Abstract

**Background:** Interspecific hybrids of frogs of the genus *Xenopus* result in sterile hybrid males and fertile hybrid females. Previous work has demonstrated a dramatic asymmetrical pattern of misexpression in hybrid males compared to the two parental species with relatively few genes misexpressed in comparisons of hybrids and the maternal species (*X. laevis*) and dramatically more genes misexpressed in hybrids compared to the paternal species (*X. muelleri*). In this work, we examine the gene expression pattern in hybrid females of *X. laevis* × *X. muelleri* to determine if this asymmetrical pattern of expression also occurs in hybrid females.

**Results:** We find a similar pattern of asymmetry in expression compared to males in that there were more genes differentially expressed between hybrids and *X. muelleri* compared to hybrids and *X. laevis*. We also found a dramatic increase in the number of misexpressed genes with hybrid females having about 20 times more genes misexpressed in ovaries compared to testes of hybrid males and therefore the match between phenotype and expression pattern is not supported.

**Conclusion:** We discuss these intriguing findings in the context of reproductive isolation and suggest that divergence in female expression may be involved in sterility of hybrid males due to the inherent sensitivity of spermatogenesis as defined by the faster male evolution hypothesis for Haldane’s rule.

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**Background**

Frogs of the genus *Xenopus* provide a striking exception to the most widespread generalization in evolutionary biology-Haldane’s rule [1-6]. Haldane’s rule states that the heterogametic sex (XY or ZW) typically suffers the most dysfunctional effects of interspecific hybridization [7] and the broad applicability of Haldane’s rule across diverse groups of organisms suggests that common mechanisms may underlie postzygotic reproductive isolation [5]. *Xenopus* have ZW sex determination and Haldane’s rule would predict that hybrid females should suffer the most dramatic effects of hybridization but contrary to expectation, F1 hybrid males are completely sterile and hybrid females are fertile [1,2].

Analyses of spermatogenesis in hybrid males of *X. laevis* × *X. muelleri* have shown that males have a dramatically lower abundance of motile sperm, increased numbers of undifferentiated sperm cells, and larger mature sperm cells compared to parental species [2]. The gene expres-
sion pattern for hybrid males shows a striking asymmetric pattern in that relatively few genes are differentially expressed between hybrids and the maternal species (X. laevis) whereas there are dramatically more genes differentially expressed between hybrid males and the paternal species, X. muelleri. These results suggest intriguing mechanisms operating on the transcriptome in hybrid males of Xenopus that may reflect strong maternal and/or species dominance effects [2].

Hybrid females are just as fertile as conspecific species [1] and given the phenotype of hybrid females, a reasonable prediction would be that gene expression should be similar compared to the two parental species. However, given the asymmetrical pattern of expression operating in hybrid males, it is of interest to investigate the pattern of gene expression in hybrid oogenesis, particularly since oogenesis in hybrids does not seem to be affected by the hybrid genetic background compared to hybrid males.

In this study, we analyzed the gene expression pattern of adult ovary in hybrid females of X. laevis × X. muelleri compared to the two parental species. Our analyses reveal a pattern of asymmetrical gene expression like that in testes of hybrid males but surprisingly there is a dramatic increase in the number of genes misexpressed in hybrid female ovaries compared to the two parental species relative to hybrid males. This increased level of gene misexpression suggests that oogenesis can tolerate dramatically more misexpression compared to spermatogenesis and points further evidence to the sensitive spermatogenesis component of the faster male evolution hypothesis for Haldane’s rule.

Results
There was a substantial amount of differential expression in hybrid ovary compared to the ovaries of the two parental species. Using adjusted significance tests (P < 0.05), about 14% (1,616/11,485) of genes were differentially expressed in hybrid females compared to females of X. laevis and 63% (7,279/11,485) of genes were differentially expressed between hybrids and X. muelleri (Fig. 1). The number of genes upregulated in hybrids relative to X. laevis compared to the number of genes upregulated in X. laevis relative to hybrids was the same (839 vs. 777; G = 2.38; df = 1; P > 0.05) but there were significantly more genes upregulated in X. muelleri compared to hybrids (4,349 vs. 2,930; G = 139.2; df = 1; P < 0.0001). Many of the top 30 most differentially expressed genes for each class of gene expression behavior are expressed sequence tags (ESTs) with little functional information but our results imply that these sequences play a role in oogenesis in Xenopus. Of the top 30 candidate genes with known function many have a documented role in oogenesis in other organisms (Table 1, 2, 3, 4). Comparing the two lists of differentially expressed genes showed that about 68% (1105/1616) were common to both X. laevis vs. hybrids and X. muelleri vs. hybrids. This common set of differentially expressed genes suggests a set of genes that are uniquely expressed in hybrids relative to the two parental species.

Gene expression between the two parental species was also dramatically different. More than 76% (8,741/11,485) of genes were differentially expressed between females of X. laevis and X. muelleri. Of these differentially expressed genes, about 60% (5,203/8,741) were upregulated in X. muelleri relative to X. laevis (5,203 vs. 3,538; G = 159.5; df = 1; P < 0.0001). Comparing the overlap in genes differentially expressed in the two hybrid contrasts to the three classes of expression behavior between X. laevis and X. muelleri (X. laevis > X. muelleri; X. laevis <X. muelleri; X. laevis = X. muelleri) shows a general pattern of semidominance in expression behavior (Table 5). For example, of the 839 genes upregulated in hybrids relative to X. laevis; 90% were upregulated in X. muelleri relative to X. laevis. Similarly, of the 2,930 genes that were upregulated in hybrids relative to X. muelleri, 91% were upregulated in X. laevis compared to X. muelleri. These results suggest a general pattern of intermediate expression in hybrids and are consistent with a semidominant model of expression difference even despite the asymmetrical pattern of misexpression in hybrids compared to the two parental species.

Discussion
Our analysis of hybrid females relative to the two parental species provides key insight into the process of oogenesis in hybrid females and the two parental species. There is an asymmetrical pattern of differential expression with about 4.5 times more genes differentially expressed between hybrids and Xenopus muelleri compared to X. laevis. This result implies that strong maternal and/or species dominance effects act in oogenesis and these are reflected in the hybrid transcriptome. Hybrid females have a general pattern of semidominance in gene expression with the majority of genes being expressed at intermediate levels compared to the two parental species. Finally, there is a dramatic divergence in gene expression in the ovary between the two parental species with more than 76% of genes differentially expressed between X. laevis and X. muelleri. This suggests that the process of oogenesis differs widely at the gene expression level between these two species of Xenopus.

It is important to consider the methodology used to gather the samples of RNA for this study. Samples of ovary (50 mg portions) were dissected and then homogenized in RNA extraction solution. Therefore, we gathered a sample of ovary rather than the entire ovary and this sample is a heterogeneous representation of oogenesis, rather than
Volcano plots of gene expression. Volcano plots from FDR corrected t-tests of statistical significance (vertical axis) against magnitude of expression change (horizontal axis), where each point corresponds to a gene/transcript. Expression change (fold-change) is defined as a log₂-transformed ratio of mean nonhybrid to mean hybrid expression level. (A) *Xenopus laevis* (L) vs. Hybrids (H); (B) *Xenopus muelleri* (M) vs. Hybrids (H). The red horizontal line denotes FDR adjusted alpha 0.05. The horizontal deviation from 0 towards the right or left reflects hybrid underexpression or overexpression, respectively.
Table 1: Candidate transcripts upregulated in *X. laevis* compared to hybrids.

| ProbeID       | GenBankID | Target Gene | Gene Symbol | Description/Molecular Function                                                                 | Mean Laevis | SD Laevis | Mean Hybrid | SD Hybrid | L-H | adj.P.Val |
|---------------|-----------|-------------|-------------|------------------------------------------------------------------------------------------------|-------------|-----------|-------------|-----------|-----|----------|
| XI.1473.1.A1_at| AW147858  | EST         |            | Weakly similar to hypothetical protein MGC3731 (H. sapiens)                                        | 10.18       | 0.56      | 4.28        | 0.43      | 5.90| 0.0041   |
| XI.1484.1.A1_at| B1051331  | EST         | MGC84382   | Intracellular signaling cascade                                                                     | 7.33        | 0.54      | 3.01        | 0.25      | 4.32| 0.0041   |
| XI.1487.4.A1_at| BM180490  | EST         |            | Highly similar to T2DL_HUMAN TRANSCRIPTION INITIATION FACTOR, TFII D 250 KD SUBUNIT                   | 7.35        | 0.35      | 3.29        | 0.54      | 4.06| 0.0041   |
| XI.1458.1.S1_at| BG730898  | EST         |            | Weakly similar to A39599 55K erythrocyte membrane protein – human (H. sapiens)                       | 10.12       | 0.30      | 6.10        | 0.18      | 4.02| 0.0041   |
| XI.1521.1.A1_at| CB562594  | EST         | LOC398314  | Dihydrofolamide acetyltransferase                                                                  | 7.34        | 0.40      | 3.45        | 0.37      | 3.88| 0.0041   |
| XI.1612.1.1_at| BJ079520  | EST         | LOC494727  |                                                                                                   | 9.51        | 0.42      | 5.70        | 1.02      | 3.81| 0.0072   |
| XI.23885.1.S1_at| BG388097  | EST         |            | Weakly similar to FWIA_HUMAN F-box WD-repeat protein                                               | 7.39        | 0.34      | 3.63        | 0.77      | 3.76| 0.0057   |
| XI.3628.1.A1_at| BG023013  | EST         |            |                                                                                                   | 7.18        | 1.34      | 3.52        | 0.77      | 3.66| 0.0325   |
| XI.24509.1.A1_at| B078115   | EST         | MGC132176  | Heme oxygenase (deacylizing) activity                                                              | 9.04        | 0.30      | 5.50        | 0.56      | 3.53| 0.0041   |
| XI.14330.2.S1_at| BG019901  | EST         | MGC85135   | Nucleic acid binding                                                                              | 8.63        | 0.27      | 5.34        | 0.36      | 3.29| 0.0044   |
| XI.10703.1.A1_at| BU051566  | EST         | LOC495457  | Weakly similar to A36368 transcription factor, CBF, CCAAT-binding – (H. sapiens)                   | 8.67        | 0.34      | 5.45        | 0.79      | 3.22| 0.0646   |
| XI.1973.1.S1_at| BG385313  | EST         | MGC78898   | Ubiquitin-protein ligase activity                                                                  | 7.41        | 0.19      | 4.19        | 0.39      | 3.22| 0.0041   |
| XI.4825.1.A1_at| BG514303  | EST         | MGC82215   |                                                                                                   | 8.16        | 0.48      | 5.64        | 0.32      | 3.15| 0.0057   |
| XI.16512.1.1_at| BJ050532  | EST         | LOC49516   |                                                                                                   | 7.03        | 0.48      | 3.91        | 1.11      | 3.12| 0.0142   |
| XI.1448.2.S1_a_at| BG408234  | EST         |            | Moderately similar to palladin; pallid (mouse) homolog, palladin (H. sapiens)                     | 7.59        | 0.44      | 4.50        | 1.91      | 3.10| 0.0325   |
| XI.16106.1.A1_at| BG161783  | EST         | LOC495116  |                                                                                                   | 8.24        | 0.23      | 5.18        | 0.69      | 3.05| 0.0057   |
| XI.25509.1.A1_at| B1084126  | EST         | LOC495259  |                                                                                                   | 6.09        | 0.34      | 3.09        | 0.23      | 3.00| 0.0044   |
| XI.25610.1.A1_at| B0225299  | EST         | LOC495295  | Similar to thyroid hormone receptor associated protein 3 (predicted) [Rattus norvegicus]           | 7.16        | 0.09      | 4.19        | 0.77      | 2.98| 0.0057   |
| XI.10828.1.A1_at| CB560349  | EST         | MGC82938   | Moderately similar to AAKG_HUMAN 5-AMP-activated protein kinase, (H. sapiens)                      | 8.37        | 0.31      | 5.42        | 0.37      | 2.95| 0.0047   |
| XI.8907.1.S1_at| AW632884  | EST         |            | Xenopus laevis, Similar to proliferation-associated 2G4                                             | 6.95        | 0.49      | 4.00        | 1.03      | 2.95| 0.0148   |
| XI.6727.1.A1_at| BG334472  | EST         | LOC780954  | Regulation of transcription, DNA-dependent                                                         | 7.28        | 0.75      | 4.33        | 0.33      | 2.95| 0.0149   |
| XI.2780.1.S1_at| BG400343  | TIA1        | tial1-a     | Nucleic acid binding                                                                              | 6.41        | 0.58      | 3.52        | 0.64      | 2.89| 0.0124   |
| XI.16426.1.A1_at| B1045456  | EST         | LOC495465  |                                                                                                   | 6.58        | 0.40      | 3.71        | 0.46      | 2.87| 0.0063   |
| XI.6421.1.A1_at| AW633197  | EST         | MGC68457   |                                                                                                   | 8.68        | 0.38      | 5.82        | 0.21      | 2.86| 0.0057   |
| XI.22941.1.A1_at| B1048949  | EST         | MGC83726   | Moderately similar to A57088 nucleoprotein-like protein Rbregulation of GTPase activity           | 6.79        | 0.15      | 3.98        | 0.43      | 2.81| 0.0041   |
| XI.25368.2.A1_at| BI444111  | EST         | MGC69123   | Weakly similar to IFR1_HUMAN INTERFERON-RELATED DEVELOPMENTAL REGULATOR I (H. sapiens)           | 6.21        | 0.61      | 3.42        | 0.56      | 2.79| 0.0139   |
| XI.1479.1.A1_at| BM179492  | EST         | MGC82526   |                                                                                                   | 7.10        | 0.69      | 4.32        | 0.68      | 2.78| 0.0182   |
| XI.19146.3.A1_at| BJ057192  | EST         | MGC81986   |                                                                                                   | 7.88        | 0.52      | 5.11        | 0.90      | 2.78| 0.0151   |

Top 30 candidate transcripts upregulated in *X. laevis* and differentially expressed between females of *X. laevis* and hybrids. Expression values are in log2 scale; SD = standard deviation of expression values. P values are adjusted according to FDR moderated t-tests.
Table 2: Candidate transcripts upregulated in hybrids compared to *X. laevis*.

| ProbeID          | GenBankID          | Target Gene       | GeneSymbol | Description/Molecular Function                                                                 | Mean Laevis | SD Laevis | Mean Hybrid | SD Hybrid | L-H   | adj.P.Val |
|------------------|--------------------|-------------------|------------|-------------------------------------------------------------------------------------------------|-------------|-----------|-------------|-----------|-------|-----------|
| Xl.4605.1.A1_at  | BG552470           | EST               | MGC83384   |                                                                                                 | 3.41        | 0.39      | 7.63        | 0.44      | -4.22 | 0.0041    |
| Xl.19075.1.A1_at | BI675584           | EST               |            |                                                                                                 | 5.53        | 1.29      | 9.67        | 0.27      | -4.14 | 0.0205    |
| Xl.24502.1.S1_at | BI098608           | EST               |            | Weakly similar to forhead box P2; (H. sapiens)                                                   | 4.56        | 0.70      | 8.65        | 0.00      | -4.09 | 0.0060    |
| Xl.23573.1.S1_at | BC041496.1         | thymine-DNA glycosylase | TDG       | Hydrolase activity, acting on glycosyl bonds                                                    | 5.55        | 1.16      | 9.22        | 0.15      | -3.67 | 0.0210    |
| Xl.16239.1.A1_at | BI039922           | EST               |            |                                                                                                 | 5.53        | 1.36      | 9.67        | 0.27      | -4.27 | 0.0405    |
| Xl.8630.1.S1_at  | BC045722.1         | MGC53990          | MGC53990   | Similar to serum-inducible kinase; protein serine/threonine kinase Act.                         | 4.74        | 0.36      | 8.01        | 0.75      | -3.26 | 0.0062    |
| Xl.21956.1.S1_at | BC042271.1         | MGC53461          | MGC53461   | Highly similar to alpha cardiac actin (H. sapiens)                                              | 8.39        | 0.22      | 11.57       | 0.00      | -3.18 | 0.0041    |
| Xl.24699.1.S1_at | CB904321           | EST               | MGC79012   |                                                                                                 | 3.65        | 0.36      | 6.51        | 1.79      | -2.85 | 0.0327    |
| Xl.10600.1.S1_at | BE491023           | EST               |            |                                                                                                 | 4.88        | 0.94      | 7.68        | 0.25      | -2.80 | 0.0256    |
| Xl.11324.1.A1_at | BG552091           | EST               |            |                                                                                                 | 3.44        | 0.46      | 6.23        | 0.11      | -2.79 | 0.0064    |
| Xl.13010.1.A1_at | BI099673           | EST               |            |                                                                                                 | 5.43        | 0.46      | 8.22        | 1.89      | -2.79 | 0.0417    |
| Xl.14915.1.A1_at | BM180884           | EST               |            |                                                                                                 | 5.11        | 0.30      | 7.72        | 0.35      | -2.61 | 0.0057    |
| Xl.13666.1.A1_at | BI019134           | EST               |            |                                                                                                 | 7.61        | 0.80      | 10.17       | 0.37      | -2.57 | 0.0230    |
| Xl.24515.1.S1_at | CB560511           | EST               | LOC495356  | Weakly similar to Apolipoprotein E precursor (H. sapiens); lipid binding                        | 6.41        | 0.60      | 8.93        | 1.00      | -2.52 | 0.0244    |
| Xl.13831.1.S1_at | BI075928           | EST               |            |                                                                                                 | 5.64        | 0.45      | 8.05        | 0.17      | -2.41 | 0.0094    |
| Xl.24058.1.S1_at | BI940804           | EST               | MGC82121   | Highly similar to histone H2A.FZ variant, isoform 1(H. sapiens)                                 | 6.99        | 0.28      | 9.40        | 0.09      | -2.41 | 0.0057    |
| Xl.16509.1.A1_at | BI084267           | EST               |            |                                                                                                 | 5.04        | 0.35      | 6.44        | 1.00      | -2.40 | 0.0185    |
| Xl.433.2.S1_at   | BC044959.1         | neurotrophin receptor B | trkB-b     | Protein amino acid phosphorylation                                                              | 3.68        | 0.40      | 6.01        | 0.00      | -2.33 | 0.0076    |
| Xl.19610.1.A1_at | BI084191           | EST               |            | Similar to Angiopoietin-1 receptor precursor (mTIE2)                                             | 4.48        | 0.05      | 6.81        | 0.36      | -2.33 | 0.0043    |
| Xl.75.1.S1_at    | D78003             | c4                | c4         | Endopeptidase inhibitor activity i fourth component of complement                               | 4.09        | 0.47      | 6.41        | 0.00      | -2.32 | 0.0101    |
| Xl.882.1.S1_at   | U07179.1           | Ldehydrogenase A  | ldda       | Oxidoreductase activity                                                                         | 5.29        | 0.94      | 7.60        | 0.06      | -2.31 | 0.0387    |
| Xl.17327.1.A1_at | BI492865           | EST               | MGC68503   |                                                                                                 | 4.77        | 0.49      | 7.07        | 0.19      | -2.29 | 0.0117    |
| Xl.747.1.S1_at   | AF170341.1         | galectin-1        | MGC64502   | Sugar binding                                                                                   | 5.51        | 0.21      | 7.80        | 0.55      | -2.29 | 0.0075    |
| Xl.545.1.S1_at   | AF170344.1         | metastasis associated 1 | mta2    | Transcription factor activity, regulation of transcription                                       | 7.85        | 0.51      | 10.14       | 0.13      | -2.29 | 0.0124    |
| Xl.23647.1.S1_at | BC047974.1         | cell death 2      | pdc2d      | Apoptosis                                                                                       | 4.11        | 0.36      | 6.34        | 0.40      | -2.23 | 0.0096    |
| Xl.19047.1.A1_at | BI478140           | Coatomerprotein   | copa       | ER to Golgi vesicle-mediated transport i                                                        | 8.05        | 0.35      | 10.28       | 0.01      | -2.23 | 0.0071    |
| Xl.9113.1.A1_at  | BG346438           | chimerin          | chn1       | Signal transduction                                                                            | 6.10        | 0.76      | 8.32        | 0.12      | -2.22 | 0.0276    |
| Xl.16847.1.A1_at | BI052360           | EST               |            |                                                                                                 | 4.22        | 0.74      | 6.44        | 0.36      | -2.22 | 0.0279    |
| Xl.13666.1.A1_at | BI019134           | EST               |            |                                                                                                 | 6.81        | 0.77      | 8.97        | 0.54      | -2.16 | 0.0350    |
| Xl.721.1.S1_at   | LO9778.1           | transcription factor DLL4 | Dlx2     | Regulation of transcription, DNA-dependent                                                     | 7.89        | 0.58      | 10.04       | 0.35      | -2.15 | 0.0199    |

Top 30 candidate transcripts upregulated in hybrids and differentially expressed between females of *X. laevis* and hybrids. Expression values are in log₂ scale; SD = standard deviation of expression values. *P* values are adjusted according to FDR moderated t-tests.
Table 3: Candidate transcripts upregulated in *X. muelleri* compared to hybrids.

| Probe ID     | GenBank ID       | Target Gene | Gene Symbol | Description/Molecular Function                                                                 | Mean Muell. | SD Muell. | Mean Hybrid | SD Hybrid | M-H     | P value |
|--------------|------------------|-------------|-------------|-------------------------------------------------------------------------------------------------|-------------|-----------|-------------|-----------|---------|---------|
| Xl.12012.2.A1_at | BM191810         | EST         |             | Weakly similar to FW1A_HUMAN F-box WD-repeat protein 1B (H. sapiens)                           | 8.50        | 0.22      | 3.16        | 0.55      | 5.35    | 0.0002  |
| Xl.7034.1.S1_at | BC043865.1       | LOC398646   | LOC398646   | Similar to pantophysin, transporter activity                                                   | 10.03       | 0.23      | 5.60        | 0.29      | 4.44    | 0.0002  |
| Xl.5802.1.A1_x_at | AW764672        | EST         | SEB-4       | Nucleic acid binding                                                                            | 9.84        | 0.32      | 5.46        | 0.27      | 4.38    | 0.0002  |
| Xl.5299.1.S1_at | BI445766         | EST         | seb4-a      | Weakly similar to myeloidlymphoid or mixed-lineage leukemia 2; ALL1-related gene (H. sapiens) | 8.67        | 0.44      | 4.47        | 1.24      | 4.19    | 0.0014  |
| Xl.17322.1.A1_y_at | BI077543       | EST         |             | Bone morphogenic protein, ossification, growth factor activity                                 | 7.00        | 0.09      | 2.90        | 0.14      | 4.10    | 0.0001  |
| Xl.3326.2.S1_a_at | X63427.1         | Bmp7        | MGC68434    | Bone morphogenic protein, ossification, growth factor activity                                 | 8.03        | 0.53      | 3.94        | 0.43      | 4.09    | 0.0005  |
| Xl.24194.1.S1_at | CD362680         | MGC89290    |             | Ribosome biogenesis and assembly                                                                  | 8.36        | 0.60      | 4.27        | 1.21      | 4.09    | 0.0019  |
| Xl.10122.1.S1_at | AF256087.1       | Xcat 2      | Xcat 2      | Highly similar to B-cell CLL lymphoma 11A (zinc finger protein); (H. sapiens); nucleic acid binding | 10.81       | 0.09      | 6.79        | 0.43      | 4.02    | 0.0002  |
| Xl.23898.1.A1_x_at | BF428365        | EST         | Bmp7        | Moderately similar to MOB-LAK (Homo sapiens)                                                    | 7.00        | 0.09      | 2.90        | 0.14      | 4.10    | 0.0001  |
| Xl.25735.1.S1_at | BE026658         | EST         |             | Highly similar to B-cell CLLlymphoma 11A (zinc finger protein); (H. sapiens); nucleic acid binding | 9.66        | 0.23      | 5.68        | 0.47      | 3.97    | 0.0002  |
| Xl.14298.1.A1_at | BQ383420         | EST         |             | Highly similar to Calcium-binding protein p22 (Calcium-binding protein CHP) (H. sapiens); calcium ion binding | 8.22        | 0.28      | 4.47        | 0.43      | 3.75    | 0.0003  |
| Xl.6272.1.A1_at | AW782701         | MGC83120    |             | Highly similar to Calcium-binding protein p22 (Calcium-binding protein CHP) (H. sapiens); calcium ion binding | 10.36       | 0.23      | 6.65        | 0.28      | 3.71    | 0.0002  |
| Xl.8805.1.S1_s_at | CB564916         | EST         | rpl-4       | Ribosomal protein L1; structural constituent of ribosome                                         | 11.11       | 0.08      | 7.22        | 0.18      | 3.88    | 0.0001  |
| Xl.23754.1.S1_at | CD324865         | Psma2       |             | Proteasome subunit XC3; ubiquitin-dependent protein catabolism                                 | 8.19        | 0.25      | 4.58        | 0.10      | 3.61    | 0.0002  |
| Xl.17949.1.S1_at | RG022283         | EST         | LOC734179   | Cytochrome-c oxidase activity                                                                   | 7.41        | 0.23      | 3.83        | 0.21      | 3.58    | 0.0002  |
| Xl.15786.1.S1_at | CB756768         | EST         |             | Moderately similar to SYQ_HUMAN Glutaminyl-tRNA synthetase, glutamate-tRNA ligase activity, protein biosynthesis | 7.67        | 0.18      | 4.09        | 0.30      | 3.58    | 0.0002  |
| Xl.7619.1.S1_at | BM193262         | EST         |             | Intracellular signaling cascade                                                                 | 9.85        | 0.09      | 6.28        | 0.41      | 3.57    | 0.0002  |
| Xl.2200.1.A1_at | BM179326         | EST         |             | Calcium ion binding                                                                            | 8.84        | 0.37      | 4.68        | 0.37      | 3.55    | 0.0004  |
| Xl.1140.1.S1_s_at | X63425.1         | Bmp2        | MGC83224    | tRNA processing                                                                                  | 8.88        | 0.19      | 5.40        | 0.02      | 3.48    | 0.0002  |

Top 30 candidate transcripts upregulated in *X. muelleri* and differentially expressed between females of *X. muelleri* and hybrids. Expression values are in log2 scale; SD = standard deviation of expression values. P values are adjusted according to FDR moderated t-tests.
Table 4: Candidate transcripts upregulated in hybrids compared to *X. muelleri*.

| ProbeID   | GenBank ID       | Target Gene           | GeneSymbol                     | Description/Molecular Function                                                                 | Mean Muell. | SD Muell. | Mean Hybrid | SD Hybrid | M-H   | P value  |
|-----------|------------------|-----------------------|--------------------------------|------------------------------------------------------------------------------------------------|-------------|-----------|-------------|-----------|-------|----------|
| XI.4744.1.S1_at | BE491637         |                       | LOC495025                      | Moderately similar to ubiquitin thiolesterase (H. sapiens); ubiquitin-dependent protein catabolism | 4.78        | 0.60      | 11.43       | 0.16      | -6.65 | 0.0002   |
| XI.6585.1.S1_at | BJ080015         | Similar to HIV-1 rev binding protein 2 | HRB2                          | RNA binding                                                                                | 3.22        | 0.20      | 9.75        | 0.14      | -6.53 | 0.0001   |
| XI.21357.2.S1_at | BG016692         |                       | LOC446970                      | Similar to axotroph; likely ortholog of mouse axotrophin (H. sapiens), protein binding        | 3.72        | 0.63      | 9.95        | 0.14      | -6.24 | 0.0002   |
| XI.1018.1.A1_at | U44950.1         | Vitelline envelope glycoprotein | Izpb-a                         | Xenopus laevis vitelline envelope 37 k glycoprotein xlZPB                                   | 4.10        | 0.53      | 10.31       | 0.29      | -6.22 | 0.0002   |
| XI.3536.2.S1_at | BF615663         |                       | LOC495200                      | Highly similar to transcription factor BTF3a – (H. sapiens)                                | 4.19        | 0.26      | 10.32       | 1.21      | -6.13 | 0.0004   |
| XI.23448.1.B1_at | BC041216.1       |                       | smarce1                        | Similar to SWISNF, actin dependent regulator of chromatin, regulation of transcription       | 3.75        | 0.62      | 9.88        | 0.23      | -6.13 | 0.0002   |
| XI.2060.1.A1_at | BJ055271         |                       |                                 |                                                                                             | 3.61        | 0.40      | 9.69        | 1.64      | -6.08 | 0.0008   |
| XI.576.1.S1_at  | AF184090.1       |                       | fatvg                          |                                                                                             | 4.94        | 0.56      | 11.02       | 0.03      | -6.08 | 0.0002   |
| XI.24785.1.A1_at | BM261081         |                       | LOC495200                      | Highly similar to transcription factor BTF3a – (H. sapiens)                                | 4.41        | 0.07      | 10.41       | 0.03      | -5.99 | 0.0001   |
| XI.4504.1.A1_at | BJ076394         | EST                    |                               |                                                                                             | 4.11        | 1.10      | 10.10       | 0.06      | -5.99 | 0.0007   |
| XI.7045.1.A1_at  | BC038421         | EST                    |                               |                                                                                             | 2.67        | 0.16      | 8.64        | 0.24      | -5.97 | 0.0001   |
| XI.7252.1.S1_at  | AY173320.1       |                       |                               |                                                                                             | 3.60        | 0.18      | 9.55        | 0.27      | -5.96 | 0.0001   |
| XI.2565.3.S1_at  | CB561588         | Similar to alpha-Tubulin at 84B | LOC398520                      | Xenopus laevis, Similar to alpha-Tubulin at 84B, microtubule-based movement                | 3.60        | 0.18      | 9.55        | 0.27      | -5.96 | 0.0001   |
| XI.783.1.A1_at   | BF322370         | EST                    |                               |                                                                                             | 2.94        | 0.43      | 8.88        | 0.02      | -5.94 | 0.0001   |
| XI.25809.1.A1_at | BE026874         | EST                    |                               | Histidine catabolism                                                                       | 4.26        | 0.49      | 10.18       | 0.36      | -5.92 | 0.0002   |
| XI.6605.1.A1_at  | AW632842         | EST                    |                               |                                                                                             | 3.59        | 0.26      | 9.50        | 0.02      | -5.91 | 0.0001   |
| XI.4170.2.A1_at  | BC039301         | LOC494857              | LOC494857                      | Cell differentiation                                                                       | 4.21        | 0.59      | 10.11       | 0.11      | -5.90 | 0.0002   |
| XI.1014.1.S1_at  | U466131.1        | Cdc21 protein          | cdc21                          | Xenopus laevis DNA replication initiator protein, DNA replication initiation, regulation of transcription | 4.14        | 0.46      | 10.00       | 0.08      | -5.86 | 0.0002   |
| XI.2839.1.S1_at  | BC041270.1       | Protein translocation complex | Sec61beta                      | Similar to protein translocation complex beta                                              | 5.91        | 0.17      | 11.76       | 0.08      | -5.85 | 0.0001   |
| XI.25336.1.A1_at | BE677987         | EST                    |                               | Weakly similar to hypothetical protein MGC2577 (H. sapiens)                               | 4.36        | 1.14      | 10.15       | 0.93      | -5.79 | 0.0012   |
| XI.14065.1.A1_at | AW147826         | EST                    |                               |                                                                                             | 3.61        | 0.15      | 9.33        | 0.12      | -5.72 | 0.0001   |

Top 30 candidate transcripts upregulated in hybrids and differentially expressed between females of *X. muelleri* and hybrids. Expression values are in log2 scale; SD = standard deviation of expression values. P values are adjusted according to FDR moderated t-tests.
Table 5: Overlap of transcripts from comparisons of hybrids and both species.

|       | L < H | L > H | M < H | M > H |
|-------|-------|-------|-------|-------|
| L > M | 24    | 626   | 2654  | 24    |
| L < M | 753   | 65    | 9     | 3997  |
| L = M | 62    | 86    | 267   | 328   |
| Total | 839   | 777   | 2930  | 4349  |

Comparison in the overlap of transcripts recovered as differentially expressed from the two contrasts with hybrids (Xenopus laevis (L) vs. hybrids (H) and X. muelleri (M) vs. hybrids) and the interspecies contrast (Xenopus laevis vs. X. muelleri). The congruence between patterns of expression behavior in hybrids compared to the interspecies comparison suggests a model of semidominance where hybrids have an intermediate level of expression compared to the two parental species.

The top 30 candidate genes for the contrasts between hybrids and the two parental species provide many genes with known roles in mitosis, meiosis, and oogenesis in general (Table 1, 2, 3, 4). One EST, MGC132176, is predicted to have heme oxygenase activity and this EST was upregulated 12 times in X. laevis relative to hybrids. Heme oxygenase plays a role in regulating ovarian steroidogenesis in rats and our results suggest this may be the case in Xenopus [8] as well. Of the genes with known function, many have been documented to play a role in oogenesis. For example, the proliferation associated protein PA2G4, which was upregulated in X. laevis about 8 times higher than in hybrids, has been previously isolated from Xenopus oocytes and is believed to play an important role in DNA replication and cell cycle progression [9]. One EST that is similar to the human transcription factor Hairy and enhancer of split 2, was upregulated 18 times in hybrids relative to X. laevis, and is known to be regulated by reproductive hormones in adult rat ovary [10]. Neurotrophin receptor B (TrkB-b) was upregulated 5 times in hybrids relative to X. laevis and plays a critical role in ovulation, steroid secretion, and follicular development in the ovary of rodents and humans [11-15].

Examining candidate gene lists for the Xenopus muelleri vs. hybrid comparison also reveal many genes involved in oogenesis. For example two bone morphogenetic proteins, Bmp7 and Bmp2, are 17 and 11 times respectively upregulated in X. muelleri relative to hybrid females. Bone morphogenetic proteins are part of a class of proteins involved in the development and patterning of the adult ovary [16,17]. Xcat-2 was upregulated 17 times more in X. muelleri relative to hybrids and is involved in the formation of germ plasm during stage I oocytes of Xenopus [18,19]. Another gene of interest is LAP2, upregulated 14 times higher in X. muelleri compared to hybrids and specific isoforms of LAP2 are expressed exclusively in the ovary of anurans and salamanders [20-22]. Cyclin A1, the most divergently expressed gene, was upregulated nearly 200 times higher in hybrids relative to X. muelleri and expressed the same in X. laevis and hybrids. Cyclin A1 plays a major role in mammalian gametogenesis and meiosis [23] and is a partner of Cdk2, a key gene involved in the cell cycle both in mitosis and meiosis. Female and male knockout Cdk2 mice are viable but both infertile [24]. Curiously, disruption of Cyclin A1 expression results in male infertility but not female infertility and specifically causes the developmental arrest of spermatogenesis during meiosis I [25,26]. A vitelline envelope glycoprotein, lzpb-a, was upregulated 75 times in hybrids relative to X. muelleri, and vitelline envelope proteins have an obvious role in the formation of oocytes during amphibian oogenesis [27]. Finally, Germes, a gene that localizes to the germ plasm during early oogenesis in Xenopus [28] was upregulated 62 times higher in hybrids compared to X. muelleri.

Perhaps the most surprising result of our analyses is that hybrid females are fertile yet have a dramatic increase in gene misexpression compared to hybrid males which are completely sterile [2]. These results would seem to contradict what we might intuitively predict; specifically that it seems reasonable to assume that normal phenotypes should have greater similarity in expression profiles and perturbed phenotypes should have greater divergence in expression. Hybrid males, which are completely sterile, have only 56 genes misexpressed in testes compared to both parental species whereas hybrid females, which are fertile, have nearly 20 times more genes misexpressed (1,105) in ovaries. However, these results are consistent with patterns of sex-biased gene expression in which female-biased genes were found to be more divergently expressed between species compared to male-biased genes [29] providing further evidence that this pattern represents real biological effects. Thus, we are left with a ques-

[Page 8 of 11]
tion, how can the process of oogenesis tolerate such dramatic differences in the level of gene expression, whereas the process of spermatogenesis in hybrid males has relatively few genes misexpressed yet results in complete sterility.

To date, there has been little exploration of this question because studies of gene expression and reproductive isolation have focused on the sterility phenotype which typically involves males. However, hybrid females of *Drosophila melanogaster* and *D. simulans*, which are sterile, have been analyzed and hybrids had a majority of genes misexpressed compared to the two species [30]. Recent work has shown that critical genes involved in mammalian female reproduction undergo rapid diversification due to retrotransposed genes in *Mus musculus* [31] and these results may provide a clue to the divergent expression pattern occurring in females of *Xenopus* which could be a general pattern of female reproduction.

*Xenopus* do not conform to a fundamental generalization in evolutionary biology-Haldane’s rule [1-7]. Patterns of sex-biased gene expression and comparisons between taxa in which the sex chromosome constitution is reversed suggest that the sensitive spermatogenesis component of the faster-male evolution hypothesis [3,32] is the best explanation for sterile males in *Xenopus* even though females are the heterogametic sex. Given the divergent pattern of expression in hybrids and females between species, we suggest the following scenario to explain hybrid male sterility in *Xenopus*.

First, oogenesis relies on a staggering amount of gene expression with up to 45% of all mouse genes and 55% of all *Drosophila* genes expressed in the mature oocyte [31,33] and additionally this abundant transcription results in maternally deposited RNAs and proteins which foster oocyte growth and early development [31,34]. In particular, much of this RNA deposition functions to localize coding and non-coding RNAs essential to germ cell development into a distinct subcellular domain that can be moved into the vegetal cortex of the oocyte. Interestingly, RNAs localized in the germ plasm may not be translated for years highlighting the importance of the germ plasm as a storage unit for RNA and furthermore many of these stored RNAs are involved in translational regulation of germ cell specific expression [35-38].

We find dramatic differences in gene expression between females of two species of *Xenopus* and many of the most dramatic differences have to do with Early/METRO pathway (e.g. *Germes, Fatig*, *Cyclin A1*) of germ plasm specification [38]. These dramatic, sometimes 200 times different, RNA abundance levels indicate a major difference in the amount of key genes involved in germ plasm specification and maternally loaded RNAs between species. This result would suggest that each species has a divergent way of completing oogenesis with regard to gene expression.

During fertilization, sperm fertilize an egg and this starts the dramatic changes that turn the mature oocyte into a functioning zygote [39]. Each sperm delivers a haploid paternal genome along with mature RNA that initiates and directs subsequent development [40]. The interaction between the paternal genome and stored maternal RNA must coevolve in such a way to ensure successful development. Consider now sperm from a different species, adapted for fertilizing eggs of its conspecific species, that now successfully fertilizes an egg from a different species. As our data suggest, the paternal genome will now interact with a radically different embryo with drastically different amounts of stored maternal RNAs. We therefore suggest the possibility that disruption of spermatogenesis in adult hybrid males occurs because of radically divergent expression in females during oogenesis. Oocytes armed with pools of RNA adapted for one species, work in the sense that they can be fertilized and develop but, the initial differences in maternally stored RNAs generate subsequent dysfunctions in males because molecular interactions that generate the adult testis and subsequent spermatogenesis are misregulated due to the differences in maternally stored RNA populations. Spermatogenesis is special in the sense that during early development key factors fail to interact properly to generate a normally functioning testis.

Several genes from our microarray results suggest directions by which this hypothesis could be tested. One example is *Cyclin A1* which was upregulated about 200 times more in hybrids and 170 times more in *X. laevis* compared to *X. muelleri*. Knocking out *Cyclin A1* in mice causes completely sterility and the interaction between *Cyclin A1* and *cdk2* is crucial for normal development [24,25]. *Cyclin A1* is also known to be maternally deposited in *Xenopus* and regulates the progression of the cell cycle and apoptosis [41]. Our hypothesis suggests that factors like *Cyclin A1* which are loaded into embryos in drastically different amounts may play a role in misdirecting the development of the hybrid testis.

**Conclusion**

Our work provides an important first glimpse into the expression pattern of hybrid females and parental species. We find an asymmetrical expression pattern similar to the pattern of expression in hybrid male *Xenopus* and allotetraploid *Arabidopsis* [2,42]. However, hybrid females have a dramatic increase in the number of misexpressed genes compared to sterile males and we suggest that this gene expression divergence plays a role in hybrid male sterility. Our results call for attention as to how divergent
expression in females plays a role in reproductive isolation between species.

Methods

Microarray Experiments

RNA was extracted from adult ovary in *Xenopus laevis* (n = 4), hybrids of *X. laevis* × *X. muelleri* (n = 2) and *X. muelleri* (n = 3). Hybrid individuals were produced by crossing maternal *X. laevis* with paternal *X. muelleri*. Origin of parents and methodology for creating hybrids has been described elsewhere [2,29]. Sufficient numbers of normal hybrid females from the reciprocal cross were unable to be produced because the reciprocal cross produces increased mortality and the offspring that survive have a high proportion of limb abnormalities [1]. Individual adults were euthanized with MS-222 and 50 mg of ovary was dissected and homogenized in RNA extraction solution using a hand held pestle. RNA was recovered using GeneHunter and Ambion RiboPure total RNA kits. Samples of RNA were checked for purity by examination of the 28S and 18S ribosomal RNA bands from denaturing gel electrophoresis, by 260/280 ratios from scans with a Nanodrop ND 1000 spectrophotometer, and by readouts of the Agilent Bioanalyzer. Total RNA samples were prepared and hybridized to Affymetrix *Xenopus laevis* GeneChip Genome Arrays at the University of Texas Southwestern Medical Center Microarray Array Core Facility following standard Affymetrix protocols. Affymetrix Microarray Analysis Suite (MAS) v.5.0 was used to scan and process each microarray chip. The signals of quality control and poly(A) transcripts revealed that hybridizations were of high quality in all chips. Quality control probe sets (i.e., spike in and housekeeping genes) were removed in subsequent statistical analyses. Hybridizing RNA from a heterospecific species to a microarray designed for a related species can have a dramatic impact on the signal recovered from microarrays [43-46]. To control for this effect, we used an electronic mask generated from hybridizing genomic DNA from *X. laevis* and *X. muelleri* onto the *X. laevis* microarray [2]. This mask which screens out probes that have significant sequence divergence in *X. muelleri* provides 11,485 probesets/genes for further analysis.

Data Analysis

We conducted three separate comparisons to uncover patterns of differential expression between *Xenopus laevis* and hybrids, *X. muelleri* compared to hybrids, and *X. laevis* compared to *X. muelleri*. First, the *Xenopus laevis* and hybrid chips were normalized using Robust Multichip Averaging (RMA) express software [47] using default parameters for background correction and quantile normalization. These RMA normalized data were then imported into the R statistical environment and tested for differences in expression between *X. laevis* and hybrids for each of the 11,485 genes using a moderated $t$-statistic based on an empirical Bayes method in the Limma package found in Bioconductor [48]. The TopTable function was then used to output the False Discovery Rate (FDR)-adjusted $p$-values and we considered genes with adjusted $p$-values less than 0.05 to be differentially expressed. Goodness of fit tests ($G$), based on the difference between the observed and the expected (under the null hypothesis of equal class probabilities) number of genes, were performed to test whether there was enrichment in the number of genes up-regulated in particular comparisons [49]. We normalized *X. muelleri* and hybrid chips together using RMA and repeated the analyses to uncover differential expression between *X. muelleri* and hybrids. Finally, we normalized *X. laevis* and *X. muelleri* chips together using RMA and repeated the analysis to uncover genes misexpressed between the two species. Separate normalizations were performed for each comparison in keeping with the assumptions of RMA normalization.

Authors’ contributions

JHM conceived of the study, collected the data, performed the statistical analyses, and wrote the manuscript. PM participated in its design and coordination and edited the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was conducted following the protocols of the University of Texas-Arlington Animal Care Committee (Protocol No. A05.001). We thank Professor R. C. Tinsley for donating specimens of *Xenopus muelleri* for use in this study. Funding was provided by a National Science Foundation Dissertation Improvement Grant (DEB-0508882) and Texas Academy of Science Student Research Grant to JHM.

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