INVESTIGATION

Testes Proteases Expression and Hybrid Male Sterility Between Subspecies of Drosophila pseudoobscura

Doaa Alhazmi, Seth Kaleb Fudyk, and Alberto Civetta
Department of Biology, University of Winnipeg, MB, Canada, R3B 2E9
ORCID ID: 0000-0002-9122-5621 (A.C.)

ABSTRACT
Hybrid male sterility (HMS) is a form of postmating postzygotic isolation among closely related species that can act as an effective barrier to gene flow. The Dobzhansky-Muller model provides a framework to explain how gene interactions can cause HMS between species. Genomics highlights the preponderance of non-coding DNA targets that could be involved in gene interactions resulting in gene expression changes and the establishment of isolating barriers. However, we have limited knowledge of changes in gene expression associated with HMS, gene interacting partners linked to HMS, and whether substitutions in DNA regulatory regions (cis) causes misexpression (i.e., expression of genes beyond levels found in parental species) of HMS genes in sterile hybrids. A previous transcriptome survey in a pair of D. pseudoobscura species found male reproductive tract (MRT) proteases as the largest class of genes misregulated in sterile hybrids. Here we assay gene expression in backcross (BC) and introgression (IG) progeny, along with site of expression within the MRT, to identify misexpression of proteases that might directly contribute to HMS. We find limited evidence of an accumulation of cis-regulatory changes upstream of such candidate HMS genes. The expression of four genes was differentially modulated by alleles of the previously characterized HMS gene Ovd.

KEYWORDS
Speciation
Drosophila
proteases
expression
gene interactions
cis-regulation

In speciation, prezygotic and postzygotic barriers evolve that isolate divergent populations. Many studies on the genetic basis of speciation have focused on postmating postzygotic (i.e., sterility/ inviability of hybrids) reproductive isolation and some general patterns have emerged from these studies. Commonly, there is a disproportionate contribution of the sex chromosome to heterogametic F₁ inviability/ sterility (the “large-X effect”) but gene interactions are needed for full establishment of isolation barriers (Coyne and Orr 1989; Masly and Presgraves 2007; Presgraves 2008; Cattani and Presgraves 2012; Dufresnes et al. 2016). The important role of gene x gene interactions in the onset of HMS is well illustrated in the Dobzhansky-Muller (DM) model of incompatibilities (Dobzhansky 1937; Muller 1942). The model draws attention to the relationship between populations’ divergence and the accumulation of incompatible allele interactions leading to phenotypes such as HMS. Several studies have mapped loci that contribute to the onset of HMS and a few single major HMS genes have been identified. For example, in Drosophila, Odysseus-site homeobox (OdsH) (Ting et al. 1998), JYAlpha (Masly et al. 2006), and Overdrive (Ovd) (Phadnis and Orr 2009); in mouse Prdm9 (Mihola et al. 2009). Many empirical studies have also highlighted that gene interactions are crucial for the manifestation of HMS, and in many cases the effectiveness of the aforementioned major HMS genes has been shown to be dependent on genetic background (Perez and Wu 1995; Orr and Irving 2001; Presgraves et al. 2003; Tao et al. 2003; Sawamura et al. 2004; Chang and Noor 2010; Phadnis 2011).

The eukaryotic genome consists mostly of non-coding genetic elements, approximately 80% of the D. melanogaster euchromatic genome is noncoding (Halligan and Keightley 2006), creating many opportunities for interactions that involve different DNA targets, like promoter elements, enhancers, and silencers, that can affect gene expression. Despite the preponderance of non-coding DNA targets potentially affecting gene expression and the well-acknowledged
The misregulation of proteases previously identified in a genome-wide screen (Gomes and Civetta 2015) could directly affect fertility, or do so indirectly by interacting with alleles at other loci. Here we use a combination of backcross genetics and gene expression assays to identify ten proteases that when misexpressed might directly contribute to F1 HMS. Among these ten proteases, seven are prioritized as candidate HMS genes based on patterns of tissue expression within the MRT. We also identify four proteases whose misexpression is affected by the allele status of the D. pseudoobscura previously identified major X-linked HMS gene Ovd (Phadnis and Orr 2009). The use sequence data analysis shows, with the exception of one gene, no evidence of enrichment in cis-regulatory divergence fueling gene-specific misexpression of HMS gene candidates.

MATERIALS AND METHODS

Drosophila stocks and crosses

All flies were maintained on cornmeal–molasses–yeast–agar (CMYA) medium at constant temperature on a 12-hour light–dark cycle. Virgin females were collected post-eclosion and flies were mass crossed in bottles containing CMYA medium. All Drosophila stocks were purchased from the Drosophila Species Stock Centre (https://stockcenter.ucsd.edu/). We used a wild-type D. p. pseudoobscura (14011-0121.139) and a wild-type D. p. bogotana (14011-0121.175) strain as well as two D. p. pseudoobscura mutant strains. One of the mutant stocks carries an X-linked yellow (y) body coloration mutation (14011-0121.06) while the other strain (14011-0121.08) carries, among others, an X-linked sepiap (se) eye color mutation.

First (BC1) and fourth (BC4) generation backcross males were created independently to sample different types of maternal-nuclear and X-autosomal interactions by crossing wild-type D. p. bogotana females to D. p. pseudoobscura males and then backcrossing the fertile F1 females to D. p. pseudoobscura males. The BC4 males were obtained by backcrossing fertile BC1b females to D. p. pseudoobscura males. BC1p males were generated by crossing wild-type D. p. pseudoobscura females to D. p. bogotana males and then backcrossing the fertile F1 females to D. p. pseudoobscura males (Figure 1A). A different backcross design was used to replace D. p. bogotana X-linked sterility alleles with the corresponding fertile D. p. pseudoobscura alleles in males with an otherwise genome composition similar to sterile F1 male hybrids (Figure 1B). For this, we took advantage of the fact that two X-linked sterility loci map near both y and se mutations (Phadnis and Orr 2009, Phadnis 2011). All backcrosses were created at different times and they represent independent events of recombination.

The average fecundity of the BC and IG males was assayed following a procedure described elsewhere (Gomes and Civetta 2014). Briefly, five seven-day-old focal males (i.e., BC1, BC4 and F8 males – Figure 1) were housed with five D. p. bogotana virgin females of the same age. Each cross was replicated six times. Flies were left together for 48 hr in vials (vials 1 to 6) containing CMYA food supplemented with yeast. After 48 hr, males were removed and five days after the original setup, females were transferred to a new vial for an additional 5 days. Fecundity was assayed as the total number of offspring (adult flies) produced from each vial. Counts were done for up to 24 days after the initial set-up to avoid overlapping generations. The same protocol was used to assay parental species male fecundity by using females and males of the same species.

Gene expression

We extracted total RNA from the entire MRT of 7 days-old focal males as well as parental wild-type strains. We also performed extractions...
of total RNA from testes (T), seminal vesicles (SV), accessory glands (AG) and the ejaculatory bulb (EB) of same age *D. p. pseudoobscura* males.

The Bio-Rad Aurum total RNA mini kit was used for RNA extractions. In all experiments, at least three biological replicates were obtained from parental species and focal males, each sample consisting of tissue from 10-15 males. RNA samples were quantified and tested for quality using a Nanodrop (Thermo Fisher Scientific). cDNA was synthesized from equivalent amounts of total RNA using the iScript cDNA synthesis kit (Bio-Rad). Primers were designed using Primer3Plus (http://www.primer3plus.com/) with the product size ranging from 75 to 85 base pairs long (Table S1). When possible, intron-spanning primers were used to monitor any genomic DNA contamination. We targeted 19 proteases previously identified as uniquely misregulated in the sterile F1 hybrid males resulting from crosses between *D. p. bogotana* females and *D. p. pseudoobscura* males (Gomes and Civetta 2015). Gene expression was quantified using the BioRad CFX96 Real-Time PCR Detection System. The reactions were performed using the IQ SYBR Green quantitative real-time PCR kit (Bio-Rad). In all assays, two reference genes (RpL32 and RpS18) were used to normalize RT-PCR results for each target gene. Cycling conditions for RT-PCR were the same for all genes: an initial denaturation step for 5 min at 95°C, followed by 35 cycles of 95°C for 45 sec, 58°C for 30 sec and 72°C for 45 sec. The efficiency of all primers was tested by creating a standard curve of threshold cycle values from a template dilution series. Gene expression was determined by calculating ΔCq as the Cq of the reference gene (RpL32 or RpS18) minus the Cq value of the target gene. Data were analyzed using (ANOVA) to test the null hypothesis that genes mean relative expression among crosses is not different. If significant differences were detected among crosses, a Scheffe’s post-hoc test was used to determine statistically significant differences between samples. FDR corrected q-values were used for all tests of significance.

**Identification of species sequence divergence in putative cis-regulatory regions**

Genomic DNA was extracted from pools of 5 flies and genes PCR amplified. PCR products were cleaned using a Bio-Tek E.Z.N.A Cycle Pure Kit (Omega) and their concentration measured using a Nanodrop spectrophotometer. Cleaned PCR products were sent for Sanger sequencing to the Centre for Applied Genomics (TCAG) at the Hospital for Sick Children in Toronto. We sequenced *D. p. pseudoobscura* (14011–0121.139) and *D. p. bogotana* (14011–0121.175) seven candidate HMS genes derived from our genetics and gene expression assays. Approximately 1Kb upstream, as well as a few hundred bases downstream of the transcription start site (TSS), was aligned using MUSCLE within MEGA to the currently available *D. p. pseudoobscura* gene sequences in FlyBase (https://flybase.org/). Fixed nucleotide substitutions between species can be overestimated if polymorphism data are not considered. We checked whether putative fixed changes could possibly be shared polymorphism by BLASTn of our sequences to the sequence reads of 43 *D. p. pseudoobscura* strains (Fuller et al. 2014) available at the Short Read Archive (SRA) at the National Center for Biotechnology Information (NCBI). We retained BLASTn matches with higher than 90% identity to the query. The location of fixed substitutions between species was mapped relative to the transcription start site.
(TSS) (Ohler et al. 2002; Roy and Singer 2015). We look for evidence of accumulation of substitutions in cis-regulatory regions by using an empirical cumulative distribution function. For any gene region, a monotonic increase in substitutions (n) was identified by calculating G, a measure of the difference between the relative occurrence of a nucleotide change and its relative position in the sequence (Tang and Lewontin 1999; Civetta et al. 2016). Differences between the values of G between any two events (ΔG) measure the differential accumulation of nucleotide substitutions. We reject uniform distribution of G with the highest absolute value (T) is higher than T*, where T* is a null random distribution of T obtained using Monte Carlo simulations to produce 100,000 samples of n events by sampling without replacement along a sequence of length N (Tang and Lewontin 1999). Because our a priori hypothesis is that there should be an accumulation of substitutions in cis-regulatory regions (hotspots) for misregulated genes, we performed one-tailed tests looking for sequence regions where ΔG is positive. A source code of the program used to implement the analysis (Civetta et al. 2016) is available in the figshare data repository (Supplementary material).

Data availability
Supplementary tables, source code for sequence data analysis and raw data for gene expression and fecundity assays have been submitted to the figshare repository. Gene sequences have been deposited in GenBank under accession numbers MK370073 to MK370086 and sequence alignments are available in the Figshare repository. Supplemental material available at Figshare: https://doi.org/10.25387/g3.7560578.

RESULTS

Misregulation of proteases in fertile BC and IG male progeny

All BC and IG males were generated through independently setup crosses and therefore represent different events of genomic recombination. Both BC1 and BC4 males were fully fertile. The presence of either D. p. bogotana or D. p. pseudoobscura alleles at the y locus in F8 IG males made no difference in terms of fecundity (Table 1). We suspect this is likely a consequence of y not being tightly linked to a previously mapped X-linked sterility QTL (Phadnis 2011). The introgression of a D. p. bogotana se allele caused the expected result of sterility with the D. p. pseudoobscura se allele restoring fertility (Table 1).

Comparisons of relative gene expression showed significant gene and reference gene effects (ANOVA: F1,1204= 141.6, P < 0.001 and F1,1204= 100.3, P < 0.001 respectively), but no gene × reference gene effect interaction (F1,1204= 0.03, P = 1.0) as relative gene expression (ΔCq) was always higher when using RpS18 as a reference. Eight genes (GA13457, GA18484, GA18944, GA24206, GA27806, GA28780, and GA30093) were found to be misregulated between fertile BC males and parental (D. p. pseudoobscura and D. p. bogotana) species (Scheffé’s post-hoc test; Table 2). The level of misexpression detected in fertile BC male progeny could reflect quantitative differences that are not equivalent to misregulation in sterile F1 males. We found that in all cases of misexpression in fertile BC progeny, at least one of the fertile BC progeny was not significantly different from sterile F1 males (Scheffé’s post-hoc test; Figure 2) indicating that the misregulation of these genes is not directly associated with F1 HMS. BC1b males have the same mitochondrial composition as sterile F1 males but a hybrid cytoplasmic and nuclear content that is different from sterile F1 males. The reciprocal BC1b fertile males are on average identical to BC1b males, except for their mitochondrial genome (Figure 1A). The fact that all genes were not differentially expressed between BC1b and reciprocal BC1p males (Figure 2) shows that the misregulation of these genes is not driven by mitochondrial-nuclear interaction. Only three genes were misregulated in BC4 fertile males relative to parental species, GA24206, GA27806 and GA28780 (Figure 2). BC4 males have only an average 3–4% content of D. p. bogotana genome in a D. p. pseudoobscura background, thus limiting opportunities for interspecies interactions that might affect gene targets expression. The fact that GA24206, GA27806, and GA28780 were misregulated in BC4 males suggests that few D. p. bogotana alleles are sufficient to trigger misregulation of these targets.

The analysis of X chromosome allele introgression progeny revealed eight genes (GA13457, GA17870, GA18484, GA18944, GA24206, GA27806, GA28780, and GA30093) misregulated in fertile IG progeny relative to parental species (Scheffé’s post-hoc test; Table 2). Except for GA24206, all these genes had at least one fertile introgression progeny misregulated at levels not significantly different from sterile F1 males (Scheffé’s post-hoc test; Figure 3). The combined results from the fertile BC (Figure 2) and IG males (Figure 3) allows us to exclude nine genes as those whose misregulation in the sterile F1 male hybrid condition could be directly associated with sterility. The analysis of the Xv and Xe allele introgressions provided evidence in support of proteases misregulated by X-autosomal unbalanced interactions. GA13457, GA18484 and GA18944 were equally overexpressed in introgressions that selected for different regions of the X chromosome (Y or se) while the other genes showed patterns of misregulation that was influenced by the nature of the X chromosome allele introgressed or the region of the X chromosome introgressed. Among this second group, the significant different expression of genes GA17870, GA20504, GA24206 and GA28780 between se vs. se+ allele introgressions (Figure 4) suggests that these genes are likely targets of Ovd.

Altogether, the backcross and introgression designs allowed us to establish that nine of our previously identified proteases uniquely misregulated in sterile F1 hybrid males are misregulated due to cyto-nuclear or X-autosomal unbalances, but their misexpression is not directly associated with the onset of HMS.

Candidate HMS proteases and tissue expression

Ten genes showed expression levels similar to parental species in fertile BC and IG males (Table 2). Only four of the nineteen genes assayed showed significantly higher expression in the male accessory glands than testes (Table S2). Because HMS is characterized by the production

| Table 1 Mean fecundity and standard error (SE) of parental species, backcross and introgression males. Reciprocal BC1 males are from D. p. bogotana (BC1b) and D. p. pseudoobscura (BC1p) parental females. BC4 males are from a fourth-generation backcross (Figure 1A). Introgression males were generated as shown in figure 1B and they are either yellow (F8+) vs. non-yellow (F8–) body or seipa (F8mv) vs. non-seipa (F8mv+) eyes males |
| Male | Mean Fecundity (SE) |
| D. p. pseudoobscura | 90.3 (4.2) |
| D. p. bogotana | 83.7 (4.2) |
| BC1b | 100.5 (1.7) |
| BC1p | 116.3 (5.3) |
| BC4 | 97.5 (3.1) |
| F8+ | 117.2 (17.4) |
| F8– | 104.5 (22.7) |
| F8mv | 107.7 (2.7) |
| F8mv+ | 0.0 (0.0) |
of mature but immotile sperm (Prakash 1972; Orr 1989; Snook 1998; Gomes and Civetta 2014), candidate HMS genes could be further prioritized as those with higher expression in the testes, where spermatogenesis takes place, relative to male reproductive tissue that contributes seminal fluids to the ejaculate. Among the ten gene candidates, we found nine genes with higher levels of expression in the testes than in the accessory glands and one gene (GA26803) with the reverse pattern (Table 3 and Table S2). Further, GA21772 was very lowly expressed (ACq < -10) and GA15722 very highly expressed (ACq > -5) in all male reproductive tissue samples (Table S2). Moreover, GA15722 is highly expressed in both males and females reproductive and non-reproductive anatomy (https://flybase.org/) suggesting GA15722 is a housekeeping gene that when disrupted could affect more than just sperm function.

**Limited interspecies sequence differences in putative cis-regulatory regions**

We sequenced over 1.5kb for the seven candidate HMS genes with higher expression in testes. The alignment of our sequences to the *D. p. pseudoobscura* gene sequences available in FlyBase are found in the figshare repository, which also shows substitutions identified as shared polymorphisms after BLASTn searches to the Sequence Read Archive (SRA). Table 4 summarizes the location of fixed substitutions between species in relation to the TSS. Overall, we found a very limited number of fixed nucleotide changes. Genes GA30092 and GA22690 had the highest proportion of fixed changes (0.004 and 0.009 respectively). GA22690 was the only gene to show a significant \( P < 0.05 \) monotonic increase in substitutions that corresponded to eight changes, from -751 to -525, in the promoter distal region (Table 4).

**DISCUSSION**

The genetic basis of reproductive isolation between species is typically addressed within the framework of the Dobzhansky-Muller model (Dobzhansky 1937; Muller 1942) and it has been previously shown that divergence in gene regulation is a major contributor to the evolution of Dobzhansky-Muller incompatibilities between species of *Drosophila* (Haerty and Singh 2006). Yet, we lack specific examples of gene \( \times \) gene interactions. Moreover, to understand variation in gene expression between species, it is crucial to map specific gene \( \times \) gene interactions and ultimately gene regulatory networks (Signor and Nuzhdin 2018).

Here, we have mapped gene \( \times \) gene interactions affecting expression of proteolytic candidates HMS genes by using a genetic approach that generated males genotypically similar to F1 sterile male hybrids but with different (i.e., *D. p. pseudoobscura* vs. *D. p. bogotana*) alleles. Four genes were differentially expressed based on the introgression of a sepia eye color marker (linked to the major HMS gene *Ovd*). Three of the four genes, GA17870, GA24206 and GA28780, were misregulated in BC or IG fertile males. Thus, the misregulation of these three genes cannot be directly linked to the onset of HMS. Why these genes are modulated by *Ovd* but not directly linked to sterility could also be explained by possible pleiotropic effects of *Ovd*. For example, *Ovd* is known to influence pregnancy sex-ratio (Phadnis and Orr 2009; Phadnis 2011) and the biological processes in which *Ovd* is involved are still unknown (https://flybase.org/). GA17870 is a male AG gene with very low expression in other MRT tissues. We know that seminal fluids play an important function in postmating reproductive female biology (LaFlamme and Wolfner 2013), but it remains to be determined whether sterile male hybrids between *D. p. bogotana* and *D. p. pseudoobscura* are impaired in terms of their postmating reproductive fitness due to the misregulation of AG genes like GA17870. GA20504 is arguably the most interesting candidate emerging from our study. The gene is differentially expressed based on the introgression of the sepia allele linked to *Ovd*, restores normal expression in fertile BC and IG males but becomes misregulated in introgressed male carriers of the *Ovd* sterile allele. Not much is currently known about GA20504 but one of its possible mammalian, including human, orthologs is the gene alanyl aminopeptidase (ANPEP) (https://flybase.org/). Overexpression of endopeptidase activity in man has been linked to sperm lack of motility and both ANPEP, and Neutral endopeptidase (NEP), are...
targetable through inhibitors to help restore sperm motility in man (Subirán et al. 2010; Bosler et al. 2014).

Our previous finding of proteases being the largest class of genes uniquely misregulated in the MRT of sterile F1 hybrid males was intriguing because proteases had not been previously identified as a possible contributor to HMS in Drosophila. Here, we show that F1 hybrid male sterility misregulation of nine previously identified proteases can be explained by differences in X-autosomal and nuclear-maternal asymmetries without a direct link to the sterility phenotype. Of the remaining ten candidates, seven can be prioritized as directly linked to the HMS sterility phenotype based on the genes’ site and level of expression within different tissue of the MRT. Except for GA19543, information available on the other six candidate HMS genes allow us to discuss them in the context of the sterility phenotype. GA20504 was discussed above as a gene of interest based on our results and its possible orthology to proteases that have shown associations between changes in gene expression and sperm motility. GA24796 is within a previously mapped third chromosome HMS sterility QTL between

Figure 2 Mean relative expression and standard error of genes in backcross males, that are misregulated relative to parental species, compared to the expression in sterile F1 male hybrids. Parental species are shown in gray, BC progeny in white and sterile F1 males are hatched. For each gene, from left to right, D. p. bogotana, D. p. pseudoobscura, BC4, BC1p (from parental D. p. pseudoobscura females), BC1b (from parental D. p. bogotana females) and sterile F1 hybrid males. Shared letters identify non-significantly different groups (post-hoc Scheffe’s test, FDR corrected q < 0.05).

Figure 3 Mean relative expression and standard error of genes misregulated in X-allele introgressions. Parental species are shown in gray, IG progeny in white and sterile F1 males are hatched. For each gene, from left to right, D. p. bogotana, D. p. pseudoobscura, F8y, F8y+, F8se, F8se+. Shared letters identify non-significantly different groups (post-hoc Scheffe’s test, FDR corrected q < 0.05).
D. p. pseudoobscura and D. p. bogotana (Phadnis 2011; Gomes and Civetta 2015). GA22690 also appears to be located within a previously identified X chromosome HMS QTL based on its genome location (https://flybase.org/), but the precise genome position of the QTL is harder to determined due to the use of an unannotated phenotypic marker (sd) in the QTL mapping study (Phadnis 2011). The transcription of genes required during spermatid differentiation is regulated by testes meiotic arrest complex (tMAC) proteins and the D. melanogaster ortholog of GA25574 and GA30092 is one such spermatid target, lending further support to the role of these two genes in HMS.

Variation in gene expression between species is characterized by a preponderance of cis changes that affects the expression of target genes (Wittkopp et al. 2008; Emerson and Li 2010; Gordon and Ruvinsky 2012; Coolon et al. 2014; Nourmohammad et al. 2017; Metzger et al. 2017). The sequencing of upstream gene regions for our seven candidate HMS proteases found no evidence, except for GA22690, of an enrichment of substitutions in cis. Although we assayed only seven genes, this information suggests that the genome-wide pattern of higher divergence in cis than trans between species might not necessarily apply to genes whose misregulation is linked to the establishment of reproductive isolation barriers. Because many of the genome-wide cis-regulatory changes

| Table 3 Mean male reproductive tissue expression for candidate proteases not misregulated in fertile BC/IG progeny. The ratio of expression in testes (sperm) relative to the accessory glands (seminal fluids) and fold change are used to identify genes as testes or accessory gland enriched |
|-----------------|------------------|-----------------|----------------|-----------------|-----------------|
| T               | AG               | Ratio           | Fold-change    | Tissue enriched |
|---               |---               |---              |---             |---              |
| GA14907         | −2.03            | −17.05          | 0.12           | 8.33            | Testes          |
| GA15722         | −2.09            | −4.53           | 0.46           | 2.17            | Testes          |
| GA19543         | −7.44            | −11.31          | 0.65           | 1.54            | Testes          |
| GA20504         | −6.47            | −19.15          | 0.34           | 2.94            | Testes          |
| GA21772         | −10.28           | −17.79          | 0.58           | 1.72            | Testes          |
| GA22690         | −8.62            | −19.15          | 0.45           | 2.22            | Testes          |
| GA24796         | −4.24            | −15.88          | 0.27           | 3.70            | Testes          |
| GA25574         | 0.40             | −11.14          | 0.04           | 25.00           | Testes          |
| GA26803         | −12.48           | −1.99           | 6.27           | 6.27            | Accessory Glands |
| GA30092         | −0.63            | −11.97          | 0.05           | 20.00           | Testes          |
phenotype. We have also established that the misregulation of most candidate genes shall be assessed by the generation of genetically engineered strains in which the activity of the candidate genes is either enhanced or silenced. Moving forward, the precise functional role of these candidate genes associated with incompatibilities have been identified (Brideau et al. 2006). Here, we have identified four partners of the Ovd gene, with GA20504 having a direct association with the sterility gene, with GA20504 having a direct association with the sterility

**ACKNOWLEDGMENTS**

We would like to thank two reviewers and the handling editor, Dr. Esther Betrán. We believe the paper has been significantly improved due to their very careful review, comments and suggestions. This work was funded by an NSERC Discovery Grant to A. Civetta. D. Alhazmi was funded by a graduate scholarship from the Government of Saudi Arabia.

**LITERATURE CITED**

Bayes, J. J., and H. S. Malik, 2009 Altered heterochromatin binding by a hybrid sterility protein in *Drosophila* sibling species. Science 326: 1538–1541. https://doi.org/10.1126/science.1181756

Bhattacharyya, T., R. Reifova, S. Gregorova, P. Simecek, V. Gergelits et al., 2014 Chromosome control of meiotic chromosome synapsis in mouse inter-subspecific hybrids. PloS Genet. 10: e1004088. https://doi.org/10.1371/journal.pgen.1004088

Bosler, J. S., K. P. Davies, and G. S. Neal-Perry, 2014 Peptides in seminal fluid and their role in infertility: a potential role for opiorphin inhibition of neutral endopeptidase activity as a clinically relevant modulator of sperm motility: A review. Reprod. Sci. 21: 1334–1340. https://doi.org/10.1177/1933719114536473

Brideau, N. J., H. A. Flores, J. Wang, S. Maheshwari, X. Wang et al., 2006 Two Dobzhansky-Muller genes interact to cause hybrid lethality in *Drosophila*. Science 314: 1292–1295. https://doi.org/10.1126/science.1133953

Brill, E., L. Kang, K. Michalak, P. Michalak, and D. K. Price, 2016 Hybrid sterility and evolution in *Hawaiian Drosophila*: differential gene and allele-specific expression analysis of backcross males. Heredity 117: 100–108. https://doi.org/10.1038/hdy.2016.31

Cattani, M. V., and D. C. Presgraves, 2012 Incompatibility between X chromosome factor and pericentric heterochromatic region causes
Strater, N., D. J. Sherratt, and S. D. Colloms, 1999  X-ray structure of aminopeptidase A from Escherichia coli and a model for the nucleoprotein complex in Xer site-specific recombination. EMBO J. 18: 4513–4522.  https://doi.org/10.1093/emboj/18.16.4513
Subirán, N., F. M. Pinto, E. Agirregoitia, L. Candenas, and J. Irazusta, 2010  Control of APN/CD13 and NEP/CD10 on sperm motility. Asian J. Androl. 12: 899–902.  https://doi.org/10.1038/aja.2010.82
Tang, H., and R. C. Lewontin, 1999  Locating regions of differential variability in DNA and protein sequences. Genetics 153: 485–495.
Tao, Y., Z. B. Zeng, J. Li, D. L. Hartl, and C. C. Laurie, 2003  Genetic dissection of hybrid incompatibilities between Drosophila simulans and D. mauritiana. II. Mapping hybrid male sterility loci on the third chromosome. Genetics 164: 1399–1418.
Theofel, I., M. Bartkuhn, T. Hundertmark, T. Boettger, S. M. K. Gärtner et al., 2014  tBRD-1 selectively controls gene activity in the Drosophila testis and interacts with two new members of the bromodomain and extra-terminal (BET) family. PLoS One 9: e108267.  https://doi.org/10.1371/journal.pone.0108267
Ting, C. T., S. C. Tsaur, M. L. Wu, and C. I. Wu, 1998  A rapidly evolving homeobox at the site of a hybrid sterility gene. Science 282: 1501–1504.  https://doi.org/10.1126/science.282.5393.1501
Wittkopp, P. J., B. K. Haerum, and A. G. Clark, 2008  Regulatory changes underlying expression differences within and between Drosophila species. Nat. Genet. 40: 346–350.  https://doi.org/10.1038/ng.77
Zhao, Y., W. Sun, P. Zhang, H. Chi, M. J. Zhang et al., 2012  Nematode sperm maturation triggered by protease involves sperm-secreted serine protease inhibitor (Serpin). Proc. Natl. Acad. Sci. USA 109: 1542–1547.  https://doi.org/10.1073/pnas.1109912109

Communicating editor: E. Betran