Received: 15 March 2017 | Revised: 27 August 2019 | Accepted: 9 September 2019

DOI: 10.1111/mec.15246

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ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

# Variation in hybrid gene expression: Implications for the evolution of genetic incompatibilities in interbreeding species

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**Funding information**
Office of the Director, National Institutes of Health, Grant/Award Number: 1 DP2 OD004436-01; NIH, Grant/Award Number: R01GM110255 and R35GM130381; National Science Foundation, Grant/Award Number: IOS-1555520

## Abstract

Interbreeding species often produce low-fitness hybrids due to genetic incompatibilities between parental genomes. Whether these incompatibilities reflect fixed allelic differences between hybridizing species, or, alternatively, standing variants that segregate within them, remains unknown for many natural systems. Yet, evaluating these alternatives is important for understanding the origins and nature of species boundaries. We examined these alternatives using spadefoot toads (genus *Spea*), which naturally hybridize. Specifically, we contrasted patterns of gene expression in hybrids relative to pure-species types in experimentally produced tadpoles from allopatric parents versus those from sympatric parents. We evaluated the prediction that segregating variation should result in gene expression differences between hybrids derived from sympatric parents versus hybrids derived from allopatric parents, and found that 24% of the transcriptome showed such differences. Our results further suggest that gene expression in hybrids has evolved in sympatry owing to evolutionary pressures associated with ongoing hybridization. Although we did not measure hybrid incompatibilities directly, we discuss the implications of our findings for understanding the nature of hybrid incompatibilities, how they might vary across populations over time, and the resulting effects on the evolutionary maintenance - or breakdown - of reproductive barriers between species.

**KEYWORDS**
gene expression, genetic incompatibilities, hybridization, reinforcement, speciation

## 1 | INTRODUCTION

Hybridization between species can lead to offspring that exhibit reduced fitness (e.g., reduced survival or fertility) relative to pure-species types (Arnold, 1997; Barton & Hewitt, 1985; Coyne & Orr, 2004). Because reduced hybrid fitness constitutes a barrier to gene flow between species and helps maintain species boundaries, understanding the causes and evolution of reduced hybrid fitness is a focus of speciation research (Abbott et al., 2013; Coyne & Orr, 2004; Wolf, Lindell, & Backstrom, 2010).

Seidl and Pfennig contributed equally to the work.

Reduced hybrid fitness can result from deleterious epistatic interactions between genetic variants in pure-species genomes (such interactions are referred to as Bateson-Dobzhansky-Muller Incompatibilities, hereafter BDMs; Coyne & Orr, 2004; Cutter, 2012; Mack & Nachman, 2017; Orr, 1995). Alleles that contribute to BDMs in hybrids are often assumed to represent fixed differences in the parent species, but this does not have to be the case (Cutter, 2012; Larson et al., 2018). In some instances, loci that contribute to BDMs might be polymorphic in one or both parent species (Cutter, 2012; Gerard & Presgraves, 2012; Larson et al., 2018; Matute, Gavin-Smyth, & Liu, 2014). In this latter scenario, the nature and extent of

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BDMs between hybridizing species could depend on the standing genetic variation present in the specific populations that undergo hybridization.

Evaluating whether BDMs arise from fixed differences between parental species or segregating variants within either parental species is difficult without knowing the loci involved in BDMs. In systems where this information is unknown or difficult to obtain because mapping populations are absent (as in natural systems with non-model organisms), gene expression in hybrids relative to pure-species types can provide insight, particularly when BDMs generate regulatory incompatibilities (Brill, Kang, Michalak, Michalak, & Price, 2016; Gomes & Civetta, 2015; Landry, Hartl, & Ranz, 2007; Lopez-Maestre et al., 2017; Mack & Nachman, 2017; Malone, Chrzanowski, & Michalak, 2007; Meiklejohn, Coolon, Hartl, & Wittkopp, 2014; Michalak & Noor, 2003, 2004; Moehring, Teeter, & Noor, 2007; Ortiz-Barrientos, Counterman, & Noor, 2007; Wolf et al., 2010). Specifically, BDMs impacting gene expression might produce over- or underexpression of genes in hybrids relative to pure-species types, which, in some cases, might reduce hybrid fitness and contribute to reproductive isolation between species (Landry et al., 2007; Mack & Nachman, 2017).

To the extent that gene expression reflects possible BDMs and the genetic variation that might contribute to them, comparing hybrids and pure-species types across populations could lend insight into the nature of BDMs. In particular, if BDMs arise from fixed allelic differences between the species, as is often assumed (Cutter, 2012), then patterns of gene expression in hybrid types should be the same regardless of the populations from which the hybrids' pure-species parents are derived. If, however, BDMs largely arise from alleles still segregating within either parental species, patterns of gene expression in hybrid types relative to the pure-species types might vary depending on the populations from which the hybrids' parents are derived. Moreover, further insights into the nature of BDMs could be gained by contrasting patterns of expression in genes that differ in expression between species versus those that do not. Specifically, if expression differences between species often result from fixed allelic differences between the species, then gene expression in hybrids should be less likely to vary across populations. By contrast, if similar gene expression in the pure species reflects segregating variation, then hybrid gene expression might be more likely to vary among populations in such genes.

Understanding whether or not BDMs involve segregating variation within the parent species is important for understanding the consequences of hybridization. In particular, if BDMs arise from segregating loci, then drift, gene flow, and/or selection acting on alleles at these loci can result in the evolution of BDMs. To the extent that BDMs impact gene expression, patterns of gene expression in hybrids relative to pure-species types could therefore change over time as a consequence of these evolutionary mechanisms. For example, selection could disfavour alleles that contribute to deleterious BDMs or selection could favour alleles that modify and ameliorate BDMs. The resulting decline in frequency of deleterious alleles (or increase in frequency of modifier alleles) would reduce the adverse fitness effects of hybridization and hybrids could become more similar to the pure-species types over time (Barton & Hewitt, 1985, 1989; Lammers et al., 2013; Ritchie, Butlin, & Hewitt, 1992; Sanderson, 1989; Schilthuizen & Lammers, 2013). Regardless of how BDMs evolve (via selection or otherwise), change in BDMs will impact the nature of introgression between species and the maintenance of species boundaries.

Evaluating whether BDMs evolve can be challenging, especially in systems where the loci involved in the BDMs are unknown. Indeed, even in cases where the loci involved in BDMs have been identified and shown to be polymorphic, that information does not indicate whether BDMs change over time (i.e., evolve). Instead, approaches that either allow direct observations of evolution (e.g., experimental evolution studies) or enable inference that evolution has occurred are needed.

One means of inferring whether BDMs evolve is to experimentally simulate initial contact between species by breeding individuals from allopatric populations (that have never experienced hybridization) and contrasting the resulting hybrids with hybrids produced by breeding individuals from sympatric populations (where hybridization has been ongoing). As described above, if BDMs arise from fixed allelic differences between species, then patterns of gene expression should not differ between sympatric and allopatric hybrids (assuming gene expression adequately captures BDMs; see above). Moreover, hybrids should be less likely to show expression differences between sympatry and allopatry in those genes that differ between the pure-species types as opposed to those genes that do not differ between the pure-species types. If, however, BDMs are caused by segregating variation in either of the pure-species types (with the potential for evolution in BDMs), then expression patterns of hybrids relative to pure-species types should differ between sympatry and allopatry.

A limit of this approach is two-fold. First, in naturally hybridizing species that occur in sympatry, introgression can homogenize the two species and their hybrids so that they become more similar to each other and increasingly different from pure-species types that occur in allopatry. Second, sympatry and allopatry might differ ecologically, thereby resulting in distinct evolutionary patterns that are unrelated to hybrid fitness and BDMs. Because of these issues, contrasts of gene expression that include both pure-species and hybrid types from sympatry and allopatry can provide necessary controls to evaluate the extent to which hybrids might vary in gene expression due to the evolution of possible BDMs as opposed to ecological adaptation or introgression.

Here, we take such an approach using spadefoot toads. In particular, we contrast pure-species and hybrid types generated by interbreeding allopatric pure-species parents and sympatric pure-species parents. This design allowed us to achieve two general goals. First, we determined if gene expression in hybrids relative to pure-species types differs between allopatry and sympatry. Second, we evaluated if and how any such population-level variation in hybrid gene expression was associated with whether or not pure-species types themselves differed in gene expression. To the extent that gene

expression captures the impacts of BDMs, our findings suggest that BDMs could depend on segregating variation within the hybridizing species and that they can therefore vary across populations and evolve over time.

## 2 | MATERIALS AND METHODS

### 2.1 | Study system

We used as our study system spadefoot toads, *Spea bombifrons* and *S. multiplicata*, which hybridize in the southwestern USA. Hybrids are viable, but $F_1$ males are sterile and females are less fecund than pure-species types (Pfennig & Simovich, 2002; Simovich, 1994; Wünsch & Pfennig, 2013). Introgression between the two species occurs because hybrid females will breed with pure-species males (Schmidt & Pfennig, 2016; Simovich, 1985), and subsequent cross types appear at least partially fertile and capable of reproducing (Pfennig, Allenby, Martin, Monroy, & Jones, 2012; Pfennig & Simovich, 2002; Pierce, Gutierrez, Rice, & Pfennig, 2017; Sattler, 1985; Simovich, 1985; Wünsch & Pfennig, 2013).

As tadpoles, the survival of $F_1$ hybrids depends on which species is maternal. When *S. multiplicata* is maternal, $F_1$ hybrids have lower survival than either *S. bombifrons* or *S. multiplicata* tadpoles. When *S. bombifrons* is maternal, $F_1$ hybrids survive as well as either pure-species type (Pfennig & Simovich, 2002). Moreover, hybrids have intermediate growth rates between the faster developing *S. multiplicata* and slower developing *S. bombifrons* tadpoles (Pfennig & Simovich, 2002). Because faster development is favoured in the ephemeral ponds where tadpoles develop, $F_1$ hybrid tadpoles have higher fitness relative to *S. bombifrons* tadpoles but not *S. multiplicata* tadpoles (Pfennig, 2007; Pfennig & Simovich, 2002).

The combination of these aspects of fitness generates opposing patterns of selection on hybridization behaviour in females of the two species (Pfennig & Simovich, 2002). Because hybrids with *S. multiplicata* mothers develop slower and have lower survival and reduced fertility than pure-*S. multiplicata* types, selection favours *S. multiplicata* females that do not hybridize. Indeed, female *S. multiplicata* mate choice shows hallmark patterns of divergent mating behaviours between sympatry and allopatry, as is expected if reinforcement has occurred (Pfennig, 2000; Pfennig & Rice, 2014), and reinforcement has potentially caused hybridization to decline in some populations (Pfennig, 2003).

In contrast to *S. multiplicata* females, *S. bombifrons* females benefit by hybridizing in ephemeral ponds where hybrids have a fitness advantage relative to pure *S. bombifrons* tadpoles (Pfennig, 2007; Pfennig & Simovich, 2002). Consequently, *S. bombifrons* females facultatively hybridize with *S. multiplicata* males in ephemeral ponds, but not long-lasting ponds (Pfennig, 2007). This context-dependent behaviour evolved in sympatry: allopatric females do not discriminate between conspecific and heterospecific males (Pfennig, 2007). Thus, hybridization, as well as the interbreeding of hybrids with pure-species types, occurs in sympatry (Pfennig et al., 2012; Pfennig & Simovich, 2002; Sattler, 1985).

Spadefoot natural history makes them well suited for addressing our goals (see Section 1). Specifically, as described below, we evaluated if gene expression in hybrids differs between crosses derived from sympatric individuals (in populations where hybridization is ongoing) and allopatric individuals (simulating hybrids produced by first contact between the species).

### 2.2 | Sample production and preparation

We crossed allopatric and sympatric *S. bombifrons* and *S. multiplicata* to generate eight pure-species and hybrid cross types. Table 1 shows these cross types, their corresponding abbreviations used throughout the paper, and the number of sequenced biological replicates per cross type.

To generate hybrid tadpoles from allopatric populations, we bred *S. multiplicata* from populations in Arizona, USA, outside the western range limit of *S. bombifrons*, with *S. bombifrons* from populations in Colorado, USA, outside the northern range limit of *S. multiplicata*. Because of the geographic distance between the populations used to create allopatric hybrids, we generated comparable sympatric hybrids by pairing *S. multiplicata* and *S. bombifrons* from sympatric populations in Arizona and Texas (i.e., sympatric hybrids were not

**TABLE 1** Cross types used in the experiment with their abbreviations used throughout text and figures

| Population | Parents (female x male) | Abbreviation used in text | Number of replicate families |
|---|---|---|---|
| Allopatry | *S. bombifrons* x *S. bombifrons* | BBa | 4 |
| Allopatry | *S. multiplicata* x *S. multiplicata* | MMa | 3 |
| Allopatry | *S. bombifrons* x *S. multiplicata* | BMa | 3 |
| Allopatry | *S. multiplicata* x *S. bombifrons* | MBa | 4 |
| Sympatry | *S. bombifrons* x *S. bombifrons* | BBs | 3 |
| Sympatry | *S. multiplicata* x *S. multiplicata* | MMs | 4 |
| Sympatry | *S. bombifrons* x *S. multiplicata* | BMs | 3 |
| Sympatry | *S. multiplicata* x *S. bombifrons* | MBs | 4 |

*Note:* In the text, the BMs and MBs hybrids are referred to as sympatric hybrids, whereas the BMa and MBa hybrids are referred to as allopatric hybrids. Note that allopatric hybrids would not be produced in nature.

derived by crossing individuals from the same population). So that species identity and population identity were not confounded, half of the families had *S. multiplicata* parents from Arizona and *S. bombifrons* from Texas, whereas half had *S. multiplicata* parents from Texas and *S. bombifrons* from Arizona.

To induce breeding, adults were injected with 0.07 ml 0.01 μg/ml gonadotropin releasing hormone (GnRH) agonist. Males and females were placed as pairs in separate aquaria with 10 L of dechlorinated water and allowed to oviposit. We generated at least three replicate families per cross type (Table 1). After egg release was complete, adults were removed from the tanks and the eggs were aerated until hatching. When tadpoles were swimming freely, we selected a subset of 16 tadpoles at random from each family. For each family we divided the tadpoles into two groups of eight and placed each group in a tank (34 cm × 21 cm × 11.5 cm) filled with dechlorinated water. All were fed shrimp and detritus (their natural diet) ad libitum. At approximately one week old, we euthanized tadpoles by placing them in MS-222 and freezing them in liquid nitrogen. Spadefoot tadpoles reach metamorphosis in as few as three weeks (Pfennig & Simovich, 2002). Thus, one-week-old tadpoles represented tadpoles that were well along in development and therefore had the potential to exhibit differential expression in genes that impact growth and survival during the tadpole stage.

To prepare samples for RNA extraction, we randomly selected a single whole frozen tadpole from each family, ground each tadpole with a mortar and pestle, and then homogenized each of our samples in 15 ml centrifuge tubes. Each tadpole had a mass of approximately 0.2 g. We extracted RNA from each tadpole sample using Invitrogen PureLink extraction columns with TRIzol reagent. We obtained RNA from 28 samples total (biological replicates; Table 1; Table S1). 3′ RNA-seq libraries were generated using the Lexogen Quantseq FWD kit and sequenced on an Illumina NextSeq500 (Illumina) using a single end 75 bp kit with an actual read length of 86 bp. Resulting read counts are reported in Table S1. Library preparation and sequencing were performed at the Cornell University Institute of Biotechnology.

## 2.3 | Measurement of gene expression

To measure gene expression across our different cross types (Table 1), we began by trimming the 3′ RNA-seq reads to remove adapter and poly-A contamination using Trimmomatic (Bolger, Lohse, & Usadel, 2014) with recommended parameters. Individual reads, as well as pooled reads from all pure individuals were mapped to the *S. multiplicata* genome, which is described in Seidl et al., (In press), using STAR aligner (Dobin et al., 2013) with default parameters. We used bedtools genome_cov (Quinlan & Hall, 2010) to generate bed coverage files at all positions. We performed peak discovery by finding all continuous windows of coverage ≥50. We defined the peak as the base with the maximum coverage in each window. We then extracted coverage of each peak for each individual using the bedtools coverage tool. Gene expression measurements within a sample were normalized by library size × $10^{-6}$ and $\log_2$ transformed. The table containing measurements from all samples was then digitally normalized using the R function normalize.quantiles() from the PREPROCESSCORE package (Bolstad, 2016). The following analyses were performed entirely in R. For each gene, we first fit the following global model to data:

$$expression = sampletype + error,$$

where *expression* corresponds to a vector of normalized $\log_2$ expression measurements for the samples, *sampletype* corresponds to each of our eight cross types (Table 1), and *error* denotes the vector of residuals. We used this model because we did not have a full-factorial design and alternative models would have inappropriately nested the parent and hybrid terms. Models were fit using the lm() function, *p*-values were extracted for each model using the summary() function, and point-wise FDR values (i.e., *q*-values) were then obtained using the *q*value package (Storey, Bass, Dabney, & Robinson, 2018). We used a significance threshold of FDR ≤ 0.05.

We identified 10,695 protein-coding genes in the transcriptome, and, of these, we found a total of 9,327 genes that showed a

**TABLE 2** Number of significant genes at $p \leq .0056$ (.05/number of contrasts) for each contrast performed as well as categories in which each contrast was included (see also Figure 1)

| Contrast 1 | Contrast 2 | Significant genes | Category |
|---|---|---|---|
| BB (BBs and BBa) | MM (MMs and MMa) | 3,094 | Pure-species |
| BMa | MBa | 344 | Hybrids |
| BMa | MBs | 978 | Hybrids |
| BMs | MBa | 248 | Hybrids |
| BMs | MBs | 1,930 | Hybrids |
| BMa | BMs | 438 | Hybrid; Sympatry vs. allopatry, hybrids |
| MBa | MBs | 2,255 | Hybrid; Sympatry vs. allopatry, hybrids |
| BBa | BBs | 232 | Sympatry vs. allopatry, pure-species |
| MMa | MMs | 80 | Sympatry vs. allopatry, pure-species |

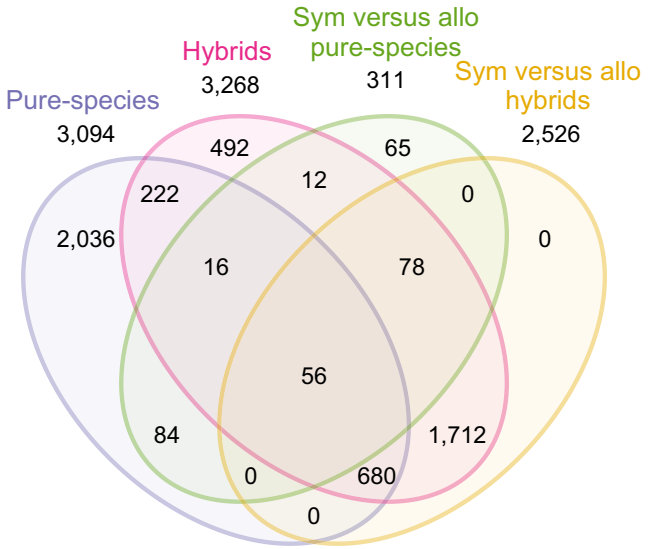


**FIGURE 1** Venn diagram showing number (and overlap) of significant genes in each set of post hoc contrasts: between pure species (BB vs. MM; Pure-species), between populations from which pure species were derived (BBa vs. BBs and MMa vs. MMs; Sym vs. allo, pure-species), between hybrids (all pairwise combinations of the hybrid cross types; Hybrids), and between populations from which hybrids were derived (BMa vs. BMs and MBa vs. MBs; Sym vs. allo, hybrids)

significant effect of sample type at an FDR of 5%. For these 9,327 genes in this initial analysis, we next ran nine post hoc contrasts aimed at identifying expression differences among our treatment groups. Specifically, we performed contrasts between the pure-species types, among the hybrid cross types, and between allopatry and sympatry (Table 2).

To perform these contrasts, we used the function lsmeans() (Lenth, 2016) followed by the contrast() function. We considered a post hoc test as significant if $p \leq .0056$ (i.e., 0.05 divided by the nine contrasts), and this procedure resulted in 5,453 genes showing at least one significant difference in the post hoc contrasts (see Section 3). This number represents 58% of the 9,327 genes initially identified as significant, and is a consequence of the conservative alpha level threshold that controls for the large number of post hoc tests performed for each gene. However, we ran the analyses using a less stringent threshold ($p \leq .05$) that did not correct for multiple post hoc tests per gene, and the results were qualitatively similar. Therefore, we report the results from the more conservative analysis here.

Genes could be significant in multiple contrasts, so we used the R package LIMMA (Ritchie et al., 2015) to generate a Venn diagram for visualizing the overlap of the gene sets (Figure 1). We then used chi-square tests to contrast the number of genes in these overlapping sets to evaluate whether patterns of expression in hybrids from different populations were consistent with the expectation that BDMs derive from fixed variation between, versus segregating variation within, either species.

## 2.4 | Gene ontology analysis in gene sets identified as significant in post hoc tests

Because the post hoc analyses revealed significant differences among our cross types (see Section 3), we evaluated if any of the gene sets identified in the post hoc contrasts (Table 2) showed enrichment or depletion of biological process gene ontology (GO) terms. Doing so allowed us to determine whether the significant genes were more likely to arise among certain processes (e.g., fertility).

To perform the GO analysis, we compared the number of genes with or without a particular GO term in a given focal set of genes to the number of genes with or without that term in those genes not in the given focal set (Cai, Mao, Li, & Wei, 2006). For each GO term, we performed a chi-square test using the chisq.test() function in R and corrected for multiple testing based on the false discovery rate using the *q*value package (Storey et al., 2018).

## 2.5 | Subsequent analysis of expression levels in subsets of genes that differed between species or hybrids

As described in the Section 1, BDMs are typically assumed to arise from fixed allelic differences, such that hybrid gene expression should not vary across populations. We found a large number of genes that were significantly different in expression between hybrids derived from sympatry versus allopatry. Moreover, hybrids were less likely to differ between sympatry and allopatry in genes that were also different between the two species (see Section 3). However, we also identified a number of significant genes that varied among hybrids but were not significantly different between the pure-species.

Based on these findings from post hoc contrasts (Table 1; see also Figure 1), we next sought to contrast gene expression among all of our cross types in three subsets of genes. Specifically we contrasted patterns of expression among all of our cross types in: (a) genes that differed between the pure-species types; (b) genes that did not differ between the species but that did differ in at least one hybrid contrast; and (c) genes that did not differ between species or within species between sympatry and allopatry, but were nevertheless different between allopatric and sympatric hybrids. The first and second subsets of genes allowed us to examine how hybrids vary relative to pure-species types as a function of whether the pure-species themselves differed in expression. The third set of genes were those genes that were seemingly invariant within and between the species, but still revealed variation in the hybrids. We took two approaches to examining gene expression in our genes of interest: a multivariate approach that contrasted overall patterns of expression among cross types, and a gene-level approach that identified which genes showed similar patterns of expression in our cross types.

For the multivariate approach, we performed separate principal component analyses (PCA) on each of these three sets of

genes using the princomp() function in R. Then, using the top two principal components (PC) scores from each PCA, we contrasted the locations of the hybrid and pure-species types in PC space for each of the three gene sets. We used the pairwise() function in the RRPP package to calculate the distance (and corresponding empirically derived *p*-values) among all cross types using a non-parametric randomized residual permutation procedure (Collyer, Sekora, & Adams, 2015).

To evaluate gene-level patterns of expression in our three subsets of genes, we used *k*-means clustering, with the kmeans() function in R, from $k = 1$ to $k = 10$ to identify clusters in our gene sets. We determined the appropriate number of clusters in a given analysis by applying the elbow method to the total within group sum of squares obtained for each level of *k*. We plotted heatmaps of the matrix of expression values ordered by the output of the *k*-means clustering using the heatmap3() function from the HEATMAP3 package in R (Zhao, Guo, Sheng, & Shyr, 2015). To determine the relationships between samples in each *k*-cluster, we calculated Euclidian distance on the matrix of per sample averaged gene expression values using the R function dist(), clustered these distance values using the R package hclust() and plotted dendrograms for each cluster of genes using the R package APE (Paradis & Schliep, 2018).

## 3 | RESULTS

Using post hoc contrasts (Table 2), we identified 5,453 genes that showed at least one significant difference among our cross types (Figure 1). Of these 5,453 genes, 3,094 (57% of the significant genes) differed between the species, and 3,268 (60% of the significant genes) differed in some way among hybrids (Figure 1). A large number of genes, 2,703, also differed between crosses derived from sympatry versus those derived from allopatry (Figure 1). Of the genes that differed between sympatric and allopatric crosses, 93% differed among hybrids whereas only 12% differed in the pure-species types (Figure 1). Excluding the 5% that differed in both, we found that hybrids were significantly more likely than the pure-species types to differ depending on whether they were derived from sympatry versus allopatry (assuming a 50:50 random expectation; $\chi^2 = 1{,}909.8$; $df = 1$; $p < .001$).

The finding that most of the genes that differ between the sympatric and allopatric crosses were in the hybrid types is consistent with the possibility that BDMs might derive from segregating variation as opposed to fixed allelic differences between the species. To further evaluate this possibility, we assessed whether sympatric and allopatric hybrids are more likely to show expression differences in genes that do not differ in the pure-species types versus those that differ between the pure-species types. We found that, of the 2,526 genes that differed between sympatric and allopatric hybrids, 736 (29%) also differed between species. By contrast, 1,790 (71%) of these 2,526 genes were not different between the two species, a pattern that was significantly different from random 50:50 expectation ($\chi^2 = 439.79$; $df = 1$; $p < .001$). Thus, hybrids are less likely to show population differences in gene expression in those genes that differ in expression between species.

Much of the overall variation among the hybrid types appears to have been driven by these differences between sympatric and allopatric hybrids. In particular, the 2,526 genes that differed between sympatric and allopatric hybrids constituted 77% of the 3,268 genes that differed among all hybrid contrasts (Figure 1; see also Table 2), whereas 742 (23%) of the genes that differed between at least one hybrid type were not associated with differences between sympatry and allopatry. This pattern was significantly different from random 50:50 expectation ($\chi^2 = 973.89$; $df = 1$; $p < .001$). Interestingly, of these 742 genes, 504 (68%) were not different between the pure-species types, whereas 238 (32%) were different between the two species, a pattern that was also significantly different from random 50:50 expectation ($\chi^2 = 95.36$; $df = 1$; $p < .001$). This result provides further evidence that our hybrid types were less likely to differ in expression in those genes that differ between species.

We evaluated whether any particular processes or pathway might be driving the significant genes in our post hoc contrasts. However, when we used gene ontology (GO) analysis corrected for multiple tests, we found no evidence for enrichment of any GO term in any of the genes identified in the four types of post hoc contrasts (i.e., Pure-species, Hybrids, Sympatry vs. allopatry, pure-species, Sympatry vs. allopatry, hybrids; Table 2).

We also sought to examine levels of expression among all of our cross types in genes that: (a) differed between the pure-species types ($N = 3{,}094$); (b) did not differ between the species, but did differ between hybrids ($N = 2{,}294$); and (c) did not differ between species or within species between sympatry and allopatry, but were nevertheless different between allopatric and sympatric hybrids ($N = 1{,}712$).

In the PCA of those 3,094 genes that differed between the species, the first two PCs combined to explain 56.0% of the variance in the data and, as expected, distinguished the *S. bombifrons* and *S. multiplicata* samples (Figure 2a; Table S2). Generally, the hybrid cross types were intermediate to the pure-species types (Figure 2a), When we compared distances among all groups, we found that BMa was not significantly different from any other cross type; MBs differed from all other groups except BMa; MBa differed from BBa and MBs; and BMs differed from both MM types and BBa, but not BBs (Figure 2a,b; Table S2). Notably, the distance, 0.552, between the sympatric hybrid types (MBs and BMs) and the distance, 0.529, between the MB cross types (MBs and MBa) were similar in magnitude to the distances (ranging from 0.515 to 0.559) among the pure-species types (BB to MM; Figure 2a,b; Table S2). Thus, in terms of multivariate patterns of gene expression, hybrid types can show differences from each other that are on the order of that seen between species.

Surprisingly, the distance between the sympatric hybrid types (BMs to MBs = 0.552), was twice that of the distance between the allopatric hybrid types (BMa to MBa = 0.221). In other words, hybrids with different species as mother actually were more dissimilar in the sympatric hybrids versus the allopatric hybrids. Part of

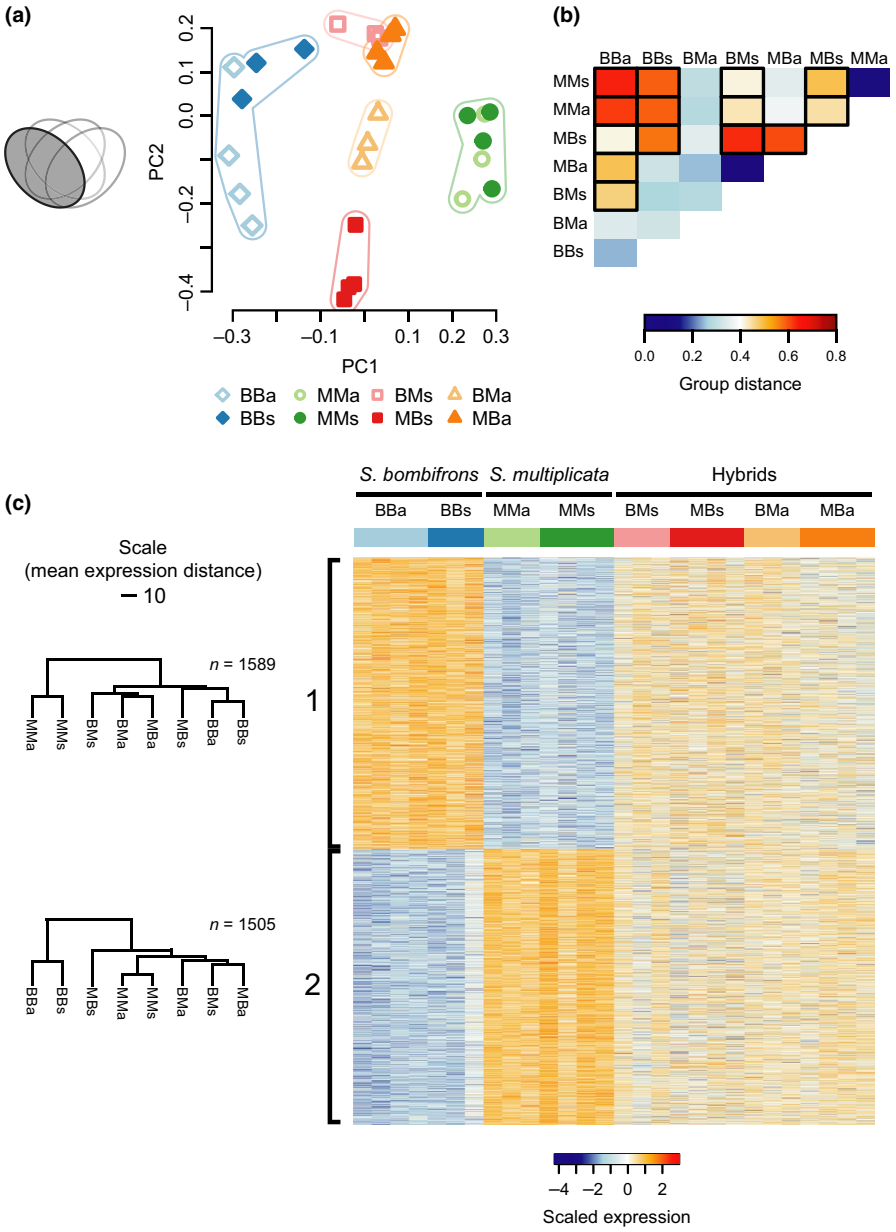


**FIGURE 2** Patterns of gene expression among cross types in those genes that differed between the species ($N$ = 3,094). Shaded portion of icon indicates where these genes correspond to Figure 1. (a) Results from PCA contrasting cross types in multivariate space. Each point represents a replicate family/tadpole for each cross type; polygons are for illustration only to indicate groups by cross type. (b) Heatmap of the magnitude of pairwise distances among groups; bold outlines denote which groups were significantly different from each other. Distances are provided in Table S2. (c) Heatmap of expression values ($\log_2$(fold coverage) scaled by Euclidian distance from row mean) each row corresponds to one gene and each column corresponds to one sample (i.e., one family/tadpole). Numbers provided to the left denote cluster membership as determined by $k$-means clustering. On the left of each cluster is a dendrogram showing the Euclidian distance of mean gene expressions between samples. Colours associated with cross types at top of heatmap correspond to those in (a)

this pattern was driven by BMs being more similar to the BBs type (0.237; Figure 2a,b; Table S2). However, part of the result was the very large difference in MBs expression relative to the other sympatric cross types (Figure 2a,b; Table S2).

The above patterns in multivariate space were recapitulated in the gene-level $k$-means analysis. We identified two clusters that were driven by the differences in expression between the species (Figure 2c). Although the hybrids were generally intermediate between the pure-species types, we did find that, in both clusters, patterns of expression relative to the pure-species types were similar for the BMa, BMs, and MBa hybrid types. By contrast, MBs was distinct from these hybrid types. Taken together with the PCA results, these findings highlight that our sympatric and allopatric hybrids differed in gene expression relative to pure-species types even for genes where the species exhibited expression differences.

When we used PCA to evaluate multivariate expression in the 2,294 genes that did not differ between the pure-species types but did vary among hybrids, we found that the first two PCs explained 49.9% of the variance. In this analysis, PC space was characterized by differences among hybrids as expected (Figure 3a). We found that MBs hybrids were significantly different from all other groups except BBa; MBa hybrids were different from BBa, BMs, and MBs; BMa hybrids were different from all groups except MBa and MMa; and BMs hybrids were different from all groups except BBs and MMs (Figure 3a,b; Table S2). For this set of genes, as with those above, the distance between the sympatric hybrid types (BMs to MBs = 0.560) was twice as large as that between allopatric hybrid types (BMa to MBa = 0.272). Thus, the hybrids produced from sympatric parents were more dissimilar in gene expression than those produced by allopatric parents. Moreover, the magnitude of difference between allopatry to sympatry for hybrids of each maternal type was similar

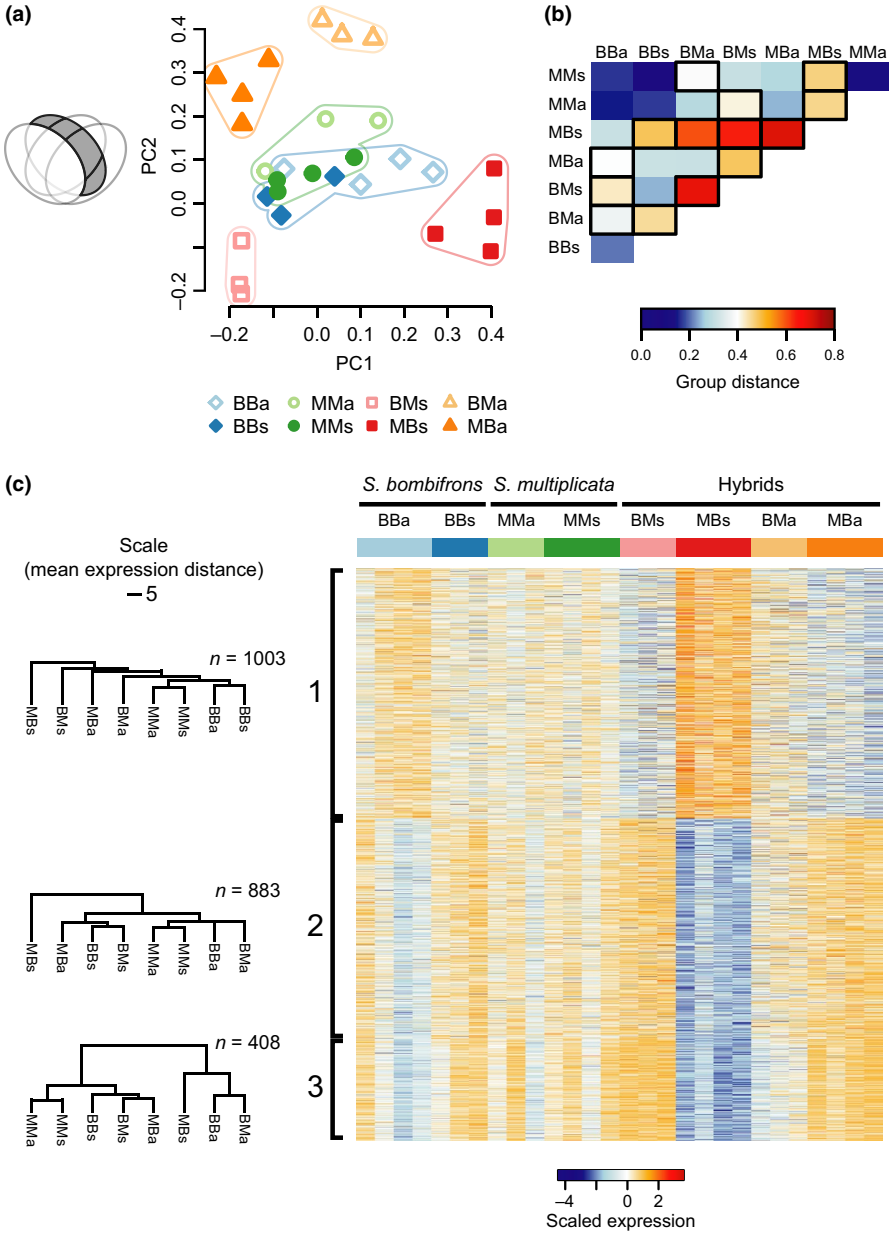


**FIGURE 3** Patterns of gene expression among cross types in those genes that did not differ between the species, but did differ between hybrids ($N$ = 2,294). Shaded portion of icon indicates where these genes correspond to Figure 1. Results from PCA and $k$-means clustering are depicted as in Figure 2

and represented the largest differences observed in the analysis (BMa to BMs = 0.605; MBa to MBs = 0.617; Table S2). However, the nature of the differences between allopatry to sympatry differed between hybrid types: the BM crosses differed along PC2, whereas the MB crosses differed along PC1 (Figure 3a).

The $k$-means clustering highlighted these different gene-level expression patterns. Three sets of genes showed distinct patterns of expression among the hybrids (Figure 3c). For most of the genes (in clusters 1 and 2; Figure 3c), MBs hybrids were distinct from the other cross types in expression. By contrast, BMs clustered with both the MBa and BBs cross types in most of the genes (clusters 2 and 3; Figure 3c), although for the 44% of genes in cluster 1, the BMs group was distinct from the pure-species types and other hybrids (Figure 3c).

Finally, we evaluated expression patterns in the 1,712 genes that differed only between sympatric and allopatric hybrids. In the PCA analysis of these genes, the first two principal components explained 54% of the variance. We found that the BMa cross type differed from all other cross types except MMa and MBa (Figure 4a,b), whereas the BMs cross type differed from both MB cross types, the BMa cross type and the BBa cross type. The MBa cross type differed from BMs, MBs and BBa, whereas the MBs cross type differed from all other cross types except BBa. A noteworthy pattern is that BMs was more similar to the sympatric pure-species types (BBs and MMs) than BMa. By contrast, MBs was more different from the pure-species types that MBa (Figure 4a,b; Table S2).

When we evaluated the gene-level patterns underlying the PCA results, we found three clusters of expression patterns (Figure 4c). In clusters 1 and 3 (representing 60% of the 1,712 genes in the analysis), MBs were distinct from the other groups, including the pure-species types. By contrast, for these same genes, BMs clustered more closely with the pure-species cross types. For the remaining

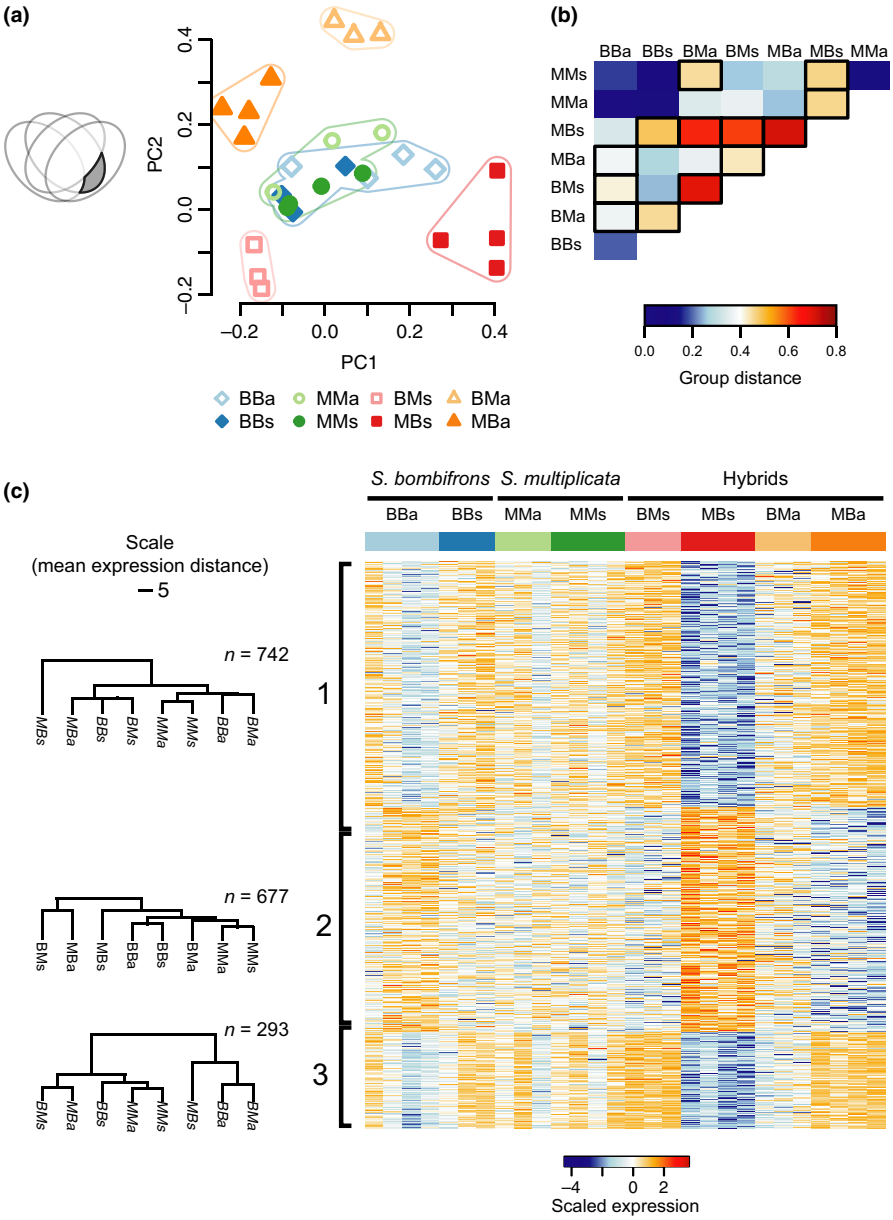


**FIGURE 4** Patterns of gene expression among cross types in those genes that did not differ between species or within species between sympatry and allopatry, but were nevertheless different between allopatric and sympatric hybrids ($N$ = 1,712). Shaded portion of icon indicates where these genes correspond to Figure 1. Results from PCA and $k$-means clustering are depicted as in Figure 2

genes in cluster 2, BMs and MBa clustered together. Nevertheless, although MBs grouped with the other cross types for this gene set, they were distinct within the group and showed notably higher expression relative to the other cross types (Figure 4c).

## 4 | DISCUSSION

Using spadefoot toads and their $F_1$ hybrids, we evaluated gene expression in pure-species and hybrid tadpoles derived from two population types: sympatry, where hybridization occurs, and allopatry, where hybridization has not occurred. If BDMs arise from fixed allelic differences between species, then sympatric and allopatric hybrids should be more likely to show similar patterns of gene expression. If, however, BDMs arise from segregating variation within the species, then hybrid gene expression should depend on the populations from which the pure-species parents derive. Perhaps more critically, any such variation in the pure species could be subject to drift, gene flow, or selection, so that BDMs could evolve. By contrasting experimentally produced hybrids from allopatric parents (thereby simulating first contact) with hybrids produced from sympatric parents, we could infer whether BDMs might have evolved in populations where hybridization is ongoing.

A major caveat of our study is the limits of using gene expression to gain insights into BDMs. Patterns of gene expression in hybrids do not directly correspond to BDMs, because even when hybrids differ from the pure-species types in gene expression, such mis-expression might not correspond to reduced fitness that would characterize BDMs (Landry et al., 2007). Moreover, the number of genes that do show expression differences provide limited insights in the quantity of BDMs that might be involved because one or a few major loci might have cascading effects that impact the expression

of many genes (Landry et al., 2007). Nevertheless, although not all patterns of gene expression represent BDMs, we assume that our data capture BDMs that impact gene expression at the tadpole stage we sampled. Using gene expression in this way lends itself to the study of BDMs (Landry et al., 2007; Mack & Nachman, 2017), especially in natural systems like ours where mapping populations do not exist and direct identification of BDMs is difficult. Indeed, despite the limitations of our approach, the general patterns of expression we observed in the sympatric hybrids broadly correspond to the known fitness consequences of hybridization in this system. Thus, to the degree that our measures of gene expression in hybrids captures BDMs, our study can provide insights into whether BDMs involve segregating variation in the pure species and the potential for BDMs to evolve over time.

We identified 2,526 protein-coding genes that exhibited expression differences between *Spea* hybrids produced from sympatric versus allopatric parents (Figure 1). Among the genes whose expression was measured in this study, these genes represented both a high proportion of the overall transcriptome (24%) and a large proportion (77%) of the 3,268 genes that varied among hybrids in some way (Figure 1, Table 2). Although we found that genes were less likely to differ in expression between sympatric and allopatric hybrids when those genes did differ in expression between the two species (as might occur if there are fixed allelic differences between the species), we also found that, even when the pure-species differed in expression, sympatric and allopatric hybrids could still differ in expression (Figure 2). Thus, segregating variation, as evidenced by population effects on hybrid gene expression, might contribute to BDMs.

One explanation for expression differences between our sympatric and allopatric hybrids is that introgression in sympatry homogenizes all types, including the pure-species. Consequently, as cross types become more similar to each other in sympatry (owing to introgression), each will become more different from its analog in allopatry. A further explanation for expression differences between sympatric and allopatric hybrids is that sympatry and allopatry constitute different habitats favouring different patterns of gene expression. Consequently, local adaptation would generate differences in gene expression between sympatry and allopatry in the species and their hybrids.

Neither of these explanations are supported by our data. In particular, we identified relatively few genes (311, representing 2.9% of the transcriptome; Figure 1) that showed expression differences between pure-species types derived from sympatry versus allopatry. That genes within the pure-species types do not show widespread population differences in expression indicates that neither introgression homogenizing the species within sympatry nor major ecological differences adequately explain the patterns we observed. Indeed, in the PCA using the genes for which the two species differ in expression, we did not observe any changes in the distance of the allopatric versus sympatric pure-species types relative to one another (Figure 2; Table S2); if the two species were converging due to introgression, then sympatric pure-species types should have been more similar to each other in expression. Moreover, we would have expected both sympatric hybrid types (BMs and MBs) to be more similar to both pure-species types and each other, which was not the case (Figure 2; Table S2).

Although introgression within sympatry does not fully explain the observed results, our finding that the sympatric hybrid types (BMs and MBs) consistently differed could be accounted for by differential introgression of the X chromosome in sympatry (Presgraves, 2018). Differential movement of the X chromosome relative to introgression in the remainder of the genome could also possibly account, at least in part, for the exaggeration (or reduction) of BDMs in sympatric hybrids relative to allopatric hybrids. Sex determination in *Spea* is currently unknown (patterns of hybrid fertility are consistent with an X–Y system), and work with other frog species that lack heteromorphic sex chromosomes reveal mixed evidence of differential introgression of the X chromosome (Dufresnes et al., 2016; Gerchen, Dufresnes, & Stock, 2018). Thus, the possibility of differential sex chromosome introgression needs further evaluation in this system. Moreover, we would expect that any such introgression would generate differences within either species between sympatry and allopatry, which was not observed (Figure 1; see also Figure 2).

A further issue with our data is that, because of introgression, the sympatric pure-species parents we used to generate the offspring in our experiment might have included alleles from the alternate species in their genomes (and the presence/location of such alleles might have varied across the parents). Interbreeding them might have thereby exposed incompatibilities that would not have been similarly exposed in the allopatric crosses (e.g., because of dominance). Consequently, sympatric families might have been more variable and such noise could have impacted our findings. Although we cannot rule out this effect entirely, we do not believe such an effect is the primary driver of our findings. Inspection of Figures 2–4, where each cross is presented as points in panel A and as columns in panel C, reveals two points that suggest this is not an issue. First, the dispersion of points in multivariate space, is not consistently greater in the families derived from sympatry than in the families derived from allopatry. Second, the heat maps do not show substantial variation within each cross type relative to the patterns among cross types. Moreover, as in the PCA, the families derived from sympatry are not consistently more variable than the families derived from allopatry. Thus, although we cannot rule out the possibility of greater variability in BDMs in our sympatric crosses relative to the allopatric crosses, the patterns of gene expression we observed does not suggest that such variability had a large impact on our results.

Despite being unable to entirely rule out these different impacts of introgression on our results, our findings nevertheless highlight two key points. First, our findings are consistent with the possibility that BDMs in hybrids are not necessarily driven by fixed differences between species (Cutter, 2012). If BDMs derive from fixed differences between the species, patterns of gene expression in hybrids should not depend on the population from which the pure-species parents were derived. Given the extensive variation in hybrid gene expression that we observed (Figure 1), our findings are more consistent with the possibility that segregating variation within either

of the pure species generates expression differences in hybrids that depend on the parents' population.

Second, our finding that so many genes differed between sympatric and allopatric hybrids emphasizes the possibility that BDMs can evolve. In particular, if BDMs arise from segregating variation within the pure-species (Cutter, 2012), then the occurrence and severity of incompatibilities produced in hybrid offspring and subsequent hybrid/hybrid or pure-species/hybrid crosses could vary over time depending on drift, gene flow, or selection. In the case of selection, for example, variation in BDMs could produce variation in phenotypes among hybrids upon which selection can act to either purge deleterious allelic combinations or favour modifier alleles that improve hybrid fitness (Barton & Hewitt, 1985, 1989; Lammers et al., 2013; Ritchie et al., 1992; Sanderson, 1989; Schilthuizen & Lammers, 2013). At the level of gene expression, selection acting in this way might result in hybrids becoming more similar in expression to either of the pure-species types over time, especially if selection favours pure-species expression patterns.

For some of the genes we identified, our results for hybrids produced by *S. bombifrons* females are consistent with the possibility that selection has ameliorated BDMs in sympatry. Specifically, the PCA and *k*-means analyses show that the BMs cross type is consistently more similar to the sympatric pure-species cross types (BBs and MMs) than it is to either allopatric pure-species cross type (BBa and MMa; Table S2). This pattern was emphasized in those genes that only differed in sympatric versus allopatric hybrids and thereby controlled for confounding variation between and within species (Figure 4; Table S2). As might be expected, the BMs cross type was more similar to pure-species patterns of expression than the first contact BMa cross type (although this varied across different gene clusters; Figure 4). If the expression differences between hybrids and pure-species types correspond to BDMs that reduce fitness in hybrid types, then our findings with the BM cross types suggest that BDMs could have been ameliorated in sympatry. Whether this is actually the case requires further study.

Although more work must be done to fully evaluate whether BDMs have been evolutionarily ameliorated in the BM cross direction, it is noteworthy that this cross direction is the one that is favoured by natural selection in some environments (Pfennig, 2007), and it is the cross direction more frequently observed in nature (Pfennig & Simovich, 2002). Indeed, *S. bombifrons* females preferentially hybridize when doing so is adaptive (Pfennig, 2007). Thus, the cross direction that is regularly exposed to selection is the one in which we observed patterns of gene expression that are consistent with the possibility that selection has acted to mitigate BDMs. Generally, any such mitigation of BDMs could reduce the strength of selection against hybrids or, if hybridization is actually favoured in some circumstances, mitigation of BDMs could broaden the conditions under which it would be favoured.

At the same time, our data also indicate that BDMs can evolve to become more exaggerated. Indeed, for many of the genes, the MBs cross type stands out as distinct from most of the other cross types, and the MBa hybrids were more similar to the pure-species types than MBs hybrids. These results diametrically contradict the prediction that selection will ameliorate incompatibilities, and pose the question of why BDMs should evolve to become more severe. One explanation is that the changes that occurred to mitigate BDMs in the BM cross direction generate more severe incompatibilities in the MB direction. A further explanation is that the directionality in hybrid production contributes to differential introgression of the sex chromosome(s) as described above, and this differential introgression contributes to the maternal effect that is observed in the differences between sympatric and allopatric hybrids. Disentangling these different explanations for the observed patterns will require additional work.

Regardless of why the MBs cross type is distinct, the expression patterns might account for the relatively low fitness of MB hybrids and, concomitantly, selection favouring *S. multiplicata* to avoid hybridizing with *S. bombifrons* males in sympatry (Pfennig, 2000; Pfennig & Rice, 2014; Pfennig & Simovich, 2002). Indeed, selection on *S. multiplicata* females to avoid hybridization appears to have driven reinforcement in this system (Pfennig, 2000, 2003; Pfennig & Rice, 2014). Generally, reinforcement is most likely when both the costs and risk of hybridization are high, but it can be impeded by gene flow between species (Coyne & Orr, 2004; Pfennig & Pfennig, 2012; Price, 2008; Servedio & Noor, 2003). If, as suggested by our results, the fitness costs of hybridization can become increasingly severe in sympatry, reinforcing selection could be maintained even in the face of introgression. Thus, our results also lend insight into the underlying mechanisms that might contribute to differential selection on hybridizing species (Pfennig & Simovich, 2002), while also indicating how reinforcement and adaptive hybridization can potentially occur in the same system (cf., Pfennig, 2003, 2007).

Taken together, the results of our study suggest the potential for BDMs to evolve after species come into contact and experience ongoing hybridization. How BDMs evolve, whether by selection, gene flow, drift, or as correlated effects of these processes acting at other loci, remains an open area of inquiry, and future work is needed to evaluate the problem. Doing so is important because BDMs constitute key reproductive isolating mechanisms between species. To the degree that they vary in space or time, so too will reproductive isolation. Speciation theory generally does not account for the possibility that BDMs vary among populations or evolve over time. Additional theoretical and empirical work is needed to understand whether BDMs do vary in space and time and what the implications of this variation might be for the origins and maintenance of diversity.

## ACKNOWLEDGEMENTS

We are grateful to A. Leichty and A. Kelly for laboratory assistance and to D. Pfennig, A. Pierce, C. Martin, G. Calabrese, S. Ingley, S. De La Serna Buzon, P. Durst, J. Pemberton, and four anonymous reviewers for comments and discussion that improved the manuscript. A New Innovator Award from the Office of the Director, National Institutes of Health (1 DP2 OD004436-01) and a grant from the National Science Foundation (IOS-1555520) to K.S.P. funded the

work; F.S. and I.M.E. were supported by an Alfred P. Sloan Fellowship (I.M.E.) and NIH grants R01GM110255 and R35GM130381 (I.M.E.). UNC's IACUC approved all procedures.

## AUTHOR CONTRIBUTIONS

K.S.P. conceived the study; K.S.P., I.M.E., F.S., and N.A.L. developed the experimental design with input from C.D.J.; K.S.P. carried out the breeding, tadpole rearing, and sample acquisition. A.M.-E., and N.A.L. performed RNA extractions and library preparations for sequencing. N.A.L. performed the GO analyses and permutation tests on PCA results and K.S.P. performed the goodness of fit contrasts. F.S. and I.M.E. performed all other analyses. K.S.P., I.M.E., and F.S. wrote the paper with input from N.A.L.; C.D.J. and A.M.-E. reviewed and approved the final versions.

## DATA AVAILABILITY STATEMENT

Raw transcriptome read data are available on the NCBI SRA database under Bioproject PRJNA545150. Our reference genome is available on NCBI under BioProject PRJNA529692. Raw expression values for 3′ RNA-seq transcription peaks and normalized expression values are included as Appendix S1.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Seidl F, Levis NA, Jones CD, Monroy-Eklund A, Ehrenreich IM, Pfennig KS. Variation in hybrid gene expression: Implications for the evolution of genetic incompatibilities in interbreeding species. *Mol Ecol*. 2019;28:4667–4679. https://doi.org/10.1111/mec.15246