Hybrid Dysfunction and Physiological Compensation in Gene Expression

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Abstract

The formation of new species is often a consequence of genetic incompatibilities accumulated between populations during allopatric divergence. When divergent taxa interbreed, these incompatibilities impact physiology and have a direct cost resulting in reduced hybrid fitness. Recent surveys of gene regulation in interspecific hybrids have revealed anomalous expression across large proportions of the genome, with 30–70% of all genes exhibiting transgressive expression (i.e., higher or lower levels compared with both parental taxa), and these were mostly in the direction of down-regulation. However, as most of these studies have focused on pairs of species exhibiting high degrees of reproductive isolation, the association between regulatory disruption and reduced hybrid fitness prior to species formation remains unclear. Within the copepod species *Tigriopus californicus*, interpopulation hybrids at F_2 or later generations show reduced fitness associated with mitochondrial dysfunction. Here we show that in contrast to studies of interspecific hybrids, only 1.2% of the transcriptome is transgressively expressed in F_{3+} interpopulation hybrids of *T. californicus*, and nearly 80% of these genes are overexpressed rather than underexpressed; remarkably, none of these genes are among those showing divergent expression between parentals, nor is magnitude of transgressive gene expression in hybrids dependent on levels of protein sequence divergence. Moreover, many genes with transgressive expression are components of functional pathways impacted by mitonuclear incompatibilities in hybrid *T. californicus* (e.g., oxidative phosphorylation and antioxidant response). Our results suggest that hybrid breakdown at early stages of speciation may result from initial incompatibilities amplified by the cost of compensatory physiological responses.

Key words: **speciation, hybridization, gene expression, RNA-seq, mitochondrial dysfunction.**

Introduction

Geographic isolation among populations promotes genomic divergence through the action of natural selection and genetic drift. Within isolated taxa, coevolution among interacting genetic elements (termed "intrinsic selection") maintains proper molecular function. Hybridization may then disrupt such interlocus coadaptations and reveal epistatic incompatibilities, as the new recombinant genome harbors allelic combinations "untested" by selection (Dobzhanksy 1936; Muller 1942). As a result, hybrids often suffer from reduced fitness, providing a strong postzygotic barrier to gene flow between divergent taxa. Identifying the genetic components involved in hybrid incompatibilities is an important step toward understanding the basis of species formation.

Most hybrid incompatibilities identified to date have involved protein-coding sequences, often between genes that show rapid structural divergence between the parental taxa (Ting et al. 1998; Presgraves et al. 2003; Maheshwari and Barbash 2011). There is emerging evidence that anomalies in gene expression are also associated with hybrid dysfunction (Ortiz-Barrientos et al. 2007; Renaut et al. 2009; Nowick et al. 2013). However, recent experiments have illustrated that lack of expression divergence between parental taxa is a very poor predictor of regulatory compatibility (Haerty and Singh 2006; Landry et al. 2007), as coadaptation between *cis*- and *trans*-regulatory elements within lineages acts to maintain optimal gene expression levels (Porter and Johnson 2002; Wittkopp et al. 2004). Regulatory dysfunctions are hence only revealed when differentiated elements are forced to interact in hybrid genomes (Haerty and Singh 2006; Landry et al. 2007). Transgressive gene expression in hybrids (i.e., expression level above or below that of parental taxa) could be due to direct incompatibilities between *cis*- and *trans*-regulatory elements (i.e., misexpression; Wittkopp et al. 2004; Graze et al. 2009; McManus et al. 2010) or they may represent cellular responses to physiological dysfunction. In the latter case, transgressive expression levels may either successfully compensate for dysfunction (leading to viable hybrids) or it may exacerbate dysfunction, ultimately contributing to hybrid inviability and promoting speciation. Most studies of gene regulation in hybrids have involved crosses between taxa that have already achieved high levels reproductive isolation; they have reported hybrid misexpression of large portions of the genome (~15–75%) (Ranz et al. 2004; Haerty and Singh 2006; Malone and Michalak 2008; McManus et al. 2010; Coloon et al. 2014). Much of this transgressive expression is likely due to genetic divergence that developed well after reproductive isolation was established. Hence, understanding the role of disruption of gene expression in speciation should ideally

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focus on earlier stages of divergence where hybrid breakdown phenotypes are less severe.

Here, we examine patterns of gene regulation in two populations of the copepod *Tigriopus californicus* and advanced generation hybrids (hereafter referred to as F_{3+} hybrids) generated by laboratory crosses. *Tigriopus californicus* populations inhabit high intertidal pools and show sharp genetic divergence along the West Coast of North America (Burton and Lee 1994; Edmands 2001). Although interpopulation F_1 hybrids show fitness vigor due to heterosis (Edmands 1999), F_2 or later generation hybrids typically suffer from reduced fitness as measured in many life-history traits. These low-fitness hybrids, however, are viable and fertile for multiple generations (Burton 1990), providing an unparalleled opportunity to uncover incompatibilities at early stages of speciation. Accumulated experimental evidence has revealed that hybrid incompatibilities in *T. californicus* occur between nuclear- and mitochondrial DNA (mtDNA)-encoded elements, reducing mtDNA transcription (Ellison and Burton 2008a) and negatively affecting the mitochondrial oxidative phosphorylation (OXPHOS) pathway (Burton et al. 2006; Ellison and Burton 2006). Yet, it remains unclear whether these mito-nuclear incompatibilities are alone responsible for reduced hybrid fitness. Hybrid breakdown might result from widespread regulatory dysfunction as seen in other systems, or alternatively, mitochondrial dysfunction in hybrids may trigger a cascade of regulatory responses in specific functional pathways that could further reduce hybrid fitness.

Although transcriptome analyses can screen for genome-wide patterns of regulatory dysfunctions, our primary hypothesis is that hybrids will exhibit transgressive expression of genes involved in mitochondrial metabolism. This hypothesis leads to three specific predictions: 1) Based on observed mito-nuclear incompatibilities (Rawson and Burton 2002; Burton et al. 2006; Harrison and Burton 2006) and the resulting impairment in mitochondrial function (Ellison and Burton 2006, 2008a), we predict that hybridization disrupts the balance of transcription between nuclear- and mtDNA-encoded subunits of OXPHOS proteins. 2) *Tigriopus californicus* hybrids have also been shown to suffer from increased oxidative damage, likely a result of mitochondrial dysfunction (Barreto and Burton 2013a). Therefore, we predict upregulation of enzymes in the antioxidant system. 3) Finally, recent molecular evolutionary analyses revealed potential incompatibilities within mitochondrial ribosomes, in which nuclear-encoded ribosomal proteins (RPs) interact with ribosomal RNA (rRNA) from the mitochondrial genome (Barreto and Burton 2013b); we predict that expression of mitochondrial ribosomal proteins (mRPs) may be upregulated in hybrids as a means of compensating for potentially suboptimal mitoribosome function.

Results and Discussion

Populations of *T. californicus* Exhibit Early Stages of Expression Divergence

Illumina transcriptome sequencing was used to quantify gene expression in two geographically isolated *T. californicus* populations (supplementary table S1 and fig. S1, Supplementary Material online). Coding sequence divergence between San Diego (SD) and Santa Cruz (SCN) is high for much of the transcriptome (Barreto et al. 2011), and additional sequencing here extended the number and length of gene sequences available. Of 13,766 coding sequences analyzed, 7,151 (51.9%) showed greater than 2% nucleotide differentiation, with a median of 2.04% and some as high as 24.1% (supplementary fig. S2, Supplementary Material online). Using a false discovery rate (FDR) threshold of 0.05, we detected significant differences between populations in the expression of 635 genes (4.6%; supplementary table S2, Supplementary Material online). Along with a recent result in lake whitefish incipient species (divergence in 1.5% of genes; Dion-Côté et al. 2014), this is a very low degree of expression divergence compared with most studies in *Drosophila* species pairs (Nuzhdin et al. 2004: 8%; Rifkin et al. 2003 and Artieri and Singh 2010: 25–35%; Coloon et al. 2014 and McManus et al. 2010: 75–78%) and strongly suggests stabilizing selection on transcriptional levels (Lemos et al. 2005; Rifkin et al. 2005; Gilad et al. 2006; Haerty and Singh 2006). Interestingly, among the 635 genes with expression divergence, we observed a strong asymmetry in the divergence, with greater than 76% of genes showing lower expression in SD (binomial test, $P < 10^{-16}$; fig. 1). Determining whether there was an evolutionary decrease in gene expression in SD or increase in SCN will require comparisons with other populations.

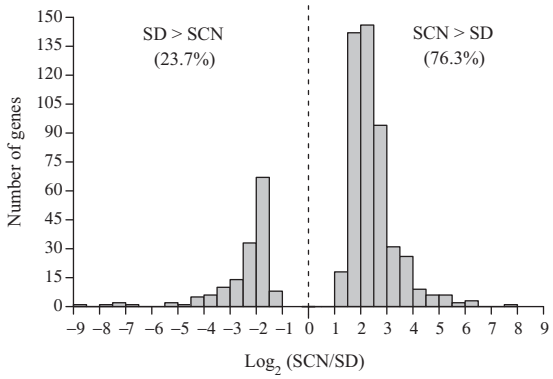

Fig. 1. Divergence in gene expression between populations of *Tigriopus californicus*. Plotted is the distribution of differential expression between SCN and SD populations across 635 genes showing significant differences (out of 13,766 assayed). Statistical significance was assessed at $\alpha = 0.05$, after correction for FDR.

Hybrid Breakdown Is Associated with Transgressive Expression of Relatively Few Genes

Consistent with previous work (Burton et al. 2006; Ellison and Burton 2008a; Barreto and Burton 2013a), reciprocal SD × SCN F_{3+} hybrids generated for this study exhibited significant fitness breakdown at several life-history traits (fig. 2). In an initial analysis, we found no significant differences in life-history phenotypes and overall gene expression between reciprocal F_{3+} hybrid crosses, so all hybrid replicates were pooled for subsequent analyses. Strong asymmetries were

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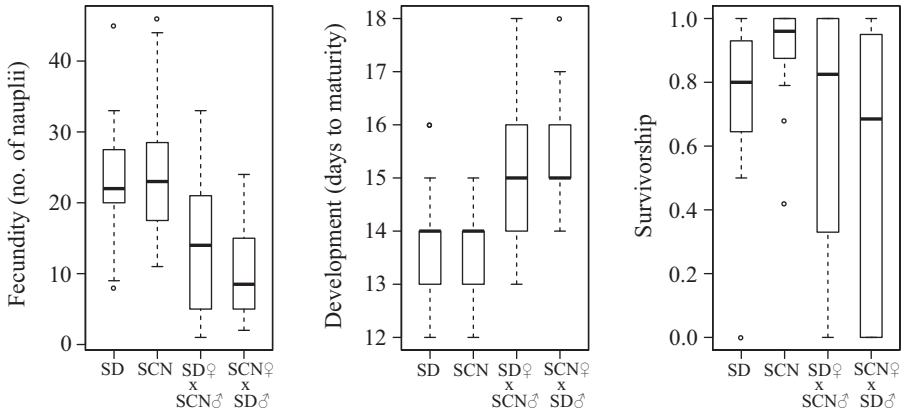

Fig. 2. Hybrid breakdown in life-history phenotypes. Fecundity was measured as the number of nauplius larvae hatched from a female's first clutch. Development was quantified as the day at which the first mature male was observed after the clutch hatched. The proportion of copepods alive at day 14 after hatching was considered a measure of survivorship. Comparisons between F_{3+} hybrids and parental samples were statistically significant in all traits (Mann–Whitney U, $P < 0.01$), whereas comparisons between hybrid crosses were not significant (all $P > 0.17$).

observed when hybrid expression profiles were compared with those from each of the parental populations. Overall, gene expression in hybrids was more similar to SCN (624 differentially expressed [DE] genes) than to SD (1,259 DE genes), at FDR $P < 0.05$. Hybrids had higher expression in 80% of DE genes relative to SD (fig. 3*A*), but elevated hybrid expression was observed in only 39.5% of DE genes compared with SCN (fig. 3*B*). These expression patterns likely reflect dominance relationships between alleles, and further efforts to elucidate these patterns are warranted.

Parental divergence is likely to contribute to population incompatibility only if hybrids exhibit a novel expression phenotype (i.e., outside the range of parental levels). Therefore, expression of a gene was considered "transgressive" when its transcript levels in hybrids were higher (i.e., overexpressed) or lower (underexpressed) than those of both parents. Despite the many differences relative to each parent (fig. 3*A* and *B*), by this definition, transgressive expression was detected in only 169 genes (1.2%; supplementary table S2, Supplementary Material online); remarkably, none of these genes is among those showing divergent expression between parental populations. This amount of transgressive gene expression is strikingly low; the vast majority of genome-scale profiling of expression in systems with hybrid breakdown observed transgressive expression in at least 10% (Dion-Côté et al. 2014) and as many as 69–80% (Ranz et al. 2004; McManus et al. 2010; Coloon et al. 2014) of total genes assayed. Although our power to detect differential expression is influenced by low RNA sequencing (RNA-seq) replication (two to four replicates per group) compared with, for instance, 4–5 replicates per group in Dion-Côté et al. (2014), our statistical threshold is less stringent (this study: FDR < 0.05; Dion-Côté et al. 2014: FDR < 0.01). In addition, our observed value is substantially lower than that of studies with no RNA-seq replication per treatment group (McManus et al. 2010; Coloon et al. 2014).

Among the transgressively expressed genes in *T. californicus*, overexpression was 3.4 × more common than underexpression (131 vs. 38 genes, respectively; binomial $P < 3 \times 10^{-13}$; fig. 4). This is in stark contrast to most of previous studies, which report consistent biases toward underexpression instead of overexpression in hybrids (reviewed in Landry et al. 2007; McManus et al. 2010; Coloon et al. 2014). Altogether, these comparisons may reflect fundamental differences among systems in genetic control of expression; for instance, transgressive expression in *Drosophila* F_1 hybrids is largely associated with effects stemming from Haldane's rule for sex chromosomes (Ranz et al. 2013), whereas polyploidy creates "transcriptome shock" in plant hybrids (Wang et al. 2006). Observed patterns in *T. californicus*, a species without morphologically distinct sex chromosomes, may also represent the earliest symptoms of regulatory divergence.

Over/Underexpressed Genes Are Implicated in Mitochondrial Function, Translation, and Cuticle Formation

Recent genome-wide screens in model species revealed hundreds to thousands of over- or underexpressed genes in interspecific hybrids (Haerty and Singh 2006; Wang et al. 2006; McManus et al. 2010; Dion-Côté et al. 2014). These genes were distributed across a wide range of functional categories, making it difficult to assess which gene classes may have played a role in early divergence. Among the 169 genes with transgressive expression in *T. californicus* F_{3+} hybrids, 101 showed homology to known proteins in GenBank. Over 30% of the annotated proteins belonged to only four categories based on their known function or pathway. These four categories were significantly overrepresented among transgressive genes (table 1 and supplementary table S3, Supplementary Material online), possibly reflecting incompatibilities among key regulatory elements throughout a network. Alternatively, altered regulation of these genes may be part of a physiological response to metabolic dysfunction initially caused by other incompatibilities. These results can be better interpreted in light of known patterns of hybrid breakdown in *T. californicus*.

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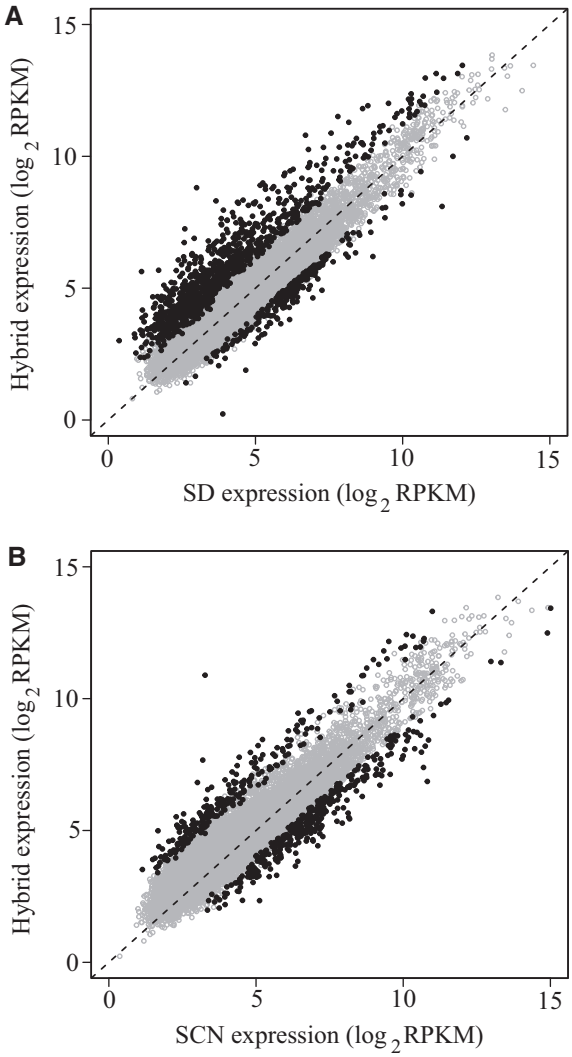

Fig. 3. Differential gene expression between parentals and hybrids. Plotted are expression levels for SD × SCN F_{3+} hybrids relative to SD (*A*) and SCN (*B*). Shown are mean values of $\log_2$ (RPKM, reads per kilobase per million mapped reads) for each gene assayed ($N = 13{,}766$). Reciprocal hybrid samples were pooled for statistical analysis. Statistical significance was assessed at $\alpha = 0.05$, after correction for FDR. Dashed line depicts identical expression (slope = 1). Black, genes significantly different between hybrids and respective parental population, with those above diagonal being upregulated in hybrids and below diagonal being downregulated in hybrids; gray, not significantly different.

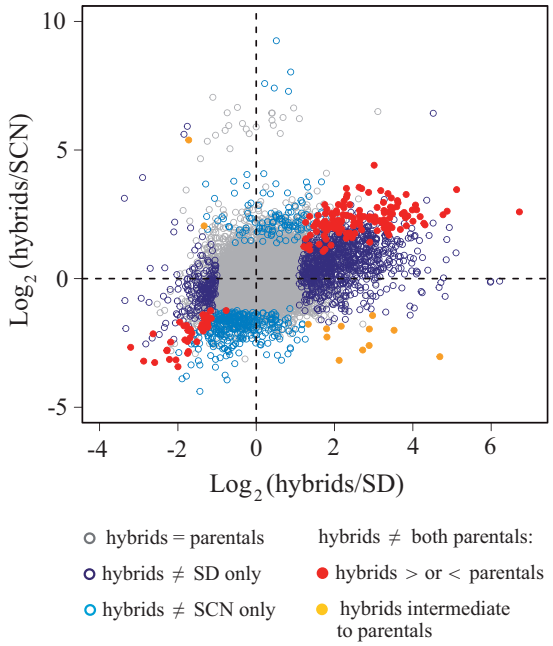

Fig. 4. Patterns of hybrid gene expression relative to both parental populations. Shown are $\log_2$-transformed mean expression ratios for comparisons between F_{3+} hybrids and parentals for all genes assayed ($N = 13{,}766$). Differential gene expression was determined statistically at an FDR threshold of 0.05.

Table 1. Categories Overrepresented among Genes with Transgressive Expression in SD × SCN Hybrids.

	cRPs[b]	Antioxidant Enzymes	OXPHOS	Cuticle Proteins
Overexpressed	7	3	4	18
Underexpressed	0	0	1	0
Total transgressive/total in transcriptome	7/75	3/30	5/57	18/43
P value[a]	1.1×10^{-5}	0.0033	0.00027	$<10^{-15}$

[a]Significance was assessed with a Fisher's exact test, considering 9,954 genes were annotated across the whole transcriptome and 101 among the transgressively expressed group.
[b]cytosolic ribosomal proteins.

Interpopulation hybrids of *T. californicus* exhibit one of the best-supported examples of how functional incompatibilities between nuclear- and mtDNA-encoded elements (mitonuclear interactions) negatively impact organismal fitness (Ellison and Burton 2008b). Most experimental evidence points to the involvement of mitonuclear mismatches in the OXPHOS (Burton et al. 2006; Harrison and Burton 2006) or mtDNA transcription (Ellison and Burton 2008a) pathways, resulting in impaired OXPHOS enzyme activity and reduced ATP synthesis (Ellison and Burton 2006). At the regulatory level, expression of nuclear and mitochondrial components of OXPHOS must be coordinated for proper function. Altered proportions of even just few interacting proteins are known to be associated with mitochondrial dysfunctions and diseases (Herrmann et al. 2003; Chae et al. 2013). In this study, *T. californicus* F_{3+} hybrids exhibited significant change in mRNA levels in some nuclear-encoded (table 1, fig. 5, and supplementary table S2, Supplementary Material online) but not in mtDNA-encoded OXPHOS subunits, indicating a potential imbalance in at least three of the five OXPHOS complexes. Also concordant with mitochondrial dysfunction was the observed 3- to 8-fold induction of three glutathione *S*-transferases (GSTs; fig. 5, table 1, and supplementary tables S2 and S3, Supplementary Material online), which are likely responding to elevated oxidative stress resulting from OXPHOS dysfunction in hybrids between these divergent populations (Barreto and Burton 2013a).

Another important (but understudied) site of mitonuclear interaction involves the synthesis of mtDNA-encoded proteins on mitochondrial ribosomes, which consist of

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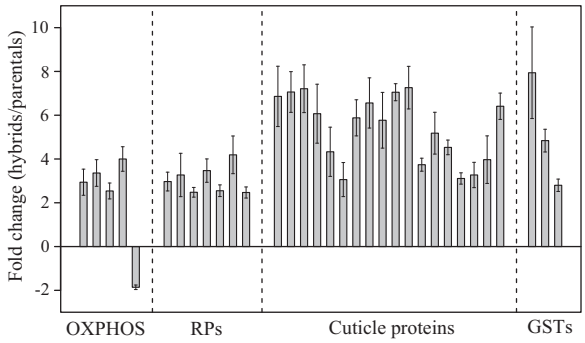

Fig. 5. Relative changes in expression in functional groups overrepresented among genes with transgressive expression. Bars show mean fold change ± SEM. OXPHOS: oxidative phosphorylation; RPs: ribosomal proteins; GSTs: glutathione *S*-transferases.

mtDNA-encoded rRNAs and a set of nuclear-encoded RPs imported into the mitochondria (mRP). High rates of divergence in mRPs (Barreto and Burton 2013b) suggest that hybrids might experience reduced performance of mitoribosomes. Therefore, we predicted significant abnormal transcription of mRP genes in hybrids. Instead, we observed significant overexpression in genes encoding cytosolic RPs (cRPs; table 1 and supplementary table S3, Supplementary Material online), which interact only with other nuclear-encoded components. Overexpression of cRPs has been consistently associated with dysfunctions in cell cycle, growth and proliferation, and tumorigenesis (Ruggero and Pandolfi 2003). Reduced expression of single RPs as a result of hybridization has been observed in other systems (Ranz et al. 2004; Moehring et al. 2007; Renaut and Bernatchez 2011), but our results contrast by showing a higher number of genes, overexpression rather than underexpression, and greater magnitude of change (fig. 5). The observed increase in cRP expression in hybrids is likely not a response to poorly assembled ribosomes, as Barreto and Burton (2013b) reported highly conserved protein and rRNA components. In the Pacific oyster (*Crassostrea gigas*), high-fitness hybrids show lowered cRP mRNA levels, which is thought to be concordant with markedly reduced protein turnover and more efficient metabolism (Hedgecock et al. 1996, 2007). We propose that upregulation of cRPs in *T. californicus* hybrids may conversely be a response to a greater need for increased translation of other proteins involved in suboptimal interactions, essentially compensating for reduced function by increased production.

Finally, hybrids showed a striking overexpression of genes encoding proteins involved in cuticle formation. This group of genes was previously unexplored in studies of hybrid incompatibilities, yet it exhibited the largest overrepresentation as well as consistently highest expression change among all genes in our study (table 1, fig. 5, and supplementary table S3, Supplementary Material online). These proteins are generally thought to have a structural role in forming the arthropod cuticle during development, molting, and adult life, but the evolution of gene number and functions across taxa varies greatly and is poorly understood. This gene family shows widespread evolutionary changes in expression among *Drosophila* lineages during metamorphosis (Rifkin et al. 2003), but we are not aware of previous reports of abnormal regulation of cuticle protein genes associated with hybrid breakdown. These genes may have a common regulatory trigger so the upregulation of 18 genes may reflect only a few divergent components. However, overexpression may again indicate a cellular response to compensate for slow development in hybrids (fig. 2).

Protein Sequence Evolution Is Correlated with Levels of Expression Divergence but Not with Transgressive Expression

Recent comparative genomic studies have shown that rates of protein sequence differentiation may be a good predictor of expression divergence between taxa (Nuzhdin et al. 2004; Jordan et al. 2005; Khaitovich et al. 2005) as well as of regulatory dysfunction in hybrids (Artieri et al. 2007; Hunt et al. 2013). This relationship may indicate an important coupling between changes in protein function and respective transcriptional levels (Castillo-Davis et al. 2004; Khaitovich et al. 2005), but it remains relatively unstudied. We took advantage of our current *T. californicus* transcriptomes to test whether rates of protein evolution parallel patterns of regulatory divergence between parental populations, and between hybrids and their parental stocks.

Among genes that showed differential expression between SD and SCN, we found a significant positive correlation between expression differences and protein divergence (d_N; Spearman's $\rho = 0.215$, $P < 0.0001$, fig. 6*A*), but not between expression differences and neutral divergence (d_S; Spearman's $\rho = 0.074$, $P = 0.152$, fig. 6*A*). The ratio d_N/d_S, which is a measure of evolutionary functional constraint on protein sequence, was also positively associated with expression differences between SD and SCN (Spearman's $\rho = 0.199$, $P < 0.001$). These correlations suggest that the magnitude of expression divergence between SD and SCN is coupled, albeit weakly, with the rate of amino acid substitutions. These results are concordant with findings in species of *Drosophila* (Nuzhdin et al. 2004), ants (Hunt et al. 2013), and apes (Khaitovich et al. 2005), and they suggest that a protein's regulatory and structural evolution may be under similar selective constraints. Although directional (positive) selection may be driving differentiation in some genes (Jordan et al. 2005; Khaitovich et al. 2005; Artieri et al. 2007), this pattern is likely explained by relaxed constraints on protein function (Castillo-Davis et al. 2004; Ortiz-Barrientos et al. 2007).

A few studies have extended these analyses to comparisons between species and their hybrids. In crosses of *Drosophila melanogaster* and *D. simulans*, Artieri et al. (2007) found a positive correlation between d_N and the magnitude of over/underexpression (i.e., the difference between hybrid expression and parental expression), whereas Hunt et al. (2013) found a strong correlation between d_N (and d_N/d_S) and transgressive expression in comparisons of the fire ants *Solenopsis invicta* and *S. richteri* and their hybrids. Moreover, the degree of transgressive expression in ant hybrids was strongly correlated with expression divergence

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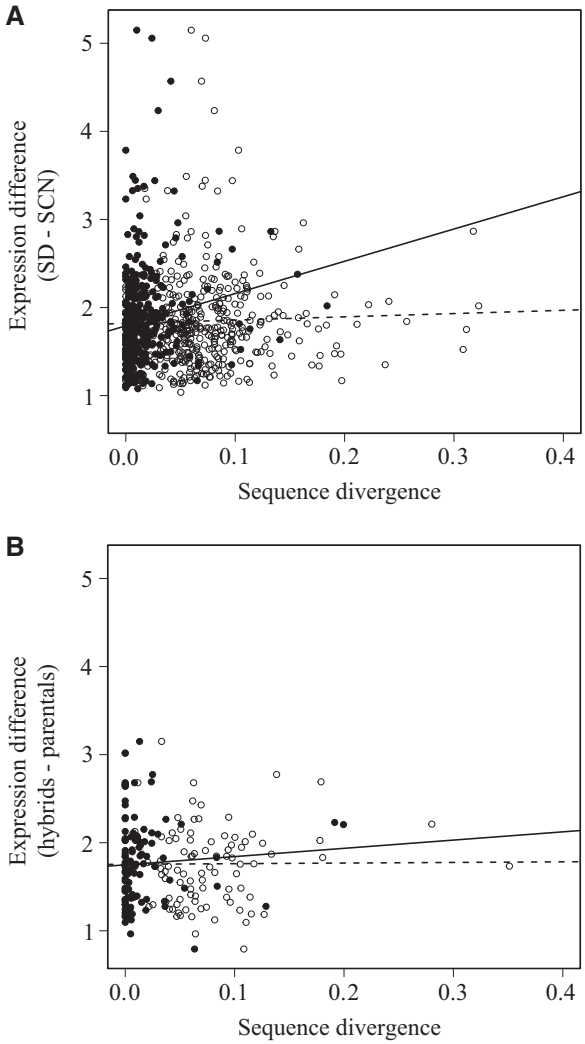

Fig. 6. Relationships between absolute expression differences and coding sequence divergence. (*A*) Analysis involving genes differentially expressed between SD and SCN ($N = 371$). (*B*) Analysis involving genes significantly over/underexpressed in F_{3+} hybrids relative to SD and SCN ($N = 101$). Structural sequence divergences were estimated as the number of synonymous (d_S, white circles and dashed lines) and nonsynonymous (d_N, black circles and solid lines) substitutions per site.

between parental species, suggesting that regulatory incompatibilities may increase directly as a result of expression divergence between species in this system (Hunt et al. 2013). Our analyses with transgressively expressed genes in *T. californicus* provide a striking contrast to these patterns. We observed no significant relationship between the magnitude of hybrid transgressive expression and estimates of sequence divergence (d_N: Spearman's $\rho = 0.096$, $P = 0.345$, fig. 6*B*; d_S: Spearman's $\rho = 0.012$, $P = 0.916$, fig. 6*B*; d_N/d_S: Spearman's $\rho = 0.128$, $P = 0.211$). As noted above, all transgressive genes in *T. californicus* hybrids occurred among genes showing no expression divergence between parental populations. Therefore, our results suggest that transgressive gene expression in *T. californicus* hybrids is a compensatory physiological response rather than a consequence of regulatory incompatibilities arising from relaxed constraints on gene expression (or associated protein divergence).

Conclusion

Abnormal transcription of genes in hybrids can result from two quite different mechanisms. Divergent regulatory elements between populations may fail to interact appropriately and lead to misregulation that may be widespread and likely without pattern related to function. Alternatively, some of the changes in expression may not be misexpression at all, but represent compensatory responses to hybrid metabolic dysfunction resulting from an initial structural or regulatory incompatibility. We propose that the patterns of overexpression of genes involved in energy production (OXPHOS), antioxidant response (GSTs), and protein synthesis (RPs) reflect a metabolic syndrome in hybrids starting with mitochondrial dysfunction leading to reduced ATP synthesis and increased oxidative damage. These metabolic symptoms of hybrid breakdown then activate compensatory cellular responses, including the altered pattern of gene regulation observed in *T. californicus* hybrids. If hybrid dysfunction is not too severe, this compensatory response may mitigate fitness loss. However, at some level of population incompatibility (as seen in the current experimental cross), mitigation fails and the cost of mounting the response may actually exacerbate physiological dysfunction and contribute to increased postzygotic isolation.

Materials and Methods

Collection and Culture of Copepods

Copepods were collected from high intertidal rocky pools in SD (32°45′N, 117°15′W) and SCN (36°57′N, 122°03′W), California, and maintained en masse in multiple 400-ml beakers filled with 0.2 -μm-filtered seawater. Cultures were kept in incubators at 20 °C with a 12-h light:dark cycle and fed powdered *Spirulina* wafers. Beakers from the same population were mixed periodically in order to maintain outbreeding.

Interpopulation crosses (SD♀ × SCN♂ and its reciprocal SCN♀ × SD♂) were performed in 100 mm Petri dishes and maintained in the same conditions as stock cultures. Mature males clasp and guard virgin females until they reach reproductive maturity (Burton 1985). Crosses were initiated by obtaining virgin females from clasped pairs from one parental population and placing them in dishes containing males from the other population. For each reciprocal cross, 250–300 pairs were created and distributed across four to six replicate dishes. Males were removed and discarded after mating occurred, and the dishes with females were monitored daily. As F_1 progeny were produced, they were mixed among replicate dishes in order to decrease the chance of inbreeding, and were then distributed among six to eight new dishes and allowed to develop, form pairs, and mate. Mated F_1 females were transferred to new dishes, which were monitored for F_2 progeny. The process was repeated until F_3 individuals were generated, and these were pooled and kept in large populations in 400-ml beakers until use. F_3 individuals were allowed to mate, and beakers were maintained until adult F_4 individuals were observed. Mixing of F_3 and F_4 generations is not problematic, as hybrid breakdown remains strong for multiple generations (Ellison and Burton 2006, 2008a; Barreto and

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Burton 2013a). The reciprocal hybrid crosses were generated independently and never mixed.

Quantifying Life-History Fitness

After generating F_{3+} hybrids, we assessed fitness of all parental and hybrid laboratory populations by measuring three life-history traits known to reflect hybrid breakdown (Burton et al. 2006). Fecundity was measured as the number of nauplii (copepod larvae) hatched from a female's first clutch. From each population, 25–30 clasped pairs were individually isolated in fresh dishes and monitored daily. When nauplii were first observed, the female was discarded and larvae were tallied under a dissecting microscope.

The nauplii were monitored daily to quantify developmental rate and survivorship. Developmental rate of each clutch was measured as the age, in days, of the first mature male observed. Finally, survivorship was defined as the fraction of progeny still alive at day 14 after hatching.

RNA Extraction and Illumina Sequencing

Our experiment included nine RNA-seq samples: 3 SD, 2 SCN, 2 F_{3+} SD♀ × SCN♂, and 2 F_{3+} SCN♀ × SD♂. Treatment of copepods and isolation of total RNA followed the same protocols for all samples. For of each sample, 400–500 copepods across all developmental stages were collected from their stock cultures by pouring from beakers into a fine mesh net and quickly transferred to fresh filtered seawater in a 50-ml Falcon tube. No efforts were taken to count individuals from each developmental stage, but this was done when stock cultures reached similar levels of mixture and density. Therefore, we expect RNA composition to vary minimally and similarly among groups. Tubes were immersed in a 20 °C water bath for 2 h. Water was then quickly removed, 4 ml of Tri-Reagent (Sigma) added, and tissue immediately disrupted using a tissue homogenizer. RNA was isolated following the manufacturer's protocol. Resuspended RNA pellets were further purified with RNeasy Mini columns (Qiagen), and final sample integrity and quantity were assessed with an Agilent 2100 BioAnalyzer.

One replicate each from SD and SCN were prepared and sequenced for an earlier study (Schoville et al. 2012). These were sequenced as 100-bp single-read fragments in an Illumina GA-II. Libraries and sequencing for all other samples were performed by the Beijing Genomics Institute. These were sequenced as 100-bp paired-end libraries in an Illumina HiSeq 2000 machine. Raw Illumina data were deposited in the NCBI Sequence Read Archive under BioProject PRJNA264820 (BioSamples SAMN03142820–SAMN03142828).

Assemblies and Annotations

Transcriptomes were developed for each parental population by combining all paired-end reads with previous 454 reads (Barreto et al. 2011); sequences were assembled de novo with the software CLC Genomics Workbench (GW) v5.1 (CLC Bio). Illumina reads were trimmed for quality by imposing a minimum Phred score of 25 and removing 5–8 terminal bases so that all reads had a minimum of 70 bp and maximum of 84 bp in length. A total of approximately 128 million high-quality reads (including ~578 k from 454) were used for the SD assembly, and approximately 49 million (~576 k from 454) were included for the SCN assembly. For assembly in CLC GW, the following parameters were used: Mismatch and insertion/deletion costs = 2, length fraction = 0.5, similarity = 0.90, and conflicts resolved by majority vote. Once assemblies were generated, we filtered them by retaining only contigs with average coverage $\geq$ 5 reads/bp. This threshold allowed us to focus on high-quality contigs without losing many reads (supplementary fig. S3, Supplementary Material online). To reduce redundancy, we performed another round of assembly by using the high-quality CLC contigs as input into the CAP3 assembler (Huang and Madan 1999). CAP3 parameters were set at default with the exception of minimum overlap and percent similarity, which we increased to 50 bp and 95%, respectively. Final assemblies comprise 36,620 SD contigs and 43,077 SCN contigs.

Predicted gene products for the SD assembly were identified by employing the Blast2GO pipeline (Conesa et al. 2005; Götz et al. 2008) to perform BLASTX searches against the "nr" protein database in NCBI, retaining best hits with $E \leq 10^{-3}$. For contigs with a significant BLAST result (N = 16,741), the best-hit taxon was identified, and only hits to metazoan species (NCBI Taxonomy ID 33208) were retained (N = 13,831). Contigs with no BLAST matches were also retained if length $\geq$150 bp. The final SD assembly comprised 33,710 contigs.

Orthology between SD and SCN was assessed by a reciprocal best-hit approach. NCBI's standalone packages (version BLAST+ 2.2.26) were used to generate searchable databases from each assembly, and reciprocal BLASTN searches performed at a threshold $E \leq 10^{-20}$. The outputs were parsed so that putative orthology was assigned only when pairs of sequences were each other's best hits and had an alignment $\geq$100 bp. This resulted in 15,625 pairs of SD–SCN orthologous contigs.

Read Mapping and Differential Expression Analysis

Because of the large degree of divergence across most of the transcriptome (supplementary fig. S2, Supplementary Material online), mapping of hybrid RNA-seq samples to only one reference assembly would likely greatly bias read counts toward the reference population. Thus, we devised a simple pipeline that incorporated both orthologous copies of each gene so that, in samples from hybrids, reads representing alleles from both populations are equally likely to find a reference mapping location (supplementary fig. S1, Supplementary Material online). Briefly, a custom Perl script was used to perform the following steps:

1) Based on the reciprocal BLAST output, orthologous contigs were reverse-complemented as needed in order to be on the same strand.
2) Each contig pair was aligned in Clustal Omega (Sievers et al. 2011).
3) The program TrimAl (Capella-Gutiérrez et al. 2009) was then used to trim off terminal gaps from the

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pairwise alignment. As the two assemblies often differed in the length of their respective orthologous contigs, this step forced both sequences to represent the same portion of the gene, preventing length differences from creating mapping biases.

4) Internal gaps introduced during alignments were removed as they may represent real insertion/deletions between orthologs.

The final reference assembly (henceforth termed "mixed assembly") was therefore composed of 15,625 pairs of trimmed orthologous contigs.

Because the original assemblies were trimmed for establishing the mixed assembly, a large portion of paired reads would likely be broken during mapping. We hence chose to use only forward reads for mapping. Read mapping was performed in CLC GW with length fraction = 0.80 and similarity = 0.95. As the mixed assembly contains two orthologous versions of each gene, many reads may map equally well to both alleles, especially when the orthologs are very similar. Forcing reads to map uniquely, as generally done, would prevent such doubly mapping reads from being counted, which could underestimate expression levels of some genes. Therefore, in order to allow such reads to be properly included in quantification, we allowed reads to be equally mappable to up to two locations. In these cases, the reads were mapped randomly to one of the two orthologs. The total read count for each gene was hence the sum of reads mapped to each ortholog (see supplementary fig. S1, Supplementary Material online, for a diagram of mixed assembly creation and mapping scheme). All samples, including pure SD and SCN libraries, were mapped to the mixed assembly. Mapping efficiency (i.e., % of reads mapped) was very similar among libraries, (range: 36.6–42.9%; supplementary table S1, Supplementary Material online), suggesting that mapping to the mixed assembly did not bias read counts from one population over the other. We note that these overall mapping % may seem low compared with other studies, but this is most likely a result of our stringently reduced reference assembly (i.e., only contigs assembled with high coverage were retained, only genes with identified SD:SCN orthologs were used, and all pairs of orthologs were trimmed to the same length).

Analysis of differential expression was performed in the Bioconductor package edgeR (Robinson et al. 2010). Genes with extremely low expression were removed by retaining only those that accumulated a minimum of one count per million in at least three samples. A total of 13,766 genes were kept, 9,954 of which had BLAST annotation. Raw read counts were then normalized using the trimmed mean of *M*-values (Robinson and Oshlack 2010) method, and tagwise dispersions applied to the data. After a negative binomial model was fitted, expression differences were assessed with exact tests followed by correction of *P* values for FDR (Benjamini and Hochberg 1995). Statistical significance for all gene expression comparisons was assessed at FDR $\alpha = 0.05$. In an initial analysis, data were partitioned into the four sample groups (two parentals and two hybrid crosses). Pairwise comparisons revealed no DE genes between the two reciprocal hybrid crosses (all FDR $P > 0.85$). Therefore, hybrid samples were pooled into one group, and the analysis was repeated with three factors (SD: $N = 3$, SCN: $N = 2$, and hybrids: $N = 4$). After all pairwise contrasts were performed, genes showing transgressive expression were recognized only when they were significantly DE in both comparisons (i.e., hybrids vs. SD and hybrids vs. SCN).

The SD and SCN assemblies used as references were submitted to the NCBI Transcriptome Shotgun Assembly database (accession numbers found on supplementary table S2, Supplementary Material online), and expression data were deposited in the Gene Expression Omnibus (accession number GSE62672). Results from differential expression analyses and normalized expression levels can be found on supplementary table S2, Supplementary Material online.

Functional Characterization

Most recent genome-level investigations highlight the use of Gene Ontology (GO) term enrichments for functional interpretation of DE genes, but recent analyses warn against relying heavily on these results (Pavlidis et al. 2012). Therefore, in addition to assessing enrichment of GO annotations, we focused on testing for overrepresentation of candidate groups of genes highlighted by previous studies in *T. californicus*.

In order to better categorize *T. californicus* genes across the functional groups of interest, we mined the annotated transcriptome using a combination of word searches and local BLAST searches. Genes of the antioxidant system were identified by name based on their original BLASTX hit and following the nomenclature described in Corona and Robinson (2006). A total of 30 genes were annotated as part of the antioxidant system. For RP genes and OXPHOS genes, names extracted from BLAST hits were highly variable and sometimes not specific enough. We hence fine-tuned their identities by performing additional BLAST searches. The complete set of RP sequences from *D. melanogaster* was obtained from the Ribosomal Protein Gene Database (Nakao et al. 2004), whereas those involved in OXPHOS were downloaded from the KEGG database (Kanehisa et al. 2012). For the latter dataset, all entries available for *D. melanogaster*, *Apis mellifera*, and *Tribolium castaneum* were obtained and pooled. These protein sets were formatted using the NCBI BLAST scripts, and the SD transcriptome was blasted against these databases using a threshold $E \leq 10^{-6}$. Significant hits were then confirmed by blasting the respective SD contigs against the entire "nr" database and comparing best hit identities between both searches. Only contigs with match to the same protein in both searches were categorized to the appropriate functional group. These analyses identified 57 nuclear-encoded OXPHOS genes, 75 cRPs, and 70 mRPs. After identifying contigs across the categories above, we employed a Fisher's exact test to test for overrepresentation of each of these groups within transgressively expressed genes relative to the set of genes with conserved expression ($N = 9{,}853$).

Finally, we used Blast2GO to assess the enrichment of any GO term in transgressive genes, in order to assess the

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overrepresentation of functions and processes not postulated by our hypotheses.

Rates of Coding Sequence Divergence

To compare levels of expression divergence or of transgressive expression with coding sequence differentiation, we estimated the number of synonymous (d_S) and of nonsynonymous (d_N) substitutions per site using the method of Yang and Nielsen (2000), implemented in the PAML package (version 4.4; Yang 2007). These analyses were restricted to genes exhibiting significant differential expression in the SD versus SCN comparison and in the hybrids versus parents comparison above.

We first used a custom Perl script to extract best open reading frames (ORFs) from SD contigs. To increase confidence in prediction of ORFs, these were predicted from the original BLASTX results; hence, only genes with a BLAST hit were included in the analysis. The SD ORFs were then translated to protein sequences, and the SCN ORFs were similarly extracted by blasting SCN contigs against the SD proteins. A Perl script was then written to automate pairwise alignments of orthologous ORFs through the program MACSE (Ranwez et al. 2011), which aligns amino acid translations before returning the corresponding nucleotide sequences in frame. All alignments were visually inspected for errors and were then used for estimation of d_N and d_S in PAML. Finally, we excluded genes with $d_S \geq 1.5$, and our final data set retained 371 genes with divergent expression between SD and SCN, and 101 genes with significant over- or underexpression in hybrids.

Nonparametric Spearman rank correlations were used to test for relationships between coding sequence divergence (d_N, d_S, and d_N/d_S) and expression differences. For analysis of parental divergence, expression differences were calculated as the absolute value of $[\log_2(\bar{x}_{SD}) - \log_2(\bar{x}_{SCN})]$. For analysis of transgressively expressed genes, expression differences were first calculated between hybrid and each parent's expressions as the absolute of $[\log_2(\bar{x}_{hybrids}) - \log_2(\bar{x}_{SD\ or\ SCN})]$, and the lower value was kept as a measure of minimum transgressive expression.

Supplementary Material

Supplementary tables S1–S3 and figures S1–S3 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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