These over-represented categories could reflect divergent evolution of function, which directly or indirectly affects hybrid sterility, or a shift in expression with no bearing on the sterile hybrid phenotype. They could also be downstream effects of sterility (e.g., a reduction in sperm production could cause a reduction in expression of certain types of genes), and it is quite possible (indeed likely) that the underexpression of downstream factors in spermatogenesis is due to failures at earlier steps in the spermatogenesis pathway. Therefore, at this stage one can only speculate as to the significance of the presence or absence of specific functional categories.

Comparison between Species Hybridizations

Although differences in gene expression between species have been studied extensively (see review in Ortiz-Barrientos et al. 2006), including several studies in *Drosophila* (e.g., Ranz et al. 2003; Rifkin et al. 2003), only one other study (Haerty and Singh 2006) has compared misexpression of a broad range of transcripts in hybrids across multiple-species hybridizations. In this study, we compared genome-wide patterns of expression in 2 species hybridizations also examined by Haerty and Singh (2006): *D. simulans–D. mauritiana* and *D. simulans–D. sechellia*.

We detected significantly more misexpressed genes (both over- and underexpressed) relative to the parental species in hybrid males of the *D. simulans–D. mauritiana* cross than in hybrids of the *D. simulans–D. sechellia* cross. This result is consistent with genetic studies of hybrid sterility in these species groups (Palopoli et al. 1996). However, the tendency for genes significantly underexpressed in hybrids of *D. simulans–D. mauritiana* to be at least nonsignificantly underexpressed in hybrids of *D. simulans–D. sechellia* suggests that we may be underestimating the number of genes misexpressed in the latter hybridization.

In contrast to the results of Haerty and Singh (2006), we found extensive overlap (128 loci) in the sets of genes significantly misexpressed in hybrids of the species pairs. Our finding suggests some commonalities in the disrupted regulatory pathways between the 2 hybridizations. There are at least 3 possible explanations for the discrepancy between our results and the previous study. First, we assayed whole bodies rather than testes alone, and much of the misexpression we detected may be specific to some tissue besides the testes. However, a disproportionate number of misexpressed genes in these hybridizations are male specific, with many involved in spermatogenesis (Michalak and Noor 2003, 2004; Haerty and Singh 2006), so this explanation seems unlikely. Second, it is possible that the inconsistency is due to the different array platforms; however, both platforms contained long cDNAs, so length of binding substrate should not be a factor. Lastly, many recent studies have shown that a minimum of 5 replicates is necessary for statistically analyzing microarray results (e.g., Pavlidis et al. 2003; Allison et al. 2006), and therefore our study with a larger sample size may have been better able to detect overlapping misexpressed genes. Consistent with this hypothesis, our real-time PCR assays of one gene, CG5762, surveyed by both studies found it to be underexpressed in hybrids relative to pure species when surveyed with RNA derived from both whole bodies and testes alone (whole bodies: see Michalak and Noor 2003, 2004; testes: *sim–mau* hybrids express this gene at about 66% pure species level; Teeter K, Noor M, unpublished data). Our microarray assays also found this gene to be underexpressed (see table 5), whereas the analyses of Haerty and Singh (2006) failed to identify significant underexpression in this gene.

Our results suggest that there are indeed significant commonalities in patterns of expression in hybrids relative to pure species between these 2 species groups, at least when whole bodies are examined. That said, commonality in patterns of misexpression does not suggest that the same genes were involved in speciation. Expression studies such as these may be sampling genes near the end of long

### Table 5

<table>
<thead>
<tr>
<th>Species Pair</th>
<th>Mean</th>
<th>Variance</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Drosophila simulans</em></td>
<td>1.065</td>
<td>0.064</td>
<td>0.254</td>
</tr>
<tr>
<td><em>Drosophila mauritana</em></td>
<td>0.982</td>
<td>0.039</td>
<td>0.197</td>
</tr>
<tr>
<td><em>F₁</em> <em>sim–mau</em></td>
<td>0.666</td>
<td>2.41E-5</td>
<td>0.005</td>
</tr>
</tbody>
</table>
regulatory networks that are disrupted at different points in the 2 hybridizations. There is no reason to assume that the panel of misexpressed genes reflects the specific failed interactions causing misexpression and possibly sterility (Noor and Feder 2006; Ortiz-Barrientos et al. 2006) but does suggest that similar pathways may be involved.

Comparison between Life Cycle Stages

Changes in gene expression occur throughout the life cycle of all species, and such changes have been studied extensively in D. melanogaster (e.g., Arbeitman et al. 2002). Differences in expression between species are also apparent within life cycle stages (e.g., Rifkin et al. 2003; Goodisman et al. 2005). Given this variation, some hybrid expression disruptions may only be observed at particular life cycle stages. Further, examining multiple stages may suggest when in development particular disruptions first occurred, possibly giving hints as to the underlying causes.

We found very little misexpression in third instar hybrid male larvae from both species crosses. Underexpression of Mst84Dc is evident this early in development, but most other disruptions are not apparent until the adult stage. We further noted a disconnection between relative expression levels in adult and larval hybrids in the D. simulans–D. sechellia hybridization (see supplementary fig. 1, Supplementary Material online). This disconnection may suggest that, especially for sim–sec hybrids, most misexpression appears later in development, but other explanations are also possible.

Based on cytological studies, some disruptions should be apparent in hybrid third instar larvae. Many postmeiotic gametes are apparent in the testes of D. simulans third instar larvae (Maside et al. 1998). However, only about half of third instar larval hybrids between D. simulans and D. mauritiana have produced sperm passing meiosis (Maside et al. 1998), suggesting that delays or defects in spermatogenesis resulting in hybrid sterility are already apparent at this life cycle stage. Transcription of genes scored on the sperm array has also been detected in pure species at this stage (Arbeitman et al. 2002), and for a few of the transcripts surveyed, even at the embryonic stage (Mukai et al. 2006), and so the opportunity for misexpression was certainly present.

Comparison between Microarray Platforms

The vast majority of studies comparing gene expression between species have been performed on single-species arrays, so that it has been impossible to distinguish differential hybridization due to sequence mismatches from underlying expression differences. Recently, Gilad et al. (2005) used a “multispecies” primate cDNA array using probes from human, chimpanzee, orangutan, and rhesus. They found a large effect of sequence divergence on hybridization signal, even in the most closely related species pair, humans and chimpanzees. They concluded that “naive use of single-species arrays in direct interspecies comparisons can yield spurious results.”

To eliminate this concern from our experiments, we used both the DGRC-1 genome-wide D. melanogaster microarray as well as a sperm array that we constructed using RT–PCRs from the same lines of D. simulans, D. mauritiana, and D. sechellia from which RNA was isolated. As a result, there was absolutely no sequence divergence between at least 1 of the 3 probe types for all of the sperm array transcripts and the hybridized RNA.

When the same normalization procedures were applied to the data from both arrays, no genes were identified as misexpressed using the genome array that were not identified as such with the sperm array, whereas the converse was not true. Several of the transcripts suggested to be misexpressed by the sperm array data but not by the genome array have been confirmed previously via real-time PCR to be misexpressed in these hybrids (e.g., Mst84Dc and Mst98Cb: see Michalak and Noor 2003), lending support for the underexpression documented by the sperm array. Taken together, this difference suggests that our sperm array was generally more sensitive to expression differences. It is tempting to speculate that this difference results in part from effects of sequence divergence from D. melanogaster, but this conclusion is not altogether fair because the platforms were constructed separately and may bear other subtle differences affecting sensitivity.

We did observe a nonsignificant trend for RNAs to hybridize better to conspecific spots than heterospecific spots among the probes on the sperm array, suggesting that despite the close relationships of the 3 species represented, there may be some slight effect of sequence divergence on hybridization. However, we did not detect substantial differences in detection of misexpression among the probes within our sperm array (see tables 3 and 4), though this negative result may have been affected by variation in the quality of the PCR products used or their spotting onto the array or the fact that these 3 species are much more closely related to each other than they are to D. melanogaster. Unlike the comparison to the genome array, significant misexpression was sometimes detected using data from “foreign” probes, which was not detected from the D. simulans probe. It is possible that analysis from our sperm array still underestimated the extent of misexpression in these hybrids and that the different probes were only sampling from a larger pool of affected transcripts. Indeed, at least 1 transcript was shown previously by real-time PCR to be underexpressed in sim–mau hybrids but was not detected as significantly underexpressed using the probes from any of our platforms (aly: see Noor 2005).

Conclusion

Several lines of evidence suggest that failures in transcriptional regulation may be associated with hybrid incompatibilities, such as hybrid sterility. In most Drosophila species hybrids, transcription of genes involved in spermatogenesis is premeiotic (Fuller 1998), whereas the cytological disruptions associated with hybrid male sterility appear postmeiotically (Wu et al. 1992; Coyne and Orr 2004). This suggests that the opportunity for transcription was present in sterile hybrids, and the observed underexpression of many transcripts may not be a simple artifact of sterility. Here, we have shown extensive commonalities in patterns of misexpression across 2 species hybridizations. We have also shown that much of the hybrid misexpression
is not apparent until late in development, though a few transcripts are underexpressed in hybrid larvae. Further examination of expression differences between sterile and fertile later-generation hybrids may help to elucidate which (if any) of the misexpressed transcripts may be directly or indirectly associated with hybrid male sterility and what upstream regulatory factors are causing misexpression (e.g., Michalak and Noor 2004).

Supplementary Material

Supplementary figures 1–3, 2 Microsoft Excel files (supplementary tables 1 and 2), and description of construction of sperm array are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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