GENE EXPRESSION DIFFERENCES IN HYBRIDS AT BEGINNING OF SPECIATION CONTINUUM

by

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Abstract

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The formation of new species results from reproductive isolation, or the barrier to successful mating between organisms of divergent populations. The degree of reproductive isolation can be measured by the reduction in fitness of hybrid offspring that can result from incompatibilities between the genotypes of each parent. In this study, genetic incompatibilities arising at the earliest stage of speciation are examined between intraspecific hybrids in which an effectively sterile hybrid phenotype termed “still” is observed at ~50% frequency in crosses between divergent populations of *Tribolium castaneum*. Timing and magnitude of gene expression regulation is examined at four stages of development, two pupal and two adult. Gene interactions are examined on a systemic level to elucidate proximal mechanisms for misregulation such as allele bias and alternative splicing. Further evidence is put forth for phosphine resistance as a possible driver of the genetic incompatibility behind “still.” Misregulated genes exhibit functional enrichment across developmental timepoints for oxidative damage, neuromuscular function, and chromosome structure.

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Differential Gene Expression Across the Timecourse of Development

Introduction

Speciation is the process by which reproductive isolation between members of divergent populations results in reduced fitness of offspring when mated. Decreases in viability and fertility of hybrid offspring follow from genetic incompatibilities between alleles present in each parent population (Bateson 1909; Dobzhansky 1936; Muller 1942). The strength of reproductive isolation is positively correlated to the completeness of speciation on a continuum of hybrid incompatibilities. Hybrid incompatibility factors can arise between any two loci that participate in the gene regulatory network and fail to produce a sufficient level of gene product to establish viability (Birchler and Veitia 2009). In regulation of gene expression, interacting loci can be broadly categorized as either regulatory or coding sequence depending on responsibility for initiating transcription or being transcribed and translated into gene products, respectively. Gene expression can be regulated by nearby sequences in *cis* if located within a defined region on the same chromosome and *trans* if this distance is exceeded (or from a different chromosome). Unless compensatory mechanisms restore gene interactions to a sufficient level (Landry et al. 2005), transgressive gene expression can manifest at the phenotypic level with reduced fitness effects (Schwarzbach et al. 2001). Divergence of gene expression regulation is an important source of evolutionary novelty, wherein regulatory sequences that initiate transcription evolve faster than regions coding for protein (Wray et al. 2003).

As a consequence of this higher turnover rate, gene regulation specific to species or subpopulations can result in widespread gene misexpression that acts as a speciation boundary (Michalak and Noor 2003). For example, parental copies of coding and regulatory sequence can effect post-transcriptional regulation (Braidotti and Barlow 1997), protein-protein interactions (Rawson and Burton 2002), or changes in transcript abundance (Zhuang and Adams 2007). In the case of altered transcript abundance, cis-regulatory sequences (enhancers and promoters) undergo differential binding by trans-acting sequences (i.e. transcription factors) with respect to background parental levels (Wittkopp et al. 2004). Post-transcriptional regulation, or the modification of the mRNA transcript before translation, can change the biochemical function of the protein by alternative splicing of exons and introns (Buljan et al 2013). Protein-protein interactions form the basis of all biochemical function of the gene network and are influenced by changes in abundance, sequence, or physical conformation of gene products. One such change to protein conformation can rise from the overexpression of one allele of a transcript, or allele-biased expression, which has been shown to mediate pathogenesis-related stress (Yan et al. 2002).

While cis-acting mutations are predicted to account for more regulatory variation between species since faster fixation rates result from fewer damaging pleiotropic effects (Coolon and Wittkopp 2013), empirical tests for the relative contribution of *cis* versus *trans* regulation in interspecific hybrids have revealed inconsistent patterns dependent on organism. Patterns of gene regulation in intraspecific hybrids exhibit more trans-acting regulation in yeast (Sung et al. 2009; Emerson et al. 2010) and equal contributions from cis and trans in plant (Zhang and Borevitz 2009). A potential source

of bias for comparing contributions of *cis*- and *trans*-regulation between model organisms is the relative proportion of each in the ancestral state (Guerrero et al. 2016).

An interesting case-study for the magnitude of misregulation in gene expression between intraspecific hybrids lies in crosses between strains of *Tribolium castaneum*, a common stored grain pest and model organism (Richards et al. 2008). Sires of a Tanzania population maintained in laboratory culture since 1989 (Drury and Wade 2011) mated with dams of an outbred laboratory population from Chicago yield a sterile, disordered phenotype seen in three out of four crosses in the F1 hybrid generation. The lack of disordered phenotype in the reciprocal cross suggests epistatic interactions between uniparentally inherited factors. Unpublished data suggest a mitonuclear incompatibility driven by segregating variation at the *dld* locus, a mitochondrially-interacting enzyme that mediates resistance to the phosphine fumigant (Schlipalius et al. 2012) widely used worldwide on Tribolium populations in stored grains. Using a combination of pooled parental DNA sequencing and pooled hybrid RNA sequencing, patterns of gene expression misregulation can be uncovered along with parent-of-origin effects. Genes are examined for differential expression at each time point between normal and still hybrids, as well as differential expression across timepoints within normal and still hybrids separately. Additionally, genes found to be significantly differentially expressed are compared to those found in a similar study by Oppert et al. 2015, in which phosphine susceptible and phosphine resistant populations of *T. castaneum* were subject to fumigant exposure and gene expression analysis.

Methods

DNA sequencing

To sequence the parental populations that hybridize to produce “still” hybrids, DNA was purified by sodium acetate and ethanol precipitation from 7-10 day old virgin sires from Dar es Salaam, Tanzania and virgin dams from Chicago. DNA was pooled in two sex-specific libraries each containing 50 individuals per parent population. A technical replicate of each pool was sequenced on a different lane on the Hi-Seq 2000 Illumina platform, yielding 8 sequencing samples across two lanes.

To sequence the F1 hybrids, eight matings were performed by placing a virgin male and a virgin female into a single vial containing 8 grams of medium (20:1 ratio of flour to brewer’s yeast) for one week to lay eggs. Then each pair was moved to a fresh vial for another week of egg-laying, for a total of four weeks. When hybrid offspring eclosed from the pupal stage, each phenotype was scored as “still” or “normal” by degree of movement on a smooth, unobstructed surface, wherein “still” hybrids move very little. One quarter of all families produced equal proportions of normal and still hybrids, from which all offspring were frozen at 20℃. DNA was extracted by sodium acetate and ethanol precipitation and pooled by equimolar amounts by phenotype. One family consisted of 57 normal and 57 still individuals, and the second family consisted of 54 normal and 54 still individuals. Each sample was split into two technical replicates and libraries were constructed for sequencing on the Illumina Hi-seq 2000 platform using the 100 base pair, paired-end protocol. Samples were barcoded and run 4 per lane.

RNA sequencing

Crosses between sires of Dar es Salaam and dams from Chicago were made by placing a single virgin mate pair into a separate well of an 8-well culture plate containing 8 grams of standard medium. Each pair laid eggs for 2 weeks at 24 h dark, 29˚C, and 70% relative humidity after which the parents were removed and the offspring allowed to reach adulthood. Each mate pair laid eggs for 12 weeks across 6 vials. From families producing either all normal or all “still” offspring in the F1 generation, hybrids from each family were frozen at -80˚C for RNA preparation at days 1 and 3 of the pupal stage and days 1 and 3 of the adult stage. For each timepoint per normal and still family, 40 individuals were pooled at a 1:1 sex ratio to create a strand-specific RNA-Seq library for sequencing. Each library was divided into technical replicates, yielding 16 samples across four timepoints.

RNA-Seq data preparation

Raw sequencing reads were error-corrected with BFC (Li 2015) and trimmed for adapters and low-quality sequence by Trim Galore! (Krueger 2015). Reads were mapped to the reference transcriptome of *T. castaneum* version Tcas3.31 using CLC Genomics Workbench 8 (CLC Bio-Qiagen, Aarhus, Denmark) with settings modified to allow up to 30% of the read to contain mismatches to account for the divergence time between reference and laboratory populations. A table of unique read counts to each transcript was exported for downstream analysis with other software. The Blast2GO plugin (Conesa et al. 2005) for CLC Genomics Workbench performs enrichment testing

for overlap with the Gene Ontology (GO) database (Gene Ontology Consortium 2004), a hierarchical format for descriptions of gene functions at three levels: the biological process (BP) level describes the pathway of multiple products, the cellular component (CC) level describes sites of gene product activity, while the molecular function (MF) level describes the biochemical process of the gene product.

Between-condition differential expression

Gene expression differences between normal and still F1 hybrid phenotypes were examined statically at each developmental time point sampled (pupa day 1, pupa day 3, adult day 1, and adult day 3). RNA sequencing reads from each pair of technical replicates per phenotype were mapped to the reference transcriptome using CLC Genomics Workbench 8 (CLC Bio-Qiagen, Aarhus, Denmark), using only reads that map unambiguously as the proxy for expression level. Differential expression analysis was performed using an extension of Fisher's exact test over a negative binomial distribution (Robinson et al. 2010). Significant tests for differential expression were performed between normal and still samples at each developmental time point. Significant enrichment testing for Gene Ontology functional terms was performed with Blast2GO.

Within-condition differential expression

Differential expression was also examined as the change in up- or down-regulation across time for the normal and still phenotype separately. With four developmental time points sampled, there are three possible state changes of up- or

down-regulation between consecutive time points. The EBSeq-HMM R software package (Leng et al. 2015) was given as input unique read counts for each transcript for each technical replicate per phenotype per timepoint. Each analysis yielded a separate list of phenotype-specific patterns of increasing or decreasing gene expression over timepoints. With the normal hybrid sample as the experimental control, those genes uniquely up- or down-regulated in the still phenotype could be found by intersecting the gene list for each pattern between normal and still and then subtracting the genes found in the still patterns.

Results

Between-condition differential expression

A total of 2263 genes are misregulated (Fishers Exact Test p-value < 0.001) in the still phenotype across all timepoints, with 88% of misregulation occurring in late adulthood (Table 1-1). Significant GO terms for late pupal and early adult stages include oxidative stress response, immune response, and transmembrane transport (Figure 1-1). Additionally, late adults experience higher upregulation than other timepoints across 1154 genes (51% of total differential expression), of which 328 genes have significant Gene Ontology enrichment terms related to proteolysis and digestive processes (Figure 1-2). An average of 3% of genes are misexpressed in still hybrids in the pupal stages whereas an average of 10.1% of genes are misexpressed in the adult.

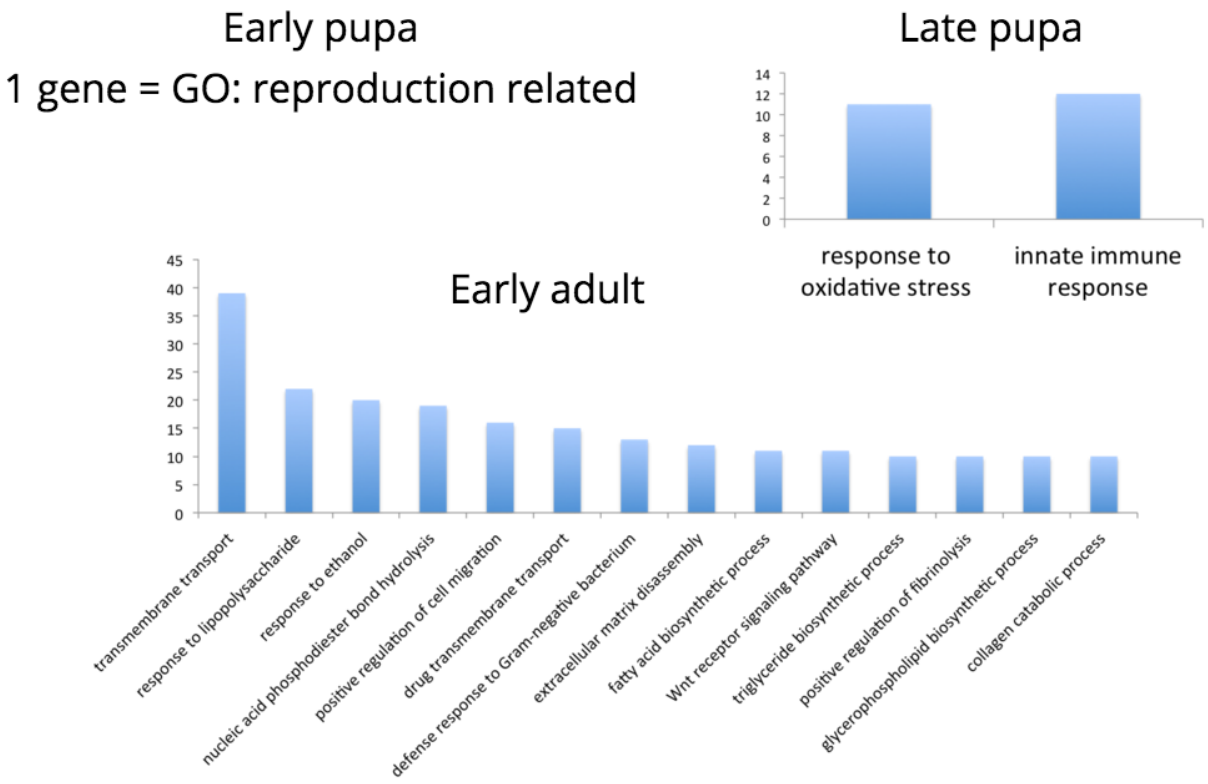


Figure 1-1 Number of genes (y-axis) for each Gene Ontology term (x-axis) with significant enrichment for the first three developmental timepoints (pupa day 1 - upper left, pupa day 3 - upper right, adult day 1 - bottom center).

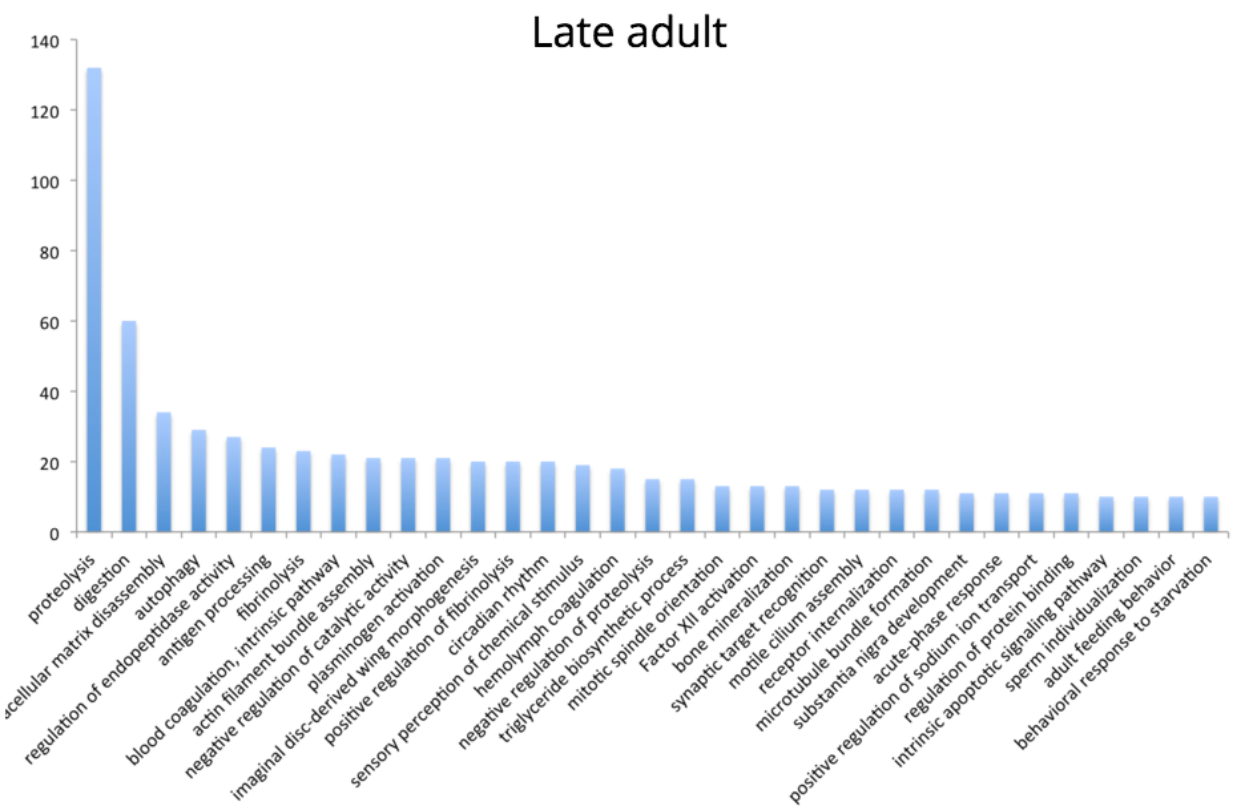


Figure 1-2 Number of genes (y-axis) for each Gene Ontology term (x-axis) with significant enrichment for the last developmental timepoint (adult day 3).

Table 1-1 Genes differentially expressed at each timepoint between normal and still F1 hybrids

Timepoint	**Total Gene Count**	**Upregulated**	**Downregulated**
Pupa (Day 1)	463	70	393
Pupa (Day 3)	537	8	529
Adult (Day 1)	1355	180	1175
Adult (Day 3)	1982	668	1314

Within-condition differential expression

There are eight expression patterns of magnitude changes in gene expression across time. With respect to the normal phenotype, “still” experiences a 6.01% reduction of total transcripts that are consistently upregulated over development (Table 1-2). The still phenotype experiences more dynamic changes in magnitude of gene expression as shown in Figure 1-3. The largest unique shift in magnitude of expression occurs in the still phenotype in the up-down-up pattern (8% of total transcripts) as shown in Table 1-2. GO term enrichment testing using Blast2GO for the still-specific up-down-up pattern members yielded highly significant matches for ‘cilium or flagellum-dependent cell motility’ and ‘monovalent inorganic cation transport’ in the Biological Process hierarchical level (Figure 1-4), ‘integral component of plasma membrane’ for Cellular

Component (Figure 1-5), and ‘metal ion transmembrane transporter activity’ in addition to ‘catecholamine binding’ for Molecular Function (Figure 1-6).

Transcription factors critical to development

Since 14% of the *T. castaneum* transcriptome undergoes differential expression in the still phenotype across the developmental window, there remains much in the way of trans-acting regulation as a potential source of misregulation. Bitra et al. 2009 elucidated the function of the basic helix–loop–helix (bHLH) family of transcription factors essential to morphogenesis through cellular proliferation, tissue differentiation, development and detoxification in *Tribolium castaneum* by performing RNA interference knockdowns of 53 candidate transcription factors at the larval stage. Of these 53 factors, 6 are found to be differentially expressed between conditions at a fold change less than 2, while 27 factors are found to be differentially expressed within-condition. 9 factors are shared across normal and still phenotypes, while each phenotype has 14 bHLH transcription factors uniquely regulated per expression pattern and phenotype (Table 1-4).

Phosphine resistance

In Oppert et al. (2015), RNA-Seq analysis was performed on susceptible and resistant *Tribolium castaneum* populations before and after exposure to sublethal doses of the phosphine fumigant. 214 genes were found to be differentially expressed between all conditions, of which 30 were found to be significantly differentially expressed in the still phenotype (Table 1-3). One transcript, LOC103314140, with GO

terms related to chromosome and peroxisome organization, was found upregulated by 2630-fold in unexposed resistant populations compared to unexposed susceptible, and upregulated by 833-fold in exposed resistant versus exposed susceptible. Our differential expression analysis shows that LOC103314140 shows the highest magnitude of upregulation at 8.6-fold in still hybrids relative to normal at the adult day 3 time point. Furthermore, LOC103314140 is found to be uniquely regulated in the still phenotype over the time-course in the down-down-up pattern.

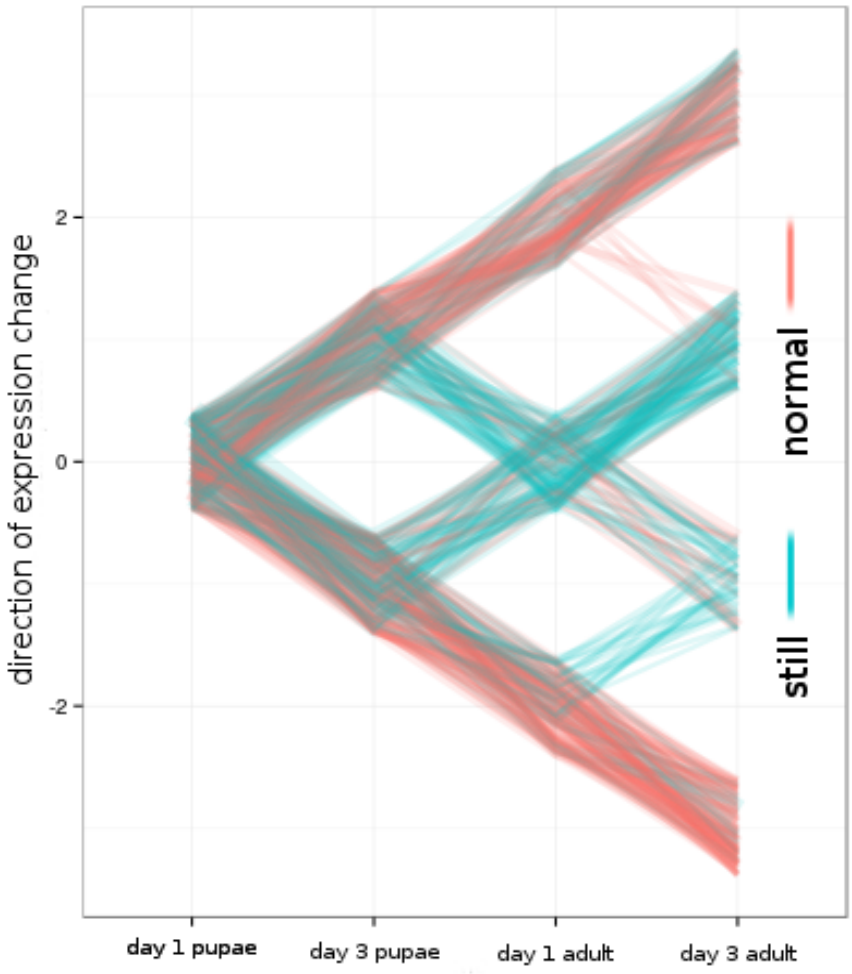


Figure 1-3 Gene expression levels plotted as log_2 fold change (y-axis) over developmental time points (x-axis) for each phenotype (red - normal, blue - still).

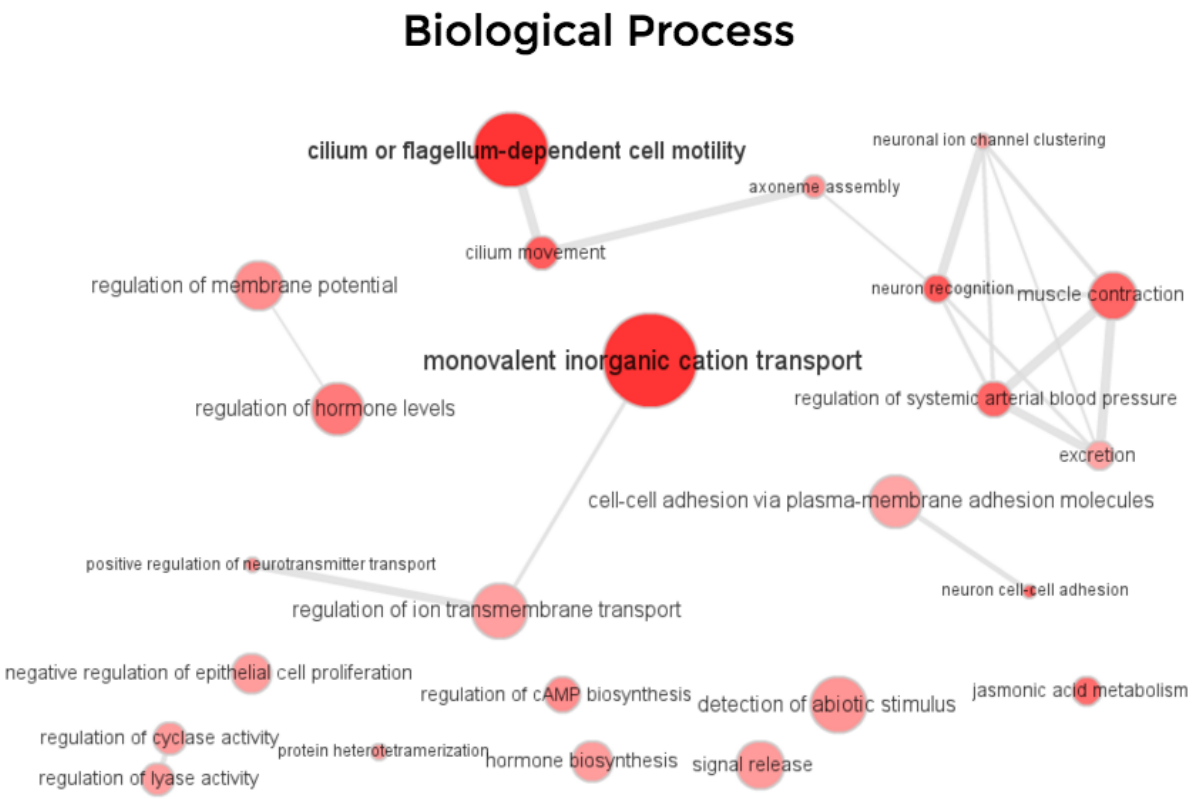


Figure 1-4 GO terms significantly enriched in the Biological Process hierarchical level for the UP-DOWN-UP pattern for genes uniquely regulated in still hybrids. Graph structure shows connected lines for associated GO terms. Darkness of color is proportional to higher significance of p-value for associated GO term.

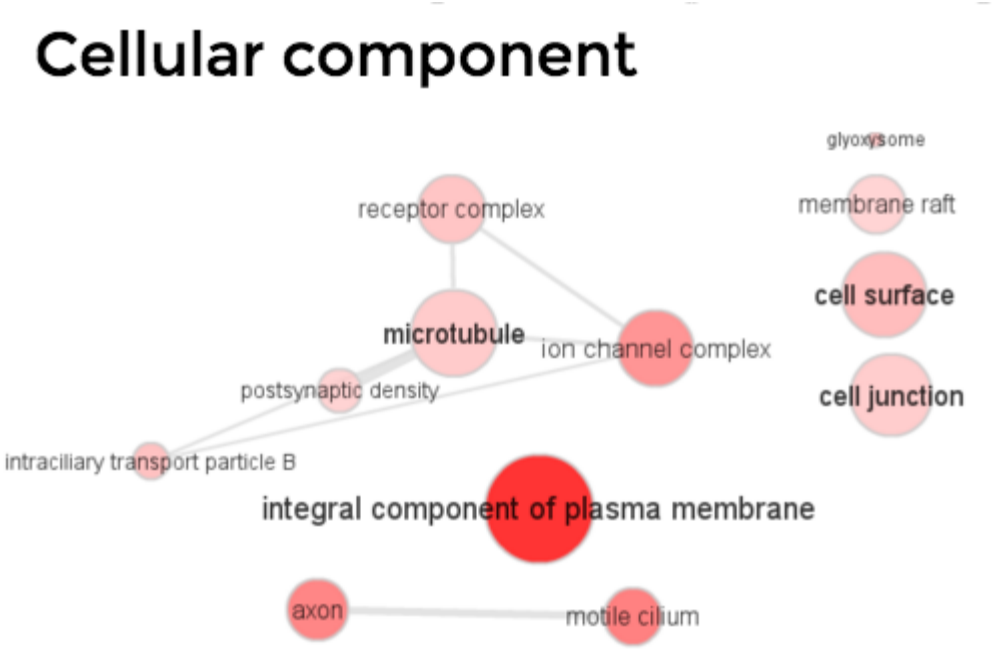


Figure 1-5 GO terms significantly enriched in the Cellular Component hierarchical level for the UP-DOWN-UP pattern for genes uniquely regulated in still hybrids. Graph structure shows connected lines for associated GO terms. Darkness of color is proportional to higher significance of p-value for associated GO term.

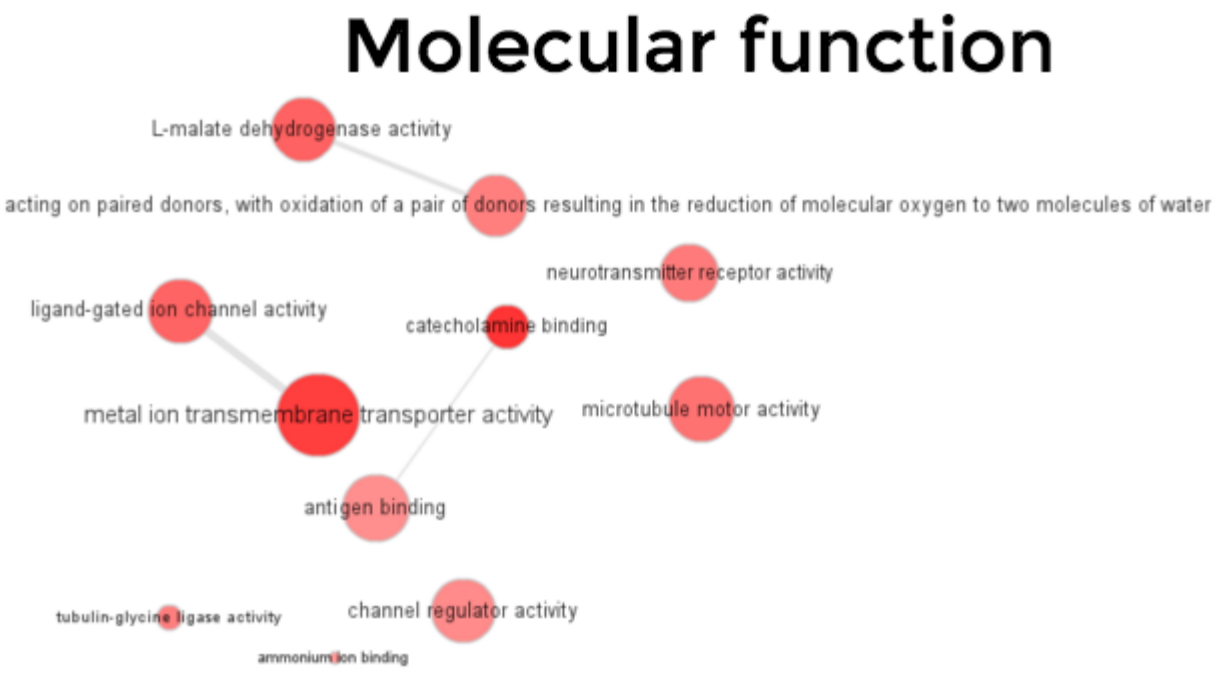
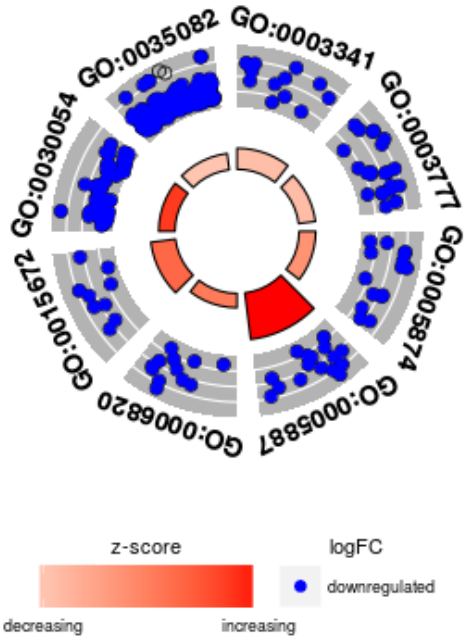


Figure 1-6 GO terms significantly enriched in the Molecular Function hierarchical level for the UP-DOWN-UP pattern for genes uniquely regulated in still hybrids. Graph structure shows connected lines for associated GO terms. Darkness of color is proportional to higher significance of p-value for associated GO term.



ID	Description
GO:0003341	cilium movement
GO:0003777	microtubule motor activity
GO:0005874	microtubule
GO:0005887	integral component of plasma membrane
GO:0006820	anion transport
GO:0015672	monovalent inorganic cation transport
GO:0030054	cell junction
GO:0035082	axoneme assembly

Figure 1-7 Genes with significant differential expression in all developmental stages and associated Gene Ontology (GO) terms. Blue circles denote downregulation of expression. Red gradient increases linearly with z-score, or number of genes belonging to each GO term. The GO:0005887 (integral component of plasma membrane) group contains the highest number of genes and the highest magnitude of downregulation found across timepoints.

Table 1-2 Genes belonging to expression patterns for each developmental transition

EXPRESSION PATTERN	UNIQUE TO STILL	UNIQUE TO NORM	SHARED
DOWN-DOWN-DOWN	254	221	101
DOWN-UP-UP	399	277	255
DOWN-UP-DOWN	361	334	300
DOWN-DOWN-UP	427	163	148
UP-DOWN-UP	1020	249	260
UP-UP-UP	189	856	142
UP-UP-DOWN	95	260	99
UP-DOWN-DOWN	276	393	322

Table 1-3 Phosphine resistance genes in Oppert et al. 2015 and their differential expression between normal and still hybrids using edgeR test (modified Fisher's exact test with negative binomial distribution). Fold change is given in logarithm base 2 (logFC) and p-value is modified for the false discovery rate (FDR). Bold entry at Adult Day 3 for LOC103314140 represents highest magnitude of upregulation found in still hybrids.

Pupa Day 1			
gene	Predicted Function	logFC	FDR
LOC661067	cytochrome P450 9e2-like	-1.6728	0.020369
LOC662197	alpha-N-acetylgalactosaminidase-like (carbohydrate-related)	-1.9531	0.04432
Pupa Day 3			
None			

Adult Day 1			
gene	Predicted Function	logFC	FDR
LOC103313179	G2/mitotic-specific cyclin-B	-5.4667	0.005572
LOC664606	intraflagellar transport protein 52 homolog	-4.4553	0.017882
LOC103312940	hypothetical protein	-5.7535	0.017882
LOC659503	venom allergen 3	-4.5497	0.017882
LOC660240	scavenger receptor class B member 1-like	-1.1051	0.01937
LOC657319	protein downstream neighbor of son homolog	-2.012	0.023987
LOC103313878	DNA ligase 1-like	1.98971	0.023987
LOC103314229	feline leukemia virus subgroup C receptor-related protein 2-like	-1.903	0.023987
Ago-2a	Argonaute-2a	-1.6934	0.041087
LOC103314418	hypothetical protein	-1.5749	0.041087

Adult Day 3			
gene	Predicted Function	logFC	FDR
LOC660240	scavenger receptor class B member 1-like	-3.3534	0.00258
LOC664606	intraflagellar transport protein 52 homolog	-4.5735	0.00258
LOC659503	venom allergen 3	4.1734	0.00258
LOC103314086	uncharacterized	2.83111	0.00258
LOC103314140 (TC004024)	**chromosome organization**	**8.46079**	**0.003503**
LOC657319	protein downstream neighbor of son homolog	-2.6428	0.006081
LOC103313179	G2/mitotic-specific cyclin-B	-3.0626	0.006081
Cyp4g7	cytochrome P450 monooxygenase CYP4G7	1.68526	0.006081
LOC103312940	hypothetical protein	-5.4091	0.006374
LOC661136	protein ST7 homolog	-1.5675	0.006374
LOC661398	DNA replication ATP-dependent helicase/nuclease DNA2	-1.7358	0.009779
LOC658236	uncharacterized	1.71952	0.011452
LOC659189	putative inorganic phosphate cotransporter	-6.2129	0.011452
Tyr1	pro-phenol oxidase subunit 1	2.3362	0.011452
LOC103313145	uncharacterized	-1.2721	0.012831

LOC103313498	protein CTLA-2-alpha-like	1.71311	0.012831
LOC657806	M-phase phosphoprotein 6	-1.4082	0.012831
LOC103315019	cuticle protein CP14.6-like	-1.7897	0.015074
LOC659404	uncharacterized	-2.1048	0.016171
LOC663534	putative fatty acyl-CoA reductase CG5065 (mitochondrial)	2.08636	0.018169
LOC661460	2',5'-phosphodiesterase 12	-1.0177	0.021029
Ago-2a	Argonaute-2a	-2.4681	0.021029
LOC103313878	DNA ligase 1-like	1.52712	0.02281
LOC659881	regulating synaptic membrane exocytosis protein 2	-1.7129	0.024516
LOC655898	pre-mRNA-splicing ATP-dependent RNA helicase PRP28-like	-0.9767	0.025847
LOC658169	tRNA-splicing ligase RtcB homolog	-1.2586	0.026529
LOC660291	spondin-1-like	1.03989	0.030358
LOC656501	splicing factor 3B subunit 4	-0.9641	0.03352
LOC100141891	glycine-rich RNA-binding protein blt801-like	1.18482	0.03352
LOC658401	uncharacterized	1.43649	0.044018

Table 1-4 Differential expression of the bHLH (basic helix–loop–helix) family of transcription factors critical to development in *Tribolium castaneum* (Bitra et al. 2009) over the timecourse of development unique to each timepoint for each phenotype.

STILL UNIQUE			NORMAL UNIQUE		
expression pattern	**gene name**	**accession**	**expression pattern**	**gene name**	**accession**
UP-DOWN-UP			**UP-DOWN-UP**		
TC004726	TcHand	XP_972310	TC007598	TcDelilah 2	XP_973102
TC011804	Side2	XP_973515	**DOWN-UP-UP**		
TC013785	TcFer2	XP_971276	TC003171	TcCato	XP_974243
TC014105	TcFer1	XP_969845	TC012851	Hairy	XP_971935

TC014225	TcMitf	XP_975837	**UP-DOWN-DOWN**		
DOWN-UP-UP			TC003170	TcAmos	XP_974297
TC005224	Deadpan	XP_967694	TC006222	TcDimmed	XP_971229
UP-DOWN-DOWN			TC013566	Dysfusion	N/A
TC014712	TcBeta3	XP_974793	**DOWN-DOWN-UP**		
TC016205	Sim1	XP_967930	TC004726	TcHand	XP_972310
DOWN-DOWN-UP			TC005224	Deadpan	XP_967694
TC005541	E(spl)mgamma2	XP_972493	**UP-UP-DOWN**		
TC008433	TcAsh	NP_001034537	TC000088	Clock	XP_967106
UP-UP-DOWN			TC002494	Cycle	NP_001107795
TC006222	TcDimmed	XP_971229	**DOWN-DOWN-DOWN**		
DOWN-DOWN-DOWN			TC000024	TcEmc	XP_970015
TC003170	TcAmos	XP_974297	TC015754	TcParaxis	XP_001808472
TC007652	TcAP-4	XP_967737	**UP-UP-UP**		
UP-UP-UP (no matches)			TC001576	TcNeuroD	N/A
DOWN-UP-DOWN			**DOWN-UP-DOWN**		
TC002494	Cycle	NP_001107795	TC002349	TcTF21	XP_968572

Table 1-5 Top enriched GO terms for genes with dynamic temporal regulation found only in the still phenotype. Expression pattern is combination of three-state change from pupa day 1 to pupa day 3, pupa day 3 to adult day 1, and adult day 1 to adult day 3. Category of GO term is listed in parentheses as BP - biological process, CC - cellular component, and MF - molecular function.

Expression Pattern	GO term number, description, and category
UP-UP-UP	GO:0072350 tricarboxylic acid metabolic process (BP) GO:0006119 oxidative phosphorylation (BP) GO:0006099 tricarboxylic acid cycle (BP) GO:0006120 mitochondrial electron transport, NADH to ubiquinone (BP) GO:0044712 single-organism catabolic process (BP) GO:0045239 tricarboxylic acid cycle enzyme complex (CC) GO:0006103 2-oxoglutarate metabolic process (BP) GO:0045254 pyruvate dehydrogenase complex (CC) GO:1901565 organonitrogen compound catabolic process (BP) GO:0051068 dihydrolipoamide metabolic process (BP) GO:0004148 dihydrolipoyl dehydrogenase activity (MF)
UP-DOWN-UP	GO:0005887 integral component of plasma membrane (CC) GO:0031514 motile cilium (CC) GO:0030424 axon (CC) GO:0034702 ion channel complex (CC) GO:0043025 neuronal cell body (CC) GO:0036064 ciliary basal body (CC) GO:0001539 cilium or flagellum-dependent cell motility (BP) GO:0015672 monovalent inorganic cation transport (BP) GO:1901338 catecholamine binding (MF) GO:0008038 neuron recognition (BP) GO:0003341 cilium movement (BP) GO:0046873 metal ion transmembrane transporter activity (MF)
UP-DOWN-DOWN	GO:0032199 reverse transcription in RNA-mediated transposition (BP) GO:0009036 Type II site-specific deoxyribonuclease activity (MF)
DOWN-DOWN-DOWN	GO:0042302 structural constituent of cuticle (MF) GO:0003682 chromatin binding (MF) GO:0051276 chromosome organization (BP) GO:0008010 structural constituent of chitin-based larval cuticle (MF) GO:0005654 nucleoplasm (CC) GO:0003677 DNA binding (MF) GO:0016568 chromatin modification (BP) GO:0005694 chromosome (CC)

	GO:0008011 structural constituent of pupal chitin-based cuticle (MF) GO:0000792 heterochromatin (CC) GO:0001106 RNA polymerase II transcription corepressor activity (MF)
DOWN-DOWN-UP	GO:0005876 spindle microtubule (CC) GO:0031616 spindle pole centrosome (CC) GO:0032133 chromosome passenger complex (CC) GO:0005550 pheromone binding (MF) GO:0051276 chromosome organization (BP) GO:0007059 chromosome segregation (BP) GO:0000070 mitotic sister chromatid segregation (BP) GO:0043138 3'-5' DNA helicase activity (MF) GO:0051302 regulation of cell division (BP) GO:0016572 histone phosphorylation (BP) GO:0000786 nucleosome (CC) GO:0043010 camera-type eye development (BP)
DOWN-UP-DOWN	GO:0005654 nucleoplasm (CC) GO:0007062 sister chromatid cohesion (BP) GO:0000404 heteroduplex DNA loop binding (MF) GO:0044454 nuclear chromosome part (CC) GO:0008094 DNA-dependent ATPase activity (MF) GO:0007131 reciprocal meiotic recombination (BP) GO:0071103 DNA conformation change (BP) GO:0000718 nucleotide-excision repair, DNA damage removal (BP) GO:0000724 double-strand break repair via homologous recombination (BP) GO:0002562 somatic diversification of immune receptors via germline recombination within a single locus (BP) GO:0031047 gene silencing by RNA (BP) GO:0006260 DNA replication (BP)
DOWN-UP-UP	GO:0005576 extracellular region (CC) GO:0007586 digestion (BP) GO:0044281 small molecule metabolic process (BP) GO:0032787 monocarboxylic acid metabolic process (BP) GO:0048252 lauric acid metabolic process (BP) GO:0005615 extracellular space (CC) GO:0018685 alkane 1-monooxygenase activity (MF) GO:1901565 organonitrogen compound catabolic process (BP)

	GO:0016042 lipid catabolic process (BP) GO:1901615 organic hydroxy compound metabolic process (BP) GO:0031349 positive regulation of defense response (BP) GO:0014070 response to organic cyclic compound (BP) GO:0055114 oxidation-reduction process (BP) GO:0017143 insecticide metabolic process (BP) GO:0004553 hydrolase activity, hydrolyzing O-glycosyl compounds (MF)

Discussion

Between-condition differential expression

Misregulation of gene expression occurs in 17% of the transcriptome between normal and still F1 hybrids, with 50% of the total number of misregulated genes occurring in adulthood. For differential expression contrasted at each timepoint (between-condition), the three most significantly enriched terms for misexpressed genes in the Gene Ontology database include oxidative-reductive processes, proteolysis, and digestion. The higher magnitude of gene expression differences in adult compared to pupa stages coincides with the developmental timing for the still phenotype to be visible. “Still” pupa and larvae are indistinguishable from normal hybrids and could reflect lower reliance on oxidative phosphorylation due to programmed mitochondrial degeneration in pupa (Shappiro and Williams 1957). In addition, an increase in bound lipids occurs during the shift from larvae to pupal stages accompanied by a shift to free lipids as the primary source of energy in pupae (Villeneuve and Lemonde 1963).

Within-condition differential expression

Gene expression changes evaluated across the developmental window within experimental conditions allows still-specific changes in temporal expression patterns to be observed by contrasting the genes observed in each expression pattern in normal. The biggest shift in temporal regulation occurs in up-down-up pattern in the still phenotype, comprising 1020 genes or 6.2% of the transcriptome (Table 1-2). This expression pattern (up-down-up) has the highest gene membership overlap with the other measure of differential expression (between-condition) at 702 of 1020 genes, with 60% of the overlap occurring in the adult stage (422 genes). Interestingly, the most functionally enriched set of GO terms for the overlapping gene set is “integral component of plasma membrane,” which is a Cellular Component level function description for protein complexes embedded in hydrophobic regions of the cellular membrane which could potentially be correlated with the second and third most enriched terms for “inorganic cation transport” and “cilium movement,” which are Biological Process level functions for movement of inorganic ions out of a cell through a transporter and the directed movement of the cilium (a key coordinator of signalling pathways during development), respectively (Figure 1-7). Evidence for oxidative stress unique to “still” can be seen in the most significant GO terms in the up-up-up pattern (consistently upregulated) (Table 1-5) that includes several mitochondrially interacting enzymes such as the dihydrolipoyl dehydrogenase complex.

High-dimensional gene expression data with several lists of Gene Ontology terms can result in a high number of candidate genes. Here it is demonstrated that Intersecting results from multiple analyses and previous studies can result in a clearer picture of the dynamics of gene expression for a physiological stress response resulting

from genetic incompatibilities that are polymorphic in the F1 generation. There is a core set of 298 genes (Figure 1-7), including 6 basic helix-loop-helix transcription factors (Table 1-4), found to be differentially expressed at all time points through development. The transcript with the highest magnitude of upregulation (accession # LOC103314140 / TC004024) in adult still hybrids was also found to have the highest differential expression between susceptible and resistant *T. castaneum* populations in Oppert et al. 2015. LOC103314140 shows an 8.6-fold change increase in still hybrids relative to normal at the adult day 3 time point. Furthermore, LOC103314140 is found to be uniquely regulated in the still phenotype over the time-course in the down-down-up pattern and represents a candidate gene for further study.

Chapter 2

Introduction

A model for genetic incompatibilities as a speciation barrier is genome shock, or any set of programmed cellular responses to stressful environments (McClintock 1983). In the most general case, genome shock can entail the activation of repair pathways in response to damage. Transcriptome shock is an extension of genome shock, wherein altered gene expression levels of hybrids result from mismatched interactions between cis and trans-acting regulatory nucleotide sequences with respect to each parental background (Hegarty et al. 2006). A well studied example of transcriptome shock is alternative splicing (AS), or the differential processing of precursor mRNAs to create multiple variations of transcripts. The most common forms of splicing variants in metazoans are the selective inclusivity of exons (cassette exons) (Kim et al. 2008) into the mature transcript, and are frequently observed to downregulate gene expression in response to changing cellular conditions by triggering nonsense-mediated decay (Lewis et al. 2003). Alternative splicing has been observed as a response to oxidative damage (Bohr et al. 2002) and low ATP levels arising from aberrant mitochondria (Maracchioni et al. 2007) by altering the subcellular destination of a transcript (Ashibe et al. 2007). Empirical tests of intraspecific differences in AS have been observed for specific instances of stress response tissue type, and developmental stage in maize (Thatcher et al. 2016) and Drosophila (Brown et al. 2014). Therefore, a specific response of AS could be observed between intraspecific F1 hybrids, given evidence of transcriptomic

shock spanning ~2200 genes across development encompassing several significant gene ontology (GO) terms enriched for oxidative stress (Table 1-5). Under the transcriptome shock model, this misregulation of gene expression could result from expression biases introduced by the differential interaction of parental alleles with regulatory elements (Hegarty et al. 2006; Cox et al. 2014). Introgressed parental alleles can recruit regulatory elements in a dominant or recessive manner to alter gene expression levels. This produces a larger mutation effect size with more potential for widespread misregulation than mutations to the coding sequences in *cis* (Meiklejohn et al. 2014). For example, cis-acting mutations can accumulate at exon/intron splicing sites in an additive fashion (Fu & Ares 2014) that can impact inclusion of exons or introns into the mature transcript, while the spliceosome machinery controls expression level when binding in trans at the exon/intron junction either dominantly or recessively (Meiklejohn et al. 2014). Parental alleles with unique sequence variation in intronic regions have potential to disrupt splice-site recognition by the spliceosome at the boundary between exon and introns (Keren et al. 2010). Mutations fixed in diverged populations of *T. castaneum* provide a potential source of cis-regulation for alternative splicing in F1 hybrids on a continuum for a fitness-reducing phenotype dubbed “still”. Further insights into the gene expression differences underlying this phenotype could reveal the variation in transcriptome shock between F1 hybrids as a result of planned responses to stress via alternative splicing.

Methods

Parental DNA-seq SNP calling

DNA sequencing samples were prepared from pooled sex-specific libraries of Chicago and Tanzania *T. castaneum* populations as detailed in Chapter 1. To account for batch effects of sequencing on different lanes of, read groups were added to each alignment using Picard (http://broadinstitute.github.io/picard) to specify which sequence library preparation, technical replicate, and sequencing lane was used.
Duplicate reads in the alignment (identical start and stop positions) were removed with Picard to eliminate bias resulting from PCR duplication during the preparation of sequence libraries. Then, four samples from each parental population (two sex-specific with technical replicates) were queried for fixed single nucleotide polymorphisms (SNPs) over transcript regions using Freebayes software (Garrison 2010) with modified settings for the ploidy of pooled samples (100 total chromosomes) and to only include alleles present in the population at 99% frequency. An annotation file in the Variant Call Format (VCF) format was produced for each parental population that was subsequently filtered to exclude sites sampled at greater than 140X depth to avoid biased calls from alignment of sequence reads arising from repetitive regions (Sims et al. 2014). To find shared sites that were heterozygous between parents, the parental VCF files were filtered to include only SNP locations that intersected. With these filtered sets of fixed SNPs, a personal haploid genome was constructed for each parental population using the Genome Analysis Toolkit (McKenna et al. 2010). From each personalized genome

for Chicago and Tanzania, transcript regions were extracted as FASTA nucleotides and concatenated into a new transcriptome containing separate alleles for each reference transcript, doubling the size to 34,160 genes.

Allele biased expression

RNA-seq reads for each hybrid phenotype and timepoint were mapped against the personalized reference transcriptome with Bowtie (Langmead 2010) with settings that allow up to 100 mapped reads at a given site, while prioritizing hits that have the least number of mismatches in the seed stage (-a --best --strata -m 100). The MMSEQ software package fits a Poisson model to the number of reads overlapping each region of transcript in the alignment that is scaled by a maximum likelihood-estimated normalization factor (Turro et al. 2011). From these read counts, an MCMC algorithm estimates the posterior probability in favor of differential expression between two treatment groups (Turro et al. 2014). Biased expression between parental transcripts is inferred from an interpretation of the posterior probability (recommended by the author) exceeding twice the prior. Magnitude of expression is represented by the posterior means.

Alternative splicing

Tophat2 (Kim et al. 2013) was used to align hybrid RNA-seq reads to the *T. castaneum* reference genome version 3.31. DEXSeq (Anders et al. 2012) is a collection of programs to infer alternative splicing by counting reads that fall into exon regions. First, *dexseq_prepare_annotation.py* takes a reference transcriptome file (Ensembl version 3.31) and collects only exon entry information to create collapsed exon counting

bins. From the alignment files, *dexseq_count.py* finds all reads overlapping these exonic bins for input to the main R script, where between-condition contrasts were performed at each time point (for example, normal and still samples at pupa day 1) over the main statistic termed “relative exon usage,” or the ratio of the number of transcripts from a gene that contain an exon to the number of all transcripts from the gene.

Inferring sources of cis-acting regulation

With a pooled sequencing strategy for parental and hybrid samples, cis-acting sources of gene expression regulation can be inferred if parental SNPs are found inside genes that are differentially expressed through other measures (between-condition, within-condition, alternative splicing, allele bias) and then ranked by SNP count and magnitude of mis-expression. Trans-acting regulation comprises the sum of gene expression differences not accounted for in *cis* (Wittkopp et al. 2004) and can be inferred indirectly based on changes in allele biased expression. Additionally, groups of allele-biased genes that form new protein-protein interactions can reveal novel perturbations in the gene regulatory network by examining protein domain functions in databases such as STRING (Szklarczyk et al. 2014).

Results

Allele biased expression

I found 22,991 fixed nucleotide differences between Chicago and Tanzania that are useful for mapping allele biased expression. From the mapping results to the combined transcriptome reference, 265 transcripts of the 17,080 allele pairs were found to have biased expression at a posterior probability > 0.2 (twice the uninformative prior of 0.1), with 182 transcripts in normal hybrids and 83 in still (32 shared between conditions). Figure 2-1 shows the distribution of these 83 still-specific transcripts at each development stage, where some genes are biased at multiple time points (107 genes total). In Table 2-1, it is demonstrated that only paternally biased alleles form novel still-specific protein-protein interactions in STRING, and have PFAM domains for developmental transcription factors, DNA repair, and chromatin modifiers. Genes with allele bias unique to normal and still hybrids but shown to be differentially expressed (between-condition) are shown in Tables 2-2 and 2-3, respectively. Table 2-4 shows the time course expression patterns of alternative splicing that are both unique to the normal phenotype, and Table 2-5 lists genes with unique temporal between-condition allele bias in still.

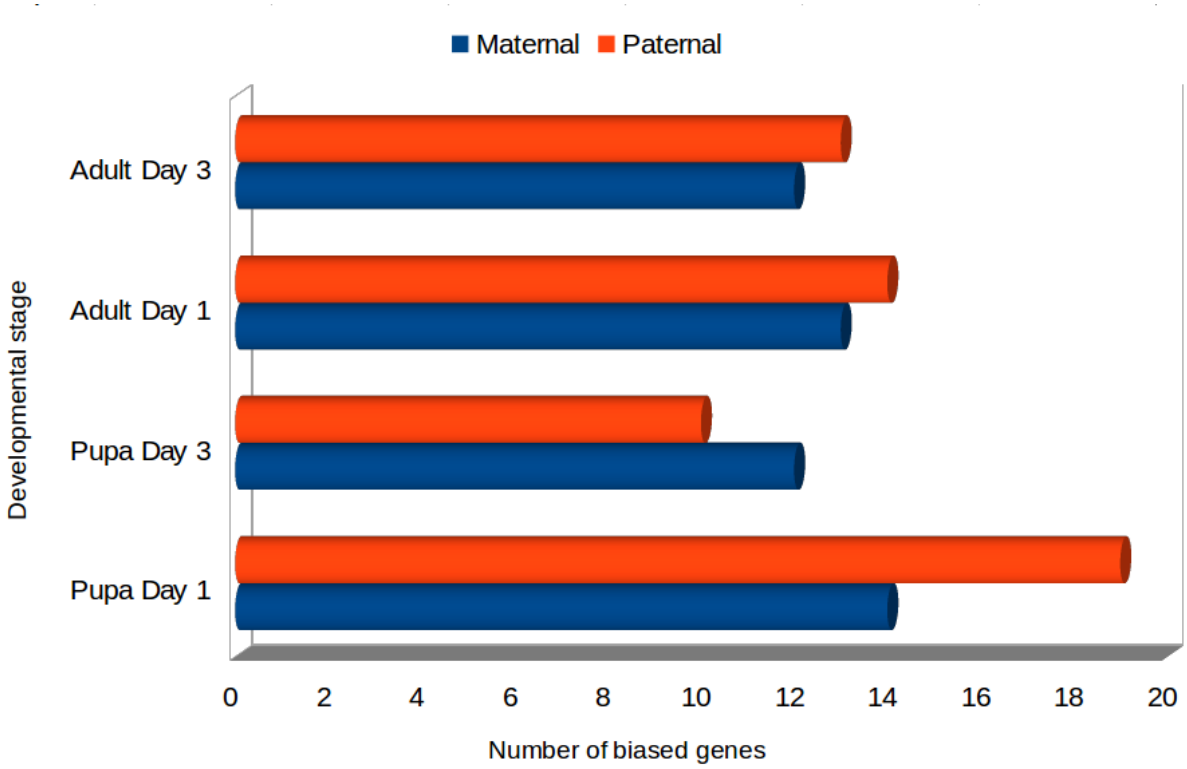


Figure 2-1 Genes with still-specific allele biased expression across developmental stages (blue – maternal - Chicago, red – paternal - Tanzania). x-axis denotes number of genes meeting significance threshold of posterior probability > 0.2. Y-axis labels each stage of development over the timecourse.

Table 2-1 Genes with still-specific paternal allele bias with predicted protein-protein interactions in STRING database at each developmental timepoint and associated PFAM domain (Finn et al. 2016) and its function. All genes listed at each time point interact according to homologous genes in other species.

Timepoint	Gene names	PFAM protein domain	Function
Pupa day 1	TC014275	CHROMO	Chromatin organization modifier domain
	TC003811	TOP4c	DNA Topoisomerase IV
Adult day 1	TC003481	COBRA	negative regulation of transcription, DNA-templated
	TC010518	RPOLA_N	RNA polymerase
	TC005866	DEXDc/HELICc	helicase
	TC002071	MutS	DNA mismatch repair
Adult day 3	TC005661	HOMEOBOX	developmental transcription factor
	TC005573	FORKHEAD	developmental transcription factor

Table 2-2 Genes with significant allele bias unique to the normal phenotype at each stage of development and differentially expressed (between-condition) with associated GO term (molecular function).

Pupa Day 1	TC008418 -- nucleotide metabolic process TC015915 -- axon guidance TC008109 -- chemosensory behavior TC030467 -- response to lipopolysaccharide TC009070 -- sodium ion transmembrane transport TC014768 -- regulation of DNA replication TC007207 -- nucleosome assembly TC011331 -- transcription, DNA-dependent TC011384 -- microtubule severing
Pupa Day 3	TC002806 -- chaperone cofactor-dependent protein refolding TC006608 -- G-protein coupled acetylcholine receptor signaling TC007207 -- nucleosome assembly TC009070 -- sodium ion transmembrane transport TC011331 -- transcription, DNA-dependent TC011384 -- microtubule severing TC013116 -- gonad development TC014768 -- regulation of DNA replication
Adult Day 1	TC014768 -- regulation of DNA replication TC002719 -- brain development TC014458 -- cellular response to phosphate starvation TC007207 -- nucleosome assembly TC006608 -- G-protein coupled acetylcholine receptor signaling TC004855 -- neurotransmitter secretion TC014016 -- response to organic cyclic compound TC002966 -- hermaphrodite genitalia development TC003198 -- neuron development TC003729 -- Leydig cell differentiation TC009950 -- collagen catabolic process TC012712 -- telomere maintenance TC007700 -- 'de novo' posttranslational protein folding TC009598 -- cellular metabolic process TC008418 -- nucleotide metabolic process TC012026 -- spleen development TC008109 -- chemosensory behavior TC002993 -- cytokinesis by cell plate formation TC030467 -- response to lipopolysaccharide TC003095 -- locomotory behavior TC006211 -- double-strand break repair TC012053 --G2M transition of mitotic cell cycle TC013116 -- gonad development

	TC002806 -- chaperone cofactor-dependent protein refolding TC009070 -- sodium ion transmembrane transport TC000245 -- mRNA splicing, via spliceosome TC011331 -- transcription, DNA-dependent TC001232 -- negative regulation of B cell activation TC011384 -- microtubule severing TC007008 -- protein refolding TC012689 -- determination of left
Adult Day 3	TC002719 -- brain development TC012053 -- G2M transition of mitotic cell cycle TC002966 -- hermaphrodite genitalia development TC003198 -- neuron development TC000117 -- oxidation-reduction process TC008418 -- nucleotide metabolic process TC006043 -- transcription, DNA-dependent TC002993 -- cytokinesis by cell plate formation TC004657 -- digestion TC009070 -- sodium ion transmembrane transport TC004855 -- neurotransmitter secretion TC014458 -- cellular response to phosphate starvation TC008109 -- chemosensory behavior TC013116 -- gonad development TC012013 -- cell projection organization TC012689 -- determination of left TC030467 -- response to lipopolysaccharide TC006211 -- double-strand break repair TC011331 -- transcription, DNA-dependent TC002806 -- chaperone cofactor-dependent protein refolding TC000245 -- mRNA splicing, via spliceosome TC012026 -- spleen development TC015161 -- oxidation-reduction process TC014016 -- response to organic cyclic compound TC001232 -- negative regulation of B cell activation TC003095 -- locomotory behavior TC007207 -- nucleosome assembly TC014768 -- regulation of DNA replication TC007008 -- protein refolding TC011384 -- microtubule severing TC003729 -- Leydig cell differentiation TC012712 -- telomere maintenance TC006608 -- G-protein coupled acetylcholine receptor signaling

	TC007700 -- 'de novo' posttranslational protein folding

Table 2-3 Genes with significant allele bias unique to the still phenotype at each stage of development and differentially expressed (between-condition) with associated GO term (molecular function).

Development timepoint	Gene accession
Pupa Day 1	TC010280 -- transposition, DNA-mediated
Pupa Day 3	TC003978 -- transcription, DNA-dependent
Adult Day 1	TC004855 -- neurotransmitter secretion TC000245 -- mRNA splicing, via spliceosome TC009090 -- hemolymph coagulation TC003978 -- transcription, DNA-dependent
Adult Day 3	TC004855 -- neurotransmitter secretion TC003978 -- transcription, DNA-dependent TC000245 -- mRNA splicing, via spliceosome TC015161 -- oxidation-reduction process TC005776 -- P granule organization TC005444 -- flight behavior TC005866 -- resolution of meiotic recombination intermediates TC009090 -- hemolymph coagulation

Table 2-4 Genes with significant allele bias unique to the normal phenotype at each expression pattern of within-condition differential expression

Expression Pattern	Gene accession
UP-UP-UP	TC002719, TC002806, TC003095, TC006316, TC006502, TC006721, TC007207, TC007493, TC008268, TC011384, TC012026, TC012051, TC012053, TC012317, TC014458, TC014768, TC015757, TC015772
UP-UP-DOWN	TC004471, TC014795
UP-DOWN-DOWN	TC002128, TC013023, TC014861
DOWN-DOWN-DOWN	TC007655, TC012717
DOWN-UP-DOWN	TC002023, TC004472, TC012017, TC012404, TC013124, TC016117

Table 2-5 Genes with significant allele bias unique to the still phenotype at each expression pattern of within-condition differential expression

Expression Pattern	Gene accession
UP-DOWN-UP	TC005732, TC009720, TC012317, TC013105, TC016090
UP-DOWN-DOWN	TC004569, TC010280, TC012885
DOWN-UP-UP	TC005444

Alternative splicing

160 exons across 108 genes were found to have significant alternative splicing in the still phenotype (Table 2-6) through differential exon usage. From these 108 genes, Table 2-7 shows the developmental timing pattern in the normal phenotype, and Table 2-8 demonstrates the unique timing patterns of splicing to still. To reduce the dimensionality of the results and examine still-specific temporal regulation of splicing, the 108 AS genes were intersected with the results of between- and within-condition differential expression to uncover potential systemic responses and the magnitude of expression change (Table 2-9).

Table 2-6 Number of genes and exons that undergo alternative splicing in the still phenotype at each timepoint.

Time Point	**Gene Count**	**Exon Count**
Pupa Day 1	5	8
Pupa Day 3	18	29
Adult Day 1	60	91
Adult Day 3	69	96

Table 2-7 Genes with significant alternative splicing between normal and still hybrids that are uniquely regulated in temporal expression patterns in the normal phenotype.

Expression Pattern	Gene accession
UP-UP-UP	TC002719, TC002806, TC003095, TC006316, TC006502, TC006721, TC007207, TC007493, TC008268, TC011384, TC012026, TC012051, TC012053, TC012317, TC014458, TC014768, TC015757, TC015772
UP-UP-DOWN	TC004471, TC014795
UP-DOWN-DOWN	TC002128, TC013023, TC014861
DOWN-DOWN-DOWN	TC007655, TC012717
DOWN-UP-DOWN	TC002023, TC004472, TC012017, TC012404, TC013124, TC016117

Table 2-8 Genes with significant alternative splicing between normal and still hybrids that are uniquely regulated in temporal expression patterns in still

Expression Pattern	Gene accession
UP-DOWN-UP	TC005732, TC009720, TC012317, TC013105, TC016090

UP-DOWN-DOWN	TC004569, TC010280, TC012885
DOWN-UP-UP	TC005444
DOWN-UP-DOWN	TC009090, TC010518, TC015161
DOWN-DOWN-UP	TC002512, TC002702, TC005573, TC005661, TC005776, TC013289, TC015550
DOWN-DOWN-DOWN	TC000100, TC000314, TC003811, TC005866

Table 2-9 Genes with significant alternative splicing and differential expression in between-condition and within-condition treatments. Expression pattern is listed by within-condition temporal patterns. Gene identifier is given along with GO term for Molecular Function. Fold change is averaged across time points with respect to still phenotype.

Expression Pattern	Gene Accession and GO term	Raw Fold Change
UP-UP-UP	TC006608 -- G-protein coupled acetylcholine receptor signaling pathway	-1.92X
UP-DOWN-UP	TC015815 -- nucleotide-excision repair, DNA gap filling TC014631 -- cGMP-mediated signaling TC000119 -- cell projection organization	-3.82X -6.34X -5.58X
DOWN-DOWN-DOWN	TC015915 -- axon guidance	1.22X
DOWN-DOWN-UP	TC009611 -- organic cation transport	-2.83X
DOWN-UP-DOWN	TC010952 -- oxidation-reduction process	0.66X
DOWN-UP-UP	TC007131 -- regulation of exocytosis	-3.98X

Discussion

Allele biased expression

Genes with significant allele bias in the normal phenotype represent a control against which allele bias can be measured in still hybrids. Nine genes (Table 2-1) have allele bias exclusive to normal hybrids and show differential expression in every time point, suggesting that the absence of this pattern in “still” suggests suppression of allele bias in favor of whole transcript down-regulation in still hybrids and may be due to a trans-acting source of regulation as part of a systemic response. From these 9 genes, GO terms for Molecular Function include regulation of DNA replication, nucleosome assembly, and microtubule severing. For allele-biased genes exclusive to still hybrids, protein-protein interactions were found only for genes exhibiting paternal (Tanzania) bias in the STRING database as shown in Figure 2-2. where all genes listed per timepoint interact directly with the potential to have unique downstream effects in the larger regulatory network. PFAM domains for interactions include developmental transcription factors, mismatch repair, and modifiers of chromosome structure modifiers (Figure 2-2). An interesting avenue for further study is the shift from the roughly equal

sex distribution of allele bias in normal hybrids (32 maternal, 30 paternal) to the clear paternal bias seen in still (9 paternal, 2 maternal).

Alternative splicing

Roughly four times as many genes are alternatively spliced between normal and still in the adult stages when contrasted with pupa (Table 2-6). Table 2-7 demonstrates that 18 genes alternatively spliced and upregulated uniquely in normal hybrids across the normal course of development are missing in still, hinting at a potential cellular response acting in *trans*. Notably, genes with alternative splicing that exhibit specificity to the normal phenotype for allele bias, upregulation at each time stage, and between-condition differential expression possess enrichment for GO terms (Molecular Function) for DNA replication, nucleosome assembly, and microtubule severing. These are the same terms that overlap each time stage for normal-specific allele bias, which is congruent with Graze et al. (2012) wherein 30% of genes with allele bias also undergo alternative splicing. Since the genotypes of normal and still hybrids differ only by alleles segregating in parent populations, this gives further evidence that allele bias in “still” could be reduced by trans-acting mechanism in response to a stressful cellular environment. Still-specific alternative splicing that undergoes temporal regulation could correlate to such a signaling response. To this end, a reduced list of genes that meet stringent criteria for alternative splicing, within- and between-condition differential expression only in still hybrids shows several possible stress signalling pathways, including neurotransmitter receptors, DNA repair, cGMP-mediated signaling, axon guidance, and oxidative phosphorylation.

Future directions

The still phenotype exhibits widespread gene expression misregulation (Table 1-1, Table 1-2). Unpublished data suggests variation at the *dld* locus as the driver of the still phenotype in response to different exposure of the parent populations to the phosphine fumigant. Additionally, the transcript LOC103314140 (TC004024) shown in Oppert et al. (2015) to have the highest expression differences between resistant and susceptible phosphine populations of *T. castaneum* also shows the highest magnitude of upregulation in still hybrids (Table 1-3). These two loci represent the strongest candidates for future functional experiments such as RNA interference while demonstrating the increased power of intersecting many analyses to reduce candidate gene lists.

Appendix A

Genes differentially expressed across time course in normal hybrids

Appendix A - Genes differentially expressed across time course in normal hybrids

Expression Pattern	GO Term	GO Function	GO Category	FDR	# genes
UP-UP-UP	GO:0098660	inorganic ion transmembrane transport	Biological Process	2.70E-06	68
UP-UP-UP	GO:0015992	proton transport	Biological Process	2.70E-06	32
UP-UP-UP	GO:0030552	cAMP binding	Molecular Function	1.00E-05	14
UP-UP-UP	GO:0005222	intracellular cAMP activated cation channel activity	Molecular Function	1.00E-05	10
UP-UP-UP	GO:0035725	sodium ion transmembrane transport	Biological Process	6.00E-05	31
UP-UP-UP	GO:0005351	sugar:proton symporter activity	Molecular Function	9.50E-05	14

UP-UP-UP	GO:0046549	retinal cone cell development	Biological Process	1.40E-04	10
UP-UP-UP	GO:0015296	anion:cation symporter activity	Molecular Function	2.60E-04	16
UP-UP-UP	GO:0009117	nucleotide metabolic process	Biological Process	2.90E-04	76
UP-UP-UP	GO:0019693	ribose phosphate metabolic process	Biological Process	3.40E-04	63
UP-UP-UP	GO:0035435	phosphate ion transmembrane transport	Biological Process	4.20E-04	12
UP-UP-UP	GO:0005316	high-affinity inorganic phosphate:sodium symporter activity	Molecular Function	4.40E-04	10
UP-UP-UP	GO:0031514	motile cilium	Cellular Component	5.80E-04	18
UP-UP-UP	GO:0071321	cellular response to cGMP	Biological Process	6.50E-04	9
UP-UP-UP	GO:0009150	purine ribonucleotide metabolic process	Biological Process	8.80E-04	56
UP-UP-UP	GO:0015136	sialic acid transmembrane transporter activity	Molecular Function	8.80E-04	10
UP-UP-UP	GO:0015739	sialic acid transport	Biological Process	8.80E-04	10
UP-UP-UP	GO:0030527	structural constituent of chromatin	Molecular Function	1.80E-03	4
UP-UP-UP	GO:1990204	oxidoreductase complex	Cellular Component	2.80E-03	18
UP-UP-UP	GO:0004016	adenylate cyclase activity	Molecular Function	3.40E-03	9
UP-UP-UP	GO:0005930	axoneme	Cellular Component	4.90E-03	17
UP-UP-UP	GO:0030505	inorganic diphosphate transport	Biological Process	6.50E-03	4
UP-UP-UP	GO:0030504	inorganic diphosphate transmembrane transporter activity	Molecular Function	6.50E-03	4
UP-UP-UP	GO:0016491	oxidoreductase activity	Molecular Function	7.70E-03	87
UP-UP-UP	GO:0005329	dopamine transmembrane transporter activity	Molecular Function	9.30E-03	7
UP-UP-UP	GO:0005313	L-glutamate transmembrane	Molecular	1.10E-	10

		transporter activity	Function	02	
UP-UP-UP	GO:0098800	inner mitochondrial membrane protein complex	Cellular Component	1.20E-02	18
UP-UP-UP	GO:0022027	interkinetic nuclear migration	Biological Process	1.20E-02	5
UP-UP-UP	GO:0005272	sodium channel activity	Molecular Function	1.30E-02	12
UP-UP-UP	GO:0003341	cilium movement	Biological Process	1.50E-02	13
UP-UP-UP	GO:0097588	archaeal or bacterial-type flagellum-dependent cell motility	Biological Process	1.60E-02	4
UP-UP-UP	GO:0015813	L-glutamate transport	Biological Process	1.80E-02	10
UP-UP-UP	GO:0022904	respiratory electron transport chain	Biological Process	1.80E-02	17
UP-UP-UP	GO:0004022	alcohol dehydrogenase (NAD) activity	Molecular Function	2.00E-02	5
UP-UP-UP	GO:0005874	microtubule	Cellular Component	2.20E-02	37
UP-UP-UP	GO:0070286	axonemal dynein complex assembly	Biological Process	2.20E-02	8
UP-UP-UP	GO:0042775	mitochondrial ATP synthesis coupled electron transport	Biological Process	2.50E-02	12
UP-UP-UP	GO:0098803	respiratory chain complex	Cellular Component	2.60E-02	13
UP-UP-UP	GO:0005333	norepinephrine transmembrane transporter activity	Molecular Function	2.70E-02	6
UP-UP-UP	GO:0030672	synaptic vesicle membrane	Cellular Component	2.90E-02	15
UP-UP-UP	GO:0051591	response to cAMP	Biological Process	3.10E-02	17
UP-UP-UP	GO:1903825	organic acid transmembrane transport	Biological Process	3.20E-02	19
UP-UP-UP	GO:0030819	positive regulation of cAMP biosynthetic process	Biological Process	3.40E-02	14
UP-UP-UP	GO:0097223	sperm part	Cellular Component	3.40E-02	19

UP-UP-UP	GO:0009624	response to nematode	Biological Process	3.40E-02	6
UP-UP-UP	GO:0042136	neurotransmitter biosynthetic process	Biological Process	3.70E-02	8
UP-UP-UP	GO:0005746	mitochondrial respiratory chain	Cellular Component	3.80E-02	13
UP-UP-UP	GO:0007286	spermatid development	Biological Process	4.70E-02	23
UP-UP-DOWN	GO:0006278	RNA-dependent DNA replication	Biological Process	1.40E-03	21
UP-UP-DOWN	GO:0003964	RNA-directed DNA polymerase activity	Molecular Function	1.40E-03	20
UP-UP-DOWN	GO:0004519	endonuclease activity	Molecular Function	1.40E-03	24
UP-UP-DOWN	GO:0090305	nucleic acid phosphodiester bond hydrolysis	Biological Process	1.30E-02	26
UP-UP-DOWN	GO:0032197	transposition, RNA-mediated	Biological Process	2.30E-02	9
UP-DOWN-UP	GO:0006805	xenobiotic metabolic process	Biological Process	1.30E-02	13
UP-DOWN-DOWN	GO:0042302	structural constituent of cuticle	Molecular Function	1.30E-09	22
UP-DOWN-DOWN	GO:0005615	extracellular space	Cellular Component	3.60E-07	61
UP-DOWN-DOWN	GO:0001523	retinoid metabolic process	Biological Process	8.90E-03	13
UP-DOWN-DOWN	GO:0020028	hemoglobin import	Biological Process	9.90E-03	6
UP-DOWN-DOWN	GO:0030492	hemoglobin binding	Molecular Function	9.90E-03	6
UP-DOWN-DOWN	GO:0042953	lipoprotein transport	Biological Process	1.10E-02	8
UP-DOWN-DOWN	GO:0030036	actin cytoskeleton organization	Biological Process	1.40E-02	37
UP-DOWN-DOWN	GO:0038024	cargo receptor activity	Molecular Function	1.60E-02	12
UP-DOWN-DOWN	GO:0043025	neuronal cell body	Cellular Component	1.90E-02	33
UP-DOWN-DOWN	GO:0004252	serine-type endopeptidase activity	Molecular Function	3.30E-02	17
UP-DOWN-	GO:0071409	cellular response to	Biological	3.40E-	3

DOWN		cycloheximide	Process	02	
UP-DOWN-DOWN	GO:0071813	lipoprotein particle binding	Molecular Function	3.40E-02	7
UP-DOWN-DOWN	GO:0005041	low-density lipoprotein receptor activity	Molecular Function	3.50E-02	6
UP-DOWN-DOWN	GO:0042954	lipoprotein transporter activity	Molecular Function	3.50E-02	5
UP-DOWN-DOWN	GO:0005578	proteinaceous extracellular matrix	Cellular Component	3.60E-02	20
DOWN-DOWN-DOWN	GO:0048863	stem cell differentiation	Biological Process	5.70E-06	24
DOWN-DOWN-DOWN	GO:0007155	cell adhesion	Biological Process	5.60E-05	45
DOWN-DOWN-DOWN	GO:2000027	regulation of organ morphogenesis	Biological Process	1.30E-04	19
DOWN-DOWN-DOWN	GO:0060021	palate development	Biological Process	1.80E-04	12
DOWN-DOWN-DOWN	GO:0001709	cell fate determination	Biological Process	2.00E-04	17
DOWN-DOWN-DOWN	GO:0007389	pattern specification process	Biological Process	3.20E-04	35
DOWN-DOWN-DOWN	GO:0051241	negative regulation of multicellular organismal process	Biological Process	7.20E-04	40
DOWN-DOWN-DOWN	GO:0051795	positive regulation of catagen	Biological Process	7.30E-04	3
DOWN-DOWN-DOWN	GO:0009953	dorsal/ventral pattern formation	Biological Process	7.30E-04	17
DOWN-DOWN-DOWN	GO:0048705	skeletal system morphogenesis	Biological Process	7.50E-04	17
DOWN-DOWN-DOWN	GO:0042659	regulation of cell fate specification	Biological Process	1.40E-03	10

DOWN-DOWN-DOWN	GO:0061180	mammary gland epithelium development	Biological Process	1.40E-03	10
DOWN-DOWN-DOWN	GO:0030198	extracellular matrix organization	Biological Process	1.40E-03	20
DOWN-DOWN-DOWN	GO:2000678	negative regulation of transcription regulatory region DNA binding	Biological Process	1.40E-03	5
DOWN-DOWN-DOWN	GO:0003203	endocardial cushion morphogenesis	Biological Process	1.60E-03	6
DOWN-DOWN-DOWN	GO:0003198	epithelial to mesenchymal transition involved in endocardial cushion formation	Biological Process	1.70E-03	5
DOWN-DOWN-DOWN	GO:0043010	camera-type eye development	Biological Process	1.80E-03	19
DOWN-DOWN-DOWN	GO:0002011	morphogenesis of an epithelial sheet	Biological Process	2.00E-03	9
DOWN-DOWN-DOWN	GO:0060536	cartilage morphogenesis	Biological Process	2.00E-03	5
DOWN-DOWN-DOWN	GO:0035880	embryonic nail plate morphogenesis	Biological Process	2.00E-03	3
DOWN-DOWN-DOWN	GO:2001055	positive regulation of mesenchymal cell apoptotic process	Biological Process	2.00E-03	3
DOWN-DOWN-DOWN	GO:0007400	neuroblast fate determination	Biological Process	2.70E-03	7
DOWN-DOWN-DOWN	GO:0042330	taxis	Biological Process	2.70E-03	32
DOWN-DOWN-DOWN	GO:0007411	axon guidance	Biological Process	3.40E-03	26
DOWN-DOWN-DOWN	GO:0042476	odontogenesis	Biological Process	3.50E-03	11

DOWN-DOWN-DOWN	GO:0045892	negative regulation of transcription, DNA-templated	Biological Process	3.50E-03	36
DOWN-DOWN-DOWN	GO:0022612	gland morphogenesis	Biological Process	3.90E-03	13
DOWN-DOWN-DOWN	GO:0010463	mesenchymal cell proliferation	Biological Process	4.20E-03	7
DOWN-DOWN-DOWN	GO:0060102	collagen and cuticulin-based cuticle extracellular matrix	Cellular Component	4.40E-03	4
DOWN-DOWN-DOWN	GO:0030857	negative regulation of epithelial cell differentiation	Biological Process	5.00E-03	6
DOWN-DOWN-DOWN	GO:0042640	anagen	Biological Process	5.50E-03	4
DOWN-DOWN-DOWN	GO:0021536	diencephalon development	Biological Process	5.60E-03	10
DOWN-DOWN-DOWN	GO:0010557	positive regulation of macromolecule biosynthetic process	Biological Process	5.60E-03	42
DOWN-DOWN-DOWN	GO:0043565	sequence-specific DNA binding	Molecular Function	5.60E-03	26
DOWN-DOWN-DOWN	GO:0060364	frontal suture morphogenesis	Biological Process	6.00E-03	3
DOWN-DOWN-DOWN	GO:0032792	negative regulation of CREB transcription factor activity	Biological Process	6.00E-03	3
DOWN-DOWN-DOWN	GO:0090427	activation of meiosis	Biological Process	6.00E-03	3

DOWN-DOWN-DOWN	GO:0005616	larval serum protein complex	Cellular Component	6.00E-03	3
DOWN-DOWN-DOWN	GO:0007398	ectoderm development	Biological Process	6.50E-03	8
DOWN-DOWN-DOWN	GO:0051254	positive regulation of RNA metabolic process	Biological Process	7.60E-03	40
DOWN-DOWN-DOWN	GO:0048754	branching morphogenesis of an epithelial tube	Biological Process	8.30E-03	16
DOWN-DOWN-DOWN	GO:0060828	regulation of canonical Wnt signaling pathway	Biological Process	8.30E-03	12
DOWN-DOWN-DOWN	GO:0035116	embryonic hindlimb morphogenesis	Biological Process	8.30E-03	6
DOWN-DOWN-DOWN	GO:0045599	negative regulation of fat cell differentiation	Biological Process	8.30E-03	6
DOWN-DOWN-DOWN	GO:0035115	embryonic forelimb morphogenesis	Biological Process	9.20E-03	6
DOWN-DOWN-DOWN	GO:0000982	transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific binding	Molecular Function	9.30E-03	12
DOWN-DOWN-DOWN	GO:0071300	cellular response to retinoic acid	Biological Process	9.40E-03	7
DOWN-DOWN-DOWN	GO:0021953	central nervous system neuron differentiation	Biological Process	9.90E-03	14
DOWN-DOWN-DOWN	GO:0090287	regulation of cellular response to growth factor stimulus	Biological Process	1.10E-02	14
DOWN-DOWN-DOWN	GO:0001667	ameboidal-type cell migration	Biological Process	1.10E-02	19

DOWN-DOWN-DOWN	GO:0003416	endochondral bone growth	Biological Process	1.20E-02	4
DOWN-DOWN-DOWN	GO:0045597	positive regulation of cell differentiation	Biological Process	1.20E-02	26
DOWN-DOWN-DOWN	GO:0043069	negative regulation of programmed cell death	Biological Process	1.20E-02	29
DOWN-DOWN-DOWN	GO:0045893	positive regulation of transcription, DNA-templated	Biological Process	1.30E-02	38
DOWN-DOWN-DOWN	GO:0005788	endoplasmic reticulum lumen	Cellular Component	1.30E-02	10
DOWN-DOWN-DOWN	GO:0042973	glucan endo-1,3-beta-D-glucosidase activity	Molecular Function	1.30E-02	3
DOWN-DOWN-DOWN	GO:0070166	enamel mineralization	Biological Process	1.30E-02	3
DOWN-DOWN-DOWN	GO:0019863	IgE binding	Molecular Function	1.30E-02	3
DOWN-DOWN-DOWN	GO:0031540	regulation of anthocyanin biosynthetic process	Biological Process	1.30E-02	3
DOWN-DOWN-DOWN	GO:0007419	ventral cord development	Biological Process	1.30E-02	7

DOWN-DOWN-DOWN	GO:0003148	outflow tract septum morphogenesis	Biological Process	1.30E-02	4
DOWN-DOWN-DOWN	GO:0060561	apoptotic process involved in morphogenesis	Biological Process	1.70E-02	6
DOWN-DOWN-DOWN	GO:0042246	tissue regeneration	Biological Process	1.70E-02	7
DOWN-DOWN-DOWN	GO:0042089	cytokine biosynthetic process	Biological Process	1.70E-02	7
DOWN-DOWN-DOWN	GO:0045667	regulation of osteoblast differentiation	Biological Process	1.70E-02	9
DOWN-DOWN-DOWN	GO:0021852	pyramidal neuron migration	Biological Process	1.70E-02	2

DOWN-DOWN-DOWN	GO:1904238	pericyte cell differentiation	Biological Process	1.70E-02	3
DOWN-DOWN-DOWN	GO:0072143	mesangial cell development	Biological Process	1.70E-02	3
DOWN-DOWN-DOWN	GO:0071392	cellular response to estradiol stimulus	Biological Process	1.80E-02	4
DOWN-DOWN-DOWN	GO:0051962	positive regulation of nervous system development	Biological Process	1.80E-02	19
DOWN-DOWN-DOWN	GO:0010812	negative regulation of cell-substrate adhesion	Biological Process	2.00E-02	6
DOWN-DOWN-DOWN	GO:0035313	wound healing, spreading of epidermal cells	Biological Process	2.10E-02	4
DOWN-DOWN-DOWN	GO:0043009	chordate embryonic development	Biological Process	2.20E-02	27
DOWN-DOWN-DOWN	GO:0045617	negative regulation of keratinocyte differentiation	Biological Process	2.30E-02	3
DOWN-DOWN-DOWN	GO:0016201	synaptic target inhibition	Biological Process	2.40E-02	4
DOWN-DOWN-DOWN	GO:0008285	negative regulation of cell proliferation	Biological Process	2.40E-02	23
DOWN-DOWN-DOWN	GO:0007498	mesoderm development	Biological Process	2.40E-02	13
DOWN-DOWN-DOWN	GO:0001738	morphogenesis of a polarized epithelium	Biological Process	2.50E-02	11
DOWN-DOWN-DOWN	GO:0048839	inner ear development	Biological Process	2.70E-02	13
DOWN-DOWN-DOWN	GO:0003208	cardiac ventricle morphogenesis	Biological Process	2.70E-02	7

DOWN-DOWN-DOWN	GO:0003705	transcription factor activity, RNA polymerase II distal enhancer sequence-specific binding	Molecular Function	2.70E-02	9
DOWN-DOWN-DOWN	GO:0009649	entrainment of circadian clock	Biological Process	2.80E-02	5

DOWN-DOWN-DOWN	GO:0014033	neural crest cell differentiation	Biological Process	2.80E-02	7
DOWN-DOWN-DOWN	GO:0007517	muscle organ development	Biological Process	2.80E-02	19
DOWN-DOWN-DOWN	GO:0002062	chondrocyte differentiation	Biological Process	2.90E-02	8
DOWN-DOWN-DOWN	GO:0090100	positive regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	Biological Process	3.40E-02	7
DOWN-DOWN-DOWN	GO:0007548	sex differentiation	Biological Process	3.50E-02	23
DOWN-DOWN-DOWN	GO:0061312	BMP signaling pathway involved in heart development	Biological Process	3.70E-02	3
DOWN-DOWN-DOWN	GO:0048771	tissue remodeling	Biological Process	3.70E-02	10
DOWN-DOWN-DOWN	GO:0014834	skeletal muscle satellite cell maintenance involved in skeletal muscle regeneration	Biological Process	3.90E-02	2
DOWN-DOWN-DOWN	GO:0060788	ectodermal placode formation	Biological Process	4.10E-02	5
DOWN-DOWN-DOWN	GO:0021772	olfactory bulb development	Biological Process	4.10E-02	7
DOWN-DOWN-DOWN	GO:0031640	killing of cells of other organism	Biological Process	4.40E-02	6
DOWN-DOWN-DOWN	GO:0045665	negative regulation of neuron differentiation	Biological Process	4.40E-02	13
DOWN-DOWN-DOWN	GO:0048514	blood vessel morphogenesis	Biological Process	4.40E-02	19
DOWN-DOWN-DOWN	GO:0072141	renal interstitial fibroblast development	Biological Process	4.40E-02	3

DOWN-DOWN-DOWN	GO:2000177	regulation of neural precursor cell proliferation	Biological Process	4.50E-02	8
DOWN-DOWN-DOWN	GO:0042706	eye photoreceptor cell fate commitment	Biological Process	4.50E-02	8
DOWN-DOWN-DOWN	GO:2000648	positive regulation of stem cell proliferation	Biological Process	4.60E-02	7
DOWN-DOWN-DOWN	GO:0042471	ear morphogenesis	Biological Process	4.70E-02	10
DOWN-DOWN-DOWN	GO:0030324	lung development	Biological Process	5.00E-02	12
DOWN-DOWN-UP	GO:0035270	endocrine system development	Biological Process	3.70E-02	12
DOWN-UP-DOWN	GO:0042381	hemolymph coagulation	Biological Process	1.50E-03	10
DOWN-UP-DOWN	GO:2000377	regulation of reactive oxygen species metabolic process	Biological Process	3.60E-03	15
DOWN-UP-DOWN	GO:0030193	regulation of blood coagulation	Biological Process	4.40E-03	15
DOWN-UP-DOWN	GO:0031639	plasminogen activation	Biological Process	9.20E-03	10
DOWN-UP-DOWN	GO:0060548	negative regulation of cell death	Biological Process	1.10E-02	43
DOWN-UP-DOWN	GO:0046909	intermembrane transport	Biological Process	1.20E-02	6
DOWN-UP-DOWN	GO:0042360	vitamin E metabolic process	Biological Process	1.30E-02	6

DOWN-UP-DOWN	GO:0090212	negative regulation of establishment of blood-brain barrier	Biological Process	1.30E-02	6
DOWN-UP-DOWN	GO:0008431	vitamin E binding	Molecular Function	1.30E-02	6
DOWN-UP-DOWN	GO:0051919	positive regulation of fibrinolysis	Biological Process	1.60E-02	9
DOWN-UP-DOWN	GO:0006885	regulation of pH	Biological Process	3.30E-02	9
DOWN-UP-DOWN	GO:0031347	regulation of defense response	Biological Process	3.30E-02	27
DOWN-UP-DOWN	GO:2000121	regulation of removal of superoxide radicals	Biological Process	3.50E-02	4
DOWN-UP-DOWN	GO:0017171	serine hydrolase activity	Molecular Function	4.60E-02	17
DOWN-UP-DOWN	GO:0007040	lysosome organization	Biological Process	4.80E-02	10
DOWN-UP-DOWN	GO:0006954	inflammatory response	Biological Process	4.80E-02	25
DOWN-UP-DOWN	GO:0080025	phosphatidylinositol-3,5-bisphosphate binding	Molecular Function	4.90E-02	7

DOWN-UP-UP	GO:0000070	mitotic sister chromatid segregation	Biological Process	5.00E-04	16
DOWN-UP-UP	GO:0031616	spindle pole centrosome	Cellular Component	6.50E-04	6
DOWN-UP-UP	GO:0051304	chromosome separation	Biological Process	1.90E-03	12
DOWN-UP-UP	GO:0032506	cytokinetic process	Biological Process	2.40E-03	12
DOWN-UP-UP	GO:0051256	mitotic spindle midzone assembly	Biological Process	2.50E-03	5
DOWN-UP-UP	GO:0005828	kinetochore microtubule	Cellular Component	7.30E-03	5
DOWN-UP-UP	GO:0051984	positive regulation of chromosome segregation	Biological Process	8.50E-03	6
DOWN-UP-UP	GO:0010075	regulation of meristem growth	Biological Process	8.50E-03	5
DOWN-UP-UP	GO:0033597	mitotic checkpoint complex	Cellular Component	8.90E-03	3
DOWN-UP-UP	GO:0000942	condensed nuclear chromosome outer kinetochore	Cellular Component	8.90E-03	3
DOWN-UP-UP	GO:0008145	phenylalkylamine binding	Molecular Function	9.20E-03	6
DOWN-UP-UP	GO:0060236	regulation of mitotic spindle organization	Biological Process	9.80E-03	5
DOWN-UP-UP	GO:0005550	pheromone binding	Molecular Function	1.20E-02	7
DOWN-UP-UP	GO:0046701	insecticide catabolic process	Biological Process	1.40E-02	5
DOWN-UP-UP	GO:0005576	extracellular region	Cellular Component	1.40E-02	86
DOWN-UP-UP	GO:0006270	DNA replication initiation	Biological Process	1.90E-02	8
DOWN-UP-UP	GO:0052689	carboxylic ester hydrolase activity	Molecular Function	1.90E-02	13
DOWN-UP-UP	GO:0071174	mitotic spindle checkpoint	Biological Process	2.00E-02	7
DOWN-UP-UP	GO:0030261	chromosome condensation	Biological Process	2.00E-02	9
DOWN-UP-UP	GO:0008608	attachment of spindle microtubules to kinetochore	Biological Process	2.30E-02	5
DOWN-UP-	GO:0045840	positive regulation of mitotic	Biological	2.40E-	7

UP		nuclear division	Process	02	
DOWN-UP-UP	GO:0045842	positive regulation of mitotic metaphase/anaphase transition	Biological Process	2.60E-02	5
DOWN-UP-UP	GO:0001556	oocyte maturation	Biological Process	3.10E-02	6
DOWN-UP-UP	GO:1901136	carbohydrate derivative catabolic process	Biological Process	3.10E-02	14
DOWN-UP-UP	GO:0006267	pre-replicative complex assembly involved in nuclear cell cycle DNA replication	Biological Process	3.10E-02	4
DOWN-UP-UP	GO:0042555	MCM complex	Cellular Component	3.10E-02	4
DOWN-UP-UP	GO:0006271	DNA strand elongation involved in DNA replication	Biological Process	3.20E-02	6
DOWN-UP-UP	GO:0005656	nuclear pre-replicative complex	Cellular Component	3.60E-02	4
DOWN-UP-UP	GO:0007019	microtubule depolymerization	Biological Process	3.80E-02	7
DOWN-UP-UP	GO:0005818	aster	Cellular Component	4.30E-02	4
DOWN-UP-UP	GO:0000911	cytokinesis by cell plate formation	Biological Process	4.70E-02	7
DOWN-UP-UP	GO:0043203	axon hillock	Cellular Component	4.90E-02	3
DOWN-UP-UP	GO:0010997	anaphase-promoting complex binding	Molecular Function	4.90E-02	3
DOWN-UP-UP	GO:0043146	spindle stabilization	Biological Process	4.90E-02	4
DOWN-UP-UP	GO:0043142	single-stranded DNA-dependent ATPase activity	Molecular Function	4.90E-02	4

Appendix B

Genes differentially expressed across time course in still hybrids

Appendix B - Genes differentially expressed across time course in still hybrids

Expression Pattern	GO Term	Name	Type	FDR	# in test group
UP-UP-UP	GO:0072350	tricarboxylic acid metabolic process	Biological Process	3.50E-06	10
UP-UP-UP	GO:0006119	oxidative phosphorylation	Biological Process	6.70E-06	11
UP-UP-UP	GO:0006099	tricarboxylic acid cycle	Biological Process	7.20E-06	9
UP-UP-UP	GO:0006120	mitochondrial electron transport, NADH to ubiquinone	Biological Process	1.70E-05	8
UP-UP-UP	GO:0044712	single-organism catabolic process	Biological Process	5.80E-05	39
UP-UP-UP	GO:0045239	tricarboxylic acid cycle enzyme complex	Cellular Component	3.50E-04	5
UP-UP-UP	GO:0006103	2-oxoglutarate metabolic process	Biological Process	3.90E-04	6
UP-UP-UP	GO:0045254	pyruvate dehydrogenase complex	Cellular Component	3.90E-04	4
UP-UP-UP	GO:1901565	organonitrogen compound catabolic process	Biological Process	4.50E-04	20
UP-UP-UP	GO:0051068	dihydrolipoamide metabolic process	Biological Process	4.80E-04	3
UP-UP-UP	GO:0004148	dihydrolipoyl dehydrogenase activity	Molecular Function	4.80E-04	3

UP-UP-UP	GO:0043544	lipoamide binding	Molecular Function	4.80E-04	3
UP-UP-UP	GO:0010510	regulation of acetyl-CoA biosynthetic process from pyruvate	Biological Process	2.00E-03	4
UP-UP-UP	GO:0005759	mitochondrial matrix	Cellular Component	2.20E-03	16
UP-UP-UP	GO:0043159	acrosomal matrix	Cellular Component	3.60E-03	3
UP-UP-UP	GO:0009106	lipoate metabolic process	Biological Process	3.60E-03	4
UP-UP-UP	GO:0045252	oxoglutarate dehydrogenase complex	Cellular Component	6.80E-03	3
UP-UP-UP	GO:1902600	hydrogen ion transmembrane transport	Biological Process	9.00E-03	8
UP-UP-UP	GO:0009152	purine ribonucleotide biosynthetic process	Biological Process	1.20E-02	13
UP-UP-UP	GO:0015078	hydrogen ion transmembrane transporter activity	Molecular Function	1.70E-02	8
UP-UP-UP	GO:0048037	cofactor binding	Molecular Function	1.70E-02	15
UP-UP-UP	GO:0007586	digestion	Biological Process	1.70E-02	14
UP-UP-UP	GO:0016645	oxidoreductase activity, acting on the CH-NH group of donors	Molecular Function	1.80E-02	5
UP-UP-UP	GO:0016655	oxidoreductase activity, acting on NAD(P)H, quinone or similar compound as acceptor	Molecular Function	1.80E-02	6
UP-UP-UP	GO:0007211	octopamine or tyramine signaling pathway	Biological Process	2.50E-02	2
UP-UP-UP	GO:0005747	mitochondrial respiratory chain complex I	Cellular Component	3.30E-02	5
UP-UP-UP	GO:0008137	NADH dehydrogenase (ubiquinone) activity	Molecular Function	3.30E-02	5
UP-UP-UP	GO:0009055	electron carrier activity	Molecular Function	3.40E-02	11
UP-UP-UP	GO:0019362	pyridine nucleotide metabolic process	Biological Process	3.50E-02	10
UP-UP-UP	GO:0046697	decidualization	Biological Process	4.10E-02	4
UP-UP-UP	GO:0006561	proline biosynthetic process	Biological	4.40E-	3

			Process	02	
UP-UP-UP	GO:0006554	lysine catabolic process	Biological Process	4.40E-02	3
UP-UP-UP	GO:0016540	protein autoprocessing	Biological Process	4.90E-02	4
UP-DOWN-UP	GO:0005887	integral component of plasma membrane	Cellular Component	2.80E-07	130
UP-DOWN-UP	GO:0031514	motile cilium	Cellular Component	8.50E-05	21
UP-DOWN-UP	GO:0030424	axon	Cellular Component	1.30E-04	81
UP-DOWN-UP	GO:0034702	ion channel complex	Cellular Component	3.80E-04	38
UP-DOWN-UP	GO:0043025	neuronal cell body	Cellular Component	8.20E-04	71
UP-DOWN-UP	GO:0036064	ciliary basal body	Cellular Component	8.20E-04	17
UP-DOWN-UP	GO:0001539	cilium or flagellum-dependent cell motility	Biological Process	1.00E-03	13
UP-DOWN-UP	GO:0015672	monovalent inorganic cation transport	Biological Process	1.00E-03	73
UP-DOWN-UP	GO:1901338	catecholamine binding	Molecular Function	3.00E-03	8
UP-DOWN-UP	GO:0008038	neuron recognition	Biological Process	3.60E-03	33
UP-DOWN-UP	GO:0003341	cilium movement	Biological Process	4.00E-03	15
UP-DOWN-UP	GO:0046873	metal ion transmembrane transporter activity	Molecular Function	4.00E-03	44
UP-DOWN-UP	GO:0031513	nonmotile primary cilium	Cellular Component	4.30E-03	24
UP-DOWN-UP	GO:0005858	axonemal dynein complex	Cellular Component	4.40E-03	9
UP-DOWN-UP	GO:0003073	regulation of systemic arterial blood pressure	Biological Process	5.40E-03	22
UP-DOWN-UP	GO:0006936	muscle contraction	Biological Process	5.40E-03	44
UP-DOWN-UP	GO:0007158	neuron cell-cell adhesion	Biological Process	5.80E-03	11

UP-DOWN-UP	GO:0030001	metal ion transport	Biological Process	5.80E-03	74
UP-DOWN-UP	GO:0009694	jasmonic acid metabolic process	Biological Process	6.90E-03	9
UP-DOWN-UP	GO:0009986	cell surface	Cellular Component	7.40E-03	80
UP-DOWN-UP	GO:0010817	regulation of hormone levels	Biological Process	1.10E-02	65
UP-DOWN-UP	GO:0030060	L-malate dehydrogenase activity	Molecular Function	1.10E-02	5
UP-DOWN-UP	GO:0030992	intraciliary transport particle B	Cellular Component	1.10E-02	6
UP-DOWN-UP	GO:0015276	ligand-gated ion channel activity	Molecular Function	1.20E-02	24
UP-DOWN-UP	GO:0043235	receptor complex	Cellular Component	1.30E-02	36
UP-DOWN-UP	GO:0042734	presynaptic membrane	Cellular Component	1.40E-02	22
UP-DOWN-UP	GO:0009514	glyoxysome	Cellular Component	1.80E-02	5
UP-DOWN-UP	GO:0003777	microtubule motor activity	Molecular Function	1.80E-02	16
UP-DOWN-UP	GO:0042391	regulation of membrane potential	Biological Process	2.10E-02	41
UP-DOWN-UP	GO:0005874	microtubule	Cellular Component	2.10E-02	40

UP-DOWN-UP	GO:0030817	regulation of cAMP biosynthetic process	Biological Process	2.10E-02	20
UP-DOWN-UP	GO:0030594	neurotransmitter receptor activity	Molecular Function	2.30E-02	14
UP-DOWN-UP	GO:0035082	axoneme assembly	Biological Process	2.30E-02	12
UP-DOWN-UP	GO:0045211	postsynaptic membrane	Cellular Component	2.40E-02	39
UP-DOWN-UP	GO:0030054	cell junction	Cellular Component	2.40E-02	108
UP-DOWN-UP	GO:0060170	ciliary membrane	Cellular Component	2.50E-02	14
UP-DOWN-UP	GO:0051590	positive regulation of neurotransmitter transport	Biological Process	2.50E-02	10
UP-DOWN-UP	GO:0003025	regulation of systemic arterial blood pressure by baroreceptor feedback	Biological Process	2.60E-02	7
UP-DOWN-UP	GO:0016717	oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water	Molecular Function	2.60E-02	7
UP-DOWN-UP	GO:0070738	tubulin-glycine ligase activity	Molecular Function	2.60E-02	3
UP-DOWN-UP	GO:0009582	detection of abiotic stimulus	Biological Process	2.70E-02	36
UP-DOWN-UP	GO:1903522	regulation of blood circulation	Biological Process	2.80E-02	34
UP-DOWN-UP	GO:0051290	protein heterotetramerization	Biological Process	3.30E-02	11
UP-DOWN-UP	GO:0007409	axonogenesis	Biological Process	3.30E-02	84
UP-DOWN-UP	GO:0009581	detection of external stimulus	Biological Process	3.30E-02	35
UP-DOWN-UP	GO:0023061	signal release	Biological Process	3.30E-02	51
UP-DOWN-UP	GO:0031279	regulation of cyclase activity	Biological Process	3.30E-02	15
UP-DOWN-UP	GO:0022843	voltage-gated cation channel activity	Molecular Function	3.30E-02	21

UP-DOWN-UP	GO:0046928	regulation of neurotransmitter secretion	Biological Process	3.40E-02	18
UP-DOWN-UP	GO:0016247	channel regulator activity	Molecular Function	3.60E-02	23
UP-DOWN-UP	GO:0042446	hormone biosynthetic process	Biological Process	3.60E-02	23
UP-DOWN-UP	GO:0050680	negative regulation of epithelial cell proliferation	Biological Process	3.60E-02	19
UP-DOWN-UP	GO:0098662	inorganic cation transmembrane transport	Biological Process	3.60E-02	51
UP-DOWN-UP	GO:2000302	positive regulation of synaptic vesicle exocytosis	Biological Process	3.70E-02	6
UP-DOWN-UP	GO:0014069	postsynaptic density	Cellular Component	3.80E-02	29
UP-DOWN-UP	GO:0034765	regulation of ion transmembrane transport	Biological Process	3.80E-02	37
UP-DOWN-UP	GO:0051339	regulation of lyase activity	Biological Process	3.80E-02	15
UP-DOWN-UP	GO:0045161	neuronal ion channel clustering	Biological Process	3.80E-02	9
UP-DOWN-UP	GO:0003823	antigen binding	Molecular Function	4.10E-02	12
UP-DOWN-UP	GO:0043198	dendritic shaft	Cellular Component	4.20E-02	15
UP-DOWN-UP	GO:0045121	membrane raft	Cellular Component	4.30E-02	42
UP-DOWN-UP	GO:0060047	heart contraction	Biological Process	4.40E-02	33
UP-DOWN-UP	GO:0098742	cell-cell adhesion via plasma-membrane adhesion molecules	Biological Process	4.80E-02	25
UP-DOWN-UP	GO:0007588	excretion	Biological Process	4.90E-02	19
UP-DOWN-UP	GO:0006820	anion transport	Biological Process	4.90E-02	57
UP-DOWN-UP	GO:0090257	regulation of muscle system process	Biological Process	4.90E-02	30
UP-DOWN-UP	GO:0070405	ammonium ion binding	Molecular Function	4.90E-02	13
UP-DOWN-DOWN	GO:0032199	reverse transcription involved in RNA-mediated transposition	Biological Process	1.80E-02	9
UP-DOWN-	GO:0009036	Type II site-specific	Molecular	1.80E-	9

DOWN		deoxyribonuclease activity	Function	02	
DOWN-DOWN-DOWN	GO:0042302	structural constituent of cuticle	Molecular Function	6.80E-13	22
DOWN-DOWN-DOWN	GO:0003682	chromatin binding	Molecular Function	5.90E-04	28
DOWN-DOWN-DOWN	GO:0051276	chromosome organization	Biological Process	1.80E-03	42
DOWN-DOWN-DOWN	GO:0008010	structural constituent of chitin-based larval cuticle	Molecular Function	4.10E-03	8
DOWN-DOWN-DOWN	GO:0005654	nucleoplasm	Cellular Component	4.30E-03	45
DOWN-DOWN-DOWN	GO:0003677	DNA binding	Molecular Function	6.90E-03	56
DOWN-DOWN-DOWN	GO:0016568	chromatin modification	Biological Process	7.90E-03	29

DOWN-DOWN-DOWN	GO:0005694	chromosome	Cellular Component	1.20E-02	34
DOWN-DOWN-DOWN	GO:0008011	structural constituent of pupal chitin-based cuticle	Molecular Function	1.40E-02	6
DOWN-DOWN-DOWN	GO:0000792	heterochromatin	Cellular Component	1.50E-02	9
DOWN-DOWN-DOWN	GO:0001106	RNA polymerase II transcription corepressor activity	Molecular Function	4.80E-02	5
DOWN-DOWN-UP	GO:0005876	spindle microtubule	Cellular Component	2.60E-04	13
DOWN-DOWN-UP	GO:0031616	spindle pole centrosome	Cellular Component	1.40E-03	6
DOWN-DOWN-UP	GO:0032133	chromosome passenger complex	Cellular Component	2.00E-03	4
DOWN-DOWN-UP	GO:0005550	pheromone binding	Molecular Function	3.00E-03	9
DOWN-DOWN-UP	GO:0051276	chromosome organization	Biological Process	4.80E-03	54
DOWN-DOWN-UP	GO:0007059	chromosome segregation	Biological Process	5.10E-03	26
DOWN-DOWN-UP	GO:0000070	mitotic sister chromatid segregation	Biological Process	1.30E-02	15
DOWN-DOWN-UP	GO:0043138	3'-5' DNA helicase activity	Molecular Function	1.30E-02	6
DOWN-DOWN-UP	GO:0051302	regulation of cell division	Biological Process	1.70E-02	25
DOWN-DOWN-UP	GO:0016572	histone phosphorylation	Biological Process	2.40E-02	8
DOWN-DOWN-UP	GO:0000786	nucleosome	Cellular Component	2.50E-02	6
DOWN-DOWN-UP	GO:0043010	camera-type eye development	Biological Process	2.50E-02	24
DOWN-DOWN-UP	GO:0043146	spindle stabilization	Biological Process	2.60E-02	5
DOWN-DOWN-UP	GO:0006270	DNA replication initiation	Biological Process	2.80E-02	9
DOWN-	GO:0001556	oocyte maturation	Biological	3.10E-	7

DOWN-UP			Process	02	
DOWN-DOWN-UP	GO:0000086	G2/M transition of mitotic cell cycle	Biological Process	3.40E-02	19
DOWN-DOWN-UP	GO:0060236	regulation of mitotic spindle organization	Biological Process	3.40E-02	5
DOWN-DOWN-UP	GO:0000780	condensed nuclear chromosome, centromeric region	Cellular Component	3.40E-02	5
DOWN-DOWN-UP	GO:0051567	histone H3-K9 methylation	Biological Process	3.40E-02	10
DOWN-DOWN-UP	GO:0090307	mitotic spindle assembly	Biological Process	3.80E-02	7
DOWN-DOWN-UP	GO:0007057	spindle assembly involved in female meiosis I	Biological Process	4.00E-02	3
DOWN-DOWN-UP	GO:0032506	cytokinetic process	Biological Process	4.00E-02	11
DOWN-DOWN-UP	GO:0000281	mitotic cytokinesis	Biological Process	4.70E-02	16
DOWN-UP-DOWN	GO:0005654	nucleoplasm	Cellular Component	8.40E-04	65
DOWN-UP-DOWN	GO:0007062	sister chromatid cohesion	Biological Process	9.90E-04	13
DOWN-UP-DOWN	GO:0000404	heteroduplex DNA loop binding	Molecular Function	2.00E-03	4

DOWN-UP-DOWN	GO:0044454	nuclear chromosome part	Cellular Component	2.10E-03	27
DOWN-UP-DOWN	GO:0008094	DNA-dependent ATPase activity	Molecular Function	3.80E-03	14
DOWN-UP-DOWN	GO:0007131	reciprocal meiotic recombination	Biological Process	4.50E-03	12
DOWN-UP-DOWN	GO:0071103	DNA conformation change	Biological Process	5.10E-03	22
DOWN-UP-DOWN	GO:0000718	nucleotide-excision repair, DNA damage removal	Biological Process	6.10E-03	6
DOWN-UP-DOWN	GO:0000724	double-strand break repair via homologous recombination	Biological Process	1.40E-02	12
DOWN-UP-DOWN	GO:0002562	somatic diversification of immune receptors via germline recombination within a single locus	Biological Process	1.50E-02	8
DOWN-UP-DOWN	GO:0031047	gene silencing by RNA	Biological Process	1.60E-02	18
DOWN-UP-DOWN	GO:0006260	DNA replication	Biological Process	1.70E-02	41
DOWN-UP-DOWN	GO:0070192	chromosome organization involved in meiosis	Biological Process	1.80E-02	9
DOWN-UP-DOWN	GO:1903047	mitotic cell cycle process	Biological Process	2.50E-02	53
DOWN-UP-DOWN	GO:0048367	shoot system development	Biological Process	2.80E-02	15
DOWN-UP-DOWN	GO:0032259	methylation	Biological Process	4.00E-02	28
DOWN-UP-DOWN	GO:0000086	G2/M transition of mitotic cell cycle	Biological Process	4.00E-02	19
DOWN-UP-DOWN	GO:0003684	damaged DNA binding	Molecular Function	4.40E-02	8
DOWN-UP-DOWN	GO:0000018	regulation of DNA recombination	Biological Process	4.60E-02	8
DOWN-UP-DOWN	GO:0051307	meiotic chromosome separation	Biological Process	4.80E-02	5
DOWN-UP-DOWN	GO:0000109	nucleotide-excision repair complex	Cellular Component	4.80E-02	5
DOWN-UP-DOWN	GO:0016568	chromatin modification	Biological Process	4.80E-02	35
DOWN-UP-	GO:0016246	RNA interference	Biological	4.80E-	11

DOWN			Process	02	
DOWN-UP-UP	GO:0005576	extracellular region	Cellular Component	5.20E-07	137
DOWN-UP-UP	GO:0007586	digestion	Biological Process	5.20E-07	34
DOWN-UP-UP	GO:0044281	small molecule metabolic process	Biological Process	2.40E-06	128
DOWN-UP-UP	GO:0032787	monocarboxylic acid metabolic process	Biological Process	2.60E-04	49
DOWN-UP-UP	GO:0048252	lauric acid metabolic process	Biological Process	2.60E-04	10
DOWN-UP-UP	GO:0005615	extracellular space	Cellular Component	2.60E-04	60
DOWN-UP-UP	GO:0018685	alkane 1-monooxygenase activity	Molecular Function	2.60E-04	10

DOWN-UP-UP	GO:1901565	organonitrogen compound catabolic process	Biological Process	2.80E-04	34
DOWN-UP-UP	GO:0016042	lipid catabolic process	Biological Process	8.70E-04	27
DOWN-UP-UP	GO:1901615	organic hydroxy compound metabolic process	Biological Process	3.10E-03	50
DOWN-UP-UP	GO:0031349	positive regulation of defense response	Biological Process	3.70E-03	24
DOWN-UP-UP	GO:0014070	response to organic cyclic compound	Biological Process	5.00E-03	57
DOWN-UP-UP	GO:0055114	oxidation-reduction process	Biological Process	7.20E-03	60
DOWN-UP-UP	GO:0017143	insecticide metabolic process	Biological Process	8.70E-03	10
DOWN-UP-UP	GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	Molecular Function	9.30E-03	17
DOWN-UP-UP	GO:0002118	aggressive behavior	Biological Process	1.40E-02	12
DOWN-UP-UP	GO:0005506	iron ion binding	Molecular Function	1.40E-02	22
DOWN-UP-UP	GO:0002237	response to molecule of bacterial origin	Biological Process	1.40E-02	19
DOWN-UP-UP	GO:0016712	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen	Molecular Function	1.70E-02	11
DOWN-UP-UP	GO:0042445	hormone metabolic process	Biological Process	1.70E-02	25
DOWN-UP-UP	GO:0042742	defense response to bacterium	Biological Process	1.80E-02	27
DOWN-UP-UP	GO:0005783	endoplasmic reticulum	Cellular Component	1.90E-02	72
DOWN-UP-UP	GO:0006954	inflammatory response	Biological Process	2.40E-02	30
DOWN-UP-UP	GO:0030574	collagen catabolic process	Biological Process	2.80E-02	12
DOWN-UP-UP	GO:0043649	dicarboxylic acid catabolic process	Biological Process	2.80E-02	5

DOWN-UP-UP	GO:0009235	cobalamin metabolic process	Biological Process	3.10E-02	7
DOWN-UP-UP	GO:0009055	electron carrier activity	Molecular Function	3.60E-02	18
DOWN-UP-UP	GO:0017144	drug metabolic process	Biological Process	4.60E-02	8

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