Rampant hybrid misexpression in craniofacial tissues within a recent adaptive radiation of *Cyprinodon* pupfishes

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Abstract

Genetic incompatibilities constitute the final stages of reproductive isolation and speciation, but little is known about incompatibilities that occur within recent adaptive radiations among closely related diverging populations. Crossing divergent species to form hybrids can break up coadapted variation, resulting in genetic incompatibilities within developmental networks shaping adaptive traits. We crossed two closely related sympatric *Cyprinodon* pupfish species – a dietary generalist and a specialized molluscivore – and measured expression levels in their F_1 hybrids to identify regulatory variation underlying the novel craniofacial morphology found in this recent microendemic adaptive radiation. We compared total mRNA expression in craniofacial tissues from four F_1 hybrids with expression in the same tissue across twelve parental species. We discovered surprisingly extensive misexpression in hybrids, with more genes showing transgressive inheritance (51.4%) than conserved inheritance (47.9%) and very few genes showing additive or dominant modes of inheritance (< 1%). Genes showing misexpression in hybrids were more pleiotropic than those showing conserved inheritance. We measured allele specific expression in hybrids across 3,360 phased heterozygous sites to identify regulatory mechanisms underlying differential expression between generalists and molluscivores and found evidence for *trans*-regulatory variation controlling expression divergence of five genes. Together, our results highlight the importance of considering misexpression as an early indicator of genetic incompatibilities in the context of rapidly diverged morphological traits and suggest that *trans*-regulatory variation is involved in expression divergence between this young species pair.

Introduction

Changes in gene expression are an important source of variation in adaptive morphological traits (Carroll 2008; Wolf et al. 2010; Indjeian et al. 2016). As genetic variation accumulates in regulatory and coding sequences, stabilizing selection on gene expression results in coevolution such that molecular functions are largely maintained (Coolon et al. 2014; Hodgins-Davis et al. 2015). Crossing divergent species to form F_1 hybrids can break up such coadapted variation, resulting in genetic incompatibilities within developmental networks responsible for adaptive evolution (Michalak and Noor 2004; Landry et al. 2007; Mack and Nachman 2017). Genetic incompatibilities that reduce hybrid fitness can drive reproductive isolation either intrinsically – causing sterility or increased embryonic mortality – or extrinsically whereby incompatibilities reduce hybrid performance in a particular environment (Schluter 2000, Coyne and Orr 2004).

Of particular importance to the process of speciation are genetic incompatibilities caused by hybrid misexpression – when gene expression levels in hybrids are transgressive and fall outside of the range of expression variation observed in either parent species (Ranz et al. 2004; Rockman and Kruglyak 2006; Renaut et al. 2009). This pattern of expression causes 'Dobzhansky-Muller' incompatibilities (DMIs) if incompatible alleles in hybrids cause misexpression that results in intrinsic post-zygotic reproductive isolation (Presgraves 2003; Coyne 2004; Sweigart et al. 2006; Ortíz-Barrientos et al. 2007; Malone and Michalak 2008; Renaut et al. 2009; Davidson and Balakrishnan 2016). Laboratory studies searching for genes that cause DMIs often search for genes causing sterility or embryonic lethality in hybrids. This approach ignores the fitness consequences of misexpression occurring at later developmental stages within diverse tissue types, thus underestimating the actual number of genetic incompatibilities distinguishing species (Fang et al. 2012; Schumer et al. 2014). Combining these studies with studies examining hybrid misexpression in adaptive morphological traits can reveal a broader view of incompatibilities that arise during speciation.

Measuring gene expression in hybrids can also implicate regulatory mechanisms underlying expression divergence between parental species, which is important to understanding how expression levels are inherited and how they shape adaptive traits (Wittkopp et al. 2004; McManus et al. 2010; Mack and Nachman 2017). Research on hybrid gene expression thus far has shown mixed results regarding patterns of inheritance (Signor and Nuzhdin 2018). Some

studies found evidence for ubiquitous transgressive expression inherited in F_1 hybrids (i.e. over- or under-dominance) (Ranz et al. 2004; Rockman and Kruglyak 2006; Roberge et al. 2008), while others found predominately additive patterns (Hughes et al. 2006; Rottscheidt and Harr 2007; Davidson and Balakrishnan 2016). Mechanisms of gene expression divergence in F_1 hybrids are characterized as interactions between *cis*-regulatory elements and *trans*-regulatory factors. *Cis* elements are often non-coding regions of DNA proximal to genes that are bound by *trans*-acting proteins and RNAs to regulate mRNA abundance. It is possible to identify mechanisms of gene expression divergence between parental species by bringing *cis* elements from both parents together in the same *trans* environment in F_1 hybrids and quantifying allele specific expression (ASE) of parental alleles at heterozygous sites (Cowles et al. 2002; Wittkopp et al. 2004). Some emerging principles of regulatory evolution suggest that *cis*-variants contribute more to interspecific divergence and show mostly additive inheritance, while *trans*-variants are often more pleiotropic, contribute more to intraspecific divergence, and show predominately recessive inheritance (Prud'homme et al. 2007; Lemos et al. 2008; Signor and Nuzhdin 2018).

Here we investigate F_1 hybrids from a cross between two closely related species of *Cyprinodon* pupfishes to understand regulatory mechanisms that led to the evolution of novel craniofacial adaptations in this group. *Cyprinodon brontotheroides* – hereafter referred to as the molluscivore – is a trophic specialist species endemic to San Salvador Island, Bahamas that has adapted to eat hard shelled prey including mollusks, gastropods, and ostracods (Martin and Wainwright 2013a,c). This species likely diverged from a generalist common ancestor within the past 10,000 years to occupy this novel niche. (Mylroie, J.E, Hagey 1995; Holtmeier 2001; Turner et al. 2008; Martin and Wainwright 2011; Martin 2016). Adapting to this niche involved extreme morphological divergence in craniofacial traits compared to its sympatric generalist sister species *Cyprinodon variegatus* (Martin and Wainwright 2013c; Lencer et al. 2016). This species – hereafter referred to as the 'generalist' – consumes mainly algae and detritus in a way that is similar to almost all other generalist Caribbean pupfish species with the exception of a novel scale-eating pupfish that is also a member of the San Salvador pupfish radiation (Martin and Wainwright 2011, 2013c) and a second sympatric radiation of trophic specialists in Laguna Chichancanab, Mexico (Humphries and Miller 1981; Strecker 2006). Molluscivores exhibit a novel skeletal protrusion on the anteriodorsal head of the maxilla not found in generalist

populations that may be used to stabilize prey items held within its oral jaws, which are shorter and more robust relative to generalist species (Fig 1A). This jaw morphology provides higher mechanical advantage for crushing mollusks and other hard-shelled prey (Wainwright and Richard 1995; Martin and Wainwright 2011).

Molluscivores and generalists readily hybridize in the laboratory to produce fertile offspring with intermediate craniofacial morphologies and no obvious sex ratio distortion (Martin and Wainwright 2011, 2013b; Martin and Feinstein 2014). These species remain largely reproductively isolated in sympatry across multiple lake populations (F_{st} = 0.08; (Martin and Feinstein 2014; West and Kodric-Brown 2015; McGirr and Martin 2017). Therefore, unlike most studies of hybrid misexpression, we are not solely concerned with identifying gene expression patterns underlying hybrid sterility or lethality. Rather, we aim to characterize misexpression within developmental gene networks shaping evolutionarily novel craniofacial phenotypes within a young species pair with ongoing gene flow. We dissected craniofacial tissue from juvenile F_1 hybrids and extracted total mRNA to quantify gene expression levels. We found extensive misexpression in hybrid cranial tissues relative to parental species. In fact, more genes showed patterns of misexpression than genes that showed conserved expression levels between hybrids and parental species. We found that genes showing misexpression showed higher pleiotropy than genes showing conserved inheritance patterns, which may suggest misexpression affects many developmental processes resulting in reduced hybrid fitness. We further categorized patterns of gene expression inheritance and found that the number of genes showing additive or dominant inheritance was far exceeded by genes showing misexpression. Finally, we quantified allele specific expression (ASE) across exome-wide phased heterozygous sites to uncover mechanisms of regulatory divergence and found evidence for *trans*-variants controlling some patterns of differential expression between generalists and molluscivores.

Materials and Methods

Study system and sample collection

We generated hybrids from a cross between a female generalist and a male molluscivore. The second-generation lab-reared female generalist used to generate hybrids was derived from wild

caught generalists from Little Lake on San Salvador Island, Bahamas from full-sib F_1 parents. The third-generation lab-reared male molluscivore used to generate hybrids was derived from wild caught molluscivores from Little Lake following two generations of full-sib mating. Twelve generalists and molluscivores that were previously used for differential expression analyses (McGirr and Martin 2018) were derived from wild caught pupfishes from Little Lake and Crescent Pond on San Salvador Island, Bahamas (three F_1 generalists and F_2 snail-eaters from Little Lake, three F_2 generalists and F_2 snail-eaters from Crescent Pond; Table S1). All fish were reared at 25–27°C, 10–15 ppt salinity, pH 8.3 and fed a mix of commercial pellet foods and frozen foods. All fry were raised exclusively on newly hatched brine shrimp after hatching and before euthanasia. Hybrid and parental species individuals were euthanized in an overdose of buffered MS-222 between 17-20 days post-fertilization. There was variation in sampling time because eggs were fertilized naturally within breeding tanks and collected on the same day or subsequent day following egg laying. We collected larvae in a haphazard manner over multiple spawning intervals and it is unlikely that sampling time varied consistently by species. This variation in sampling times and across lakes for the parental populations should increase transcript variation overall in the parents and reduce the amount of transgressive expression observed in hybrids. All samples were stored in RNA later (Ambion, Inc.) at 4° C for one day, followed by long-term storage at - 20°C for up to 18 months.

RNA sequencing and alignment

Our methods for dissecting craniofacial tissue, extracting RNA, quality filtering reads, and mapping reads to features for hybrids were the same as previously described methods used for the parental species (McGirr and Martin 2018).We dissected four F_1 hybrid larvae to isolate tissues from the anterior craniofacial region containing the dentary, angular, articular, maxilla, premaxilla, palatine, and associated craniofacial connective tissues using fine-tipped tweezers washed with RNase AWAY (Molecular BioProducts). We extracted total RNA from these tissues using RNeasy Mini Kits (Qiagen). Library preparation and sequencing was performed at The Vincent J. Coates Genomics Sequencing Laboratory at the University of California, Berkeley. Libraries were constructed using the Illumina stranded Truseq RNA kit (Illumina RS-122-2001) and pooled on a single lane. Sequencing on an Illumina HiSeq4000 produced a total

of 121,625,542 raw reads (150bp paired-end). We filtered raw reads using Trim Galore (v. 4.4, Babraham Bioinformatics) to remove Illumina adaptors and low-quality reads (mean Phred score < 20) and removed duplicate reads using the MarkDuplicates command of the Picard software package (http://broadinstitute.github.io/picard/). We mapped 106,503,040 filtered reads to the *Cyprinodon* reference genome (NCBI, C. variegatus Annotation Release 100, total sequence length = 1,035,184,475; number of scaffold = 9259, scaffold N50, = 835,301; contig N50 = 20,803; (Lencer et al. 2017)) using the RNA-seq aligner STAR (v. 2.5 (Dobin et al. 2013)). We used the featureCounts function of the Rsubread package (Liao et al. 2014) requiring paired-end and reverse stranded options to generate read counts across 24,952 previously annotated features (Lencer et al. 2017) with an average coverage depth of 322 reads. We assessed mapping and count quality using MultiQC (Ewels et al. 2016), finding a similar number of reads mapped to features for all individuals.

Hybrid inheritance of expression patterns

To determine whether genes showed additive, dominant, or transgressive patterns of inheritance, we quantified differences in cranial gene expression between hybrids vs. parental species and compared them to genes differentially expressed between generalists vs. molluscivores. We used DESeq2 (v. 3.5 (Love et al. 2014)) to normalize read counts across features and performed pairwise tests to identify differentially expressed genes between hybrids vs. parental species (n = 4 vs. n = 12), hybrids vs. generalists (n = 4 vs. n = 6), and hybrids vs. molluscivores (n = 4 vs. n = 6) (Table S1). Gene features with less than 10 individuals showing normalized counts greater than or equal to 5 were discarded leaving 12,989 for analyses. We used Wald tests to calculate significant differences in expression between groups by comparing normalized posterior log fold change estimates and correcting for multiple testing using the Benjamini–Hochberg procedure with a false discovery rate of 0.05 (Benjamini and Hochberg 1995).

Hybrid inheritance was considered additive if gene expression was intermediate between generalists and molluscivores with significant differential expression between generalists and molluscivores. Inheritance was dominant if expression was intermediate between parental species and hybrid expression was significantly different from one parent but not the other. Genes showing misexpression in hybrids showed transgressive inheritance, where hybrid gene

expression was significantly higher (overdominant) or lower (underdominant) than parental populations.

Measuring pleiotropy

We reasoned that genes showing no difference in expression between hybrids and parental species showed conserved expression patterns because they were constrained by negative pleiotropy (Wagner and Zhang 2011; Papakostas et al. 2014). Craniofacial phenotypes are tightly integrated and controlled by a complex web of regulatory networks (Richtsmeier and DeLeon 2009; Santos et al. 2011; Ahi 2016; Parsons et al. 2018). Given this highly pleiotropic architecture, adaptive changes in gene expression are expected to move along genetic lines of least resistance (Schluter 1996; Smith 2016; Parsons et al. 2018). Since coadapted regulatory changes contributing to cranial divergence are revealed by F_1 hybrid misexpression, we predicted genes showing misexpression would exhibit lower pleiotropy than genes showing conserved expression. High gene pleiotropy is correlated with participation in more protein–protein interactions (PPIs), which in turn affect multiple biological processes (He and Zhang 2006; Safari-alighiarloo et al. 2014). We estimated pleiotropy for genes showing conserved and transgressive expression in F_1 hybrid craniofacial tissues based on their number of associated biological processes and PPIs known for zebrafish orthologs (previously described in McGirr and Martin 2018). We used GO Consortium resources to determine the number of biological processes ontologies associated with each gene. We examined biological process annotations only for genes from ZFIN (zfin.org) with experimental evidence (GO evidence code EXP). We used the String database (v. 10; (Szklarczyk et al. 2015)) to quantify PPIs for protein products of genes showing conserved and transgressive expression in hybrids, focusing only on interactions with high confidence experimental evidence (experimental evidence scores in the top 95 percentile).

The *Cyprinodon* reference genome is annotated for genomic features (NCBI, *C. variegatus* Annotation Release 100, (Lencer et al. 2017)), and many annotated genes share the same name as their zebrafish orthologs. We performed gene ontology (GO) enrichment analyses for genes showing conserved, overdominant, and underdominant patterns of inheritance that shared the same name as zebrafish orthologs using GO Consortium resources available at

geneontology.org (Ashburner et al. 2000; GO Consortium 2017). We searched for enrichment across biological process ontologies curated for zebrafish. We also identified genes within each inheritance category (conserved, over-, and underdominant) that were annotated for effects on craniofacial skeleton development (GO:1904888).

We tested whether genes showing transgressive inheritance patterns in hybrids showed different levels of pleiotropy than genes showing conserved inheritance patterns by fitting a generalized linear model on count data for pleiotropy estimates (negative binomial family; glm.nb function in the R library “MASS”).

Allele specific expression and mechanisms of regulatory divergence

We partitioned hybrid gene expression divergence into patterns that could be attributed to *cis*-regulatory variation in cases where linked genetic variation acting within proximal non-coding DNA affected expression levels, and *trans*-regulatory variation in cases where genetic variation in coding proteins bound to *cis*-regulatory elements affected expression levels. *Cis* and *trans* regulatory variants often interact to affect expression divergence of the same gene (Wittkopp et al. 2004; McManus et al. 2010). However, we could only detect expression divergence due to solely *cis* or *trans* variation because the exact maternal and paternal alleles within F_1 hybrids were unknown.

We followed the best practices guide recommended by the Genome Analysis Toolkit (DePristo et al. 2011 (v. 3.5)) in order to call and refine SNP variants within coding gene regions using the Haplotype Caller function. We called SNPs using conservative hard-filtering parameters (DePristo et al. 2011; Marsden et al. 2014): Phred-scaled variant confidence divided by the depth of nonreference samples >2.0, Phred-scaled *P*-value using Fisher's exact test to detect strand bias > 60, Mann–Whitney rank-sum test for mapping qualities (z > 12.5), Mann–Whitney rank-sum test for distance from the end of a read for those with the alternate allele (z > 8.0). We used the ReadBackedPhasing function with a phase quality threshold > 20 to identify phased variants. We used the VariantsToTable function (with genotypeFilterExpression "isHet == 1") to output phased heterozygous variants for each parental species and hybrid individual. We counted the number of reads covering heterozygous sites using the

ASEReadCounter (with -U ALLOW_N_CIGAR_READS -minDepth 20 --minMappingQuality 10 --minBaseQuality 20 -drf DuplicateRead). In total we identified 3,360 phased heterozygous sites with sequencing coverage >= 20× that fell within 1,205 genes used for differential expression analyses.

We assigned each heterozygous allele as the reference allele, alternate allele, or second alternate allele and matched each allele to its corresponding read depth. This allowed us to identify allele specific expression (ASE) by measuring expression variation between the two haplotypes of each gene distinguished by heterozygous sites. We used a binomal test in R (binom.test) to determine if a heterozygous site showed significantly biased expression of one allele over another ($P < 0.05$; McManus et al. 2010; Mack and Nachman 2016). A gene was considered to show ASE if every phased heterozygous allele within that gene showed consistent biased expression in the same direction. We measured ASE across 1,205 genes expressed in parental species and hybrids. A gene was considered to show ASE in hybrids if every phased heterozygous allele within that gene showed consistent biased expression in the same direction in all four hybrid samples and did not show ASE in any of the twelve parental samples.

We paired our differential expression analyses with genes showing ASE to determine mechanisms of regulatory divergence. A gene showing allele specific expression in F_1 hybrids that is differentially expressed between parental species is the result of *cis*-regulatory divergence (Wittkopp et al. 2004). We identified expression divergence due to *cis*-regulation if genes showed significant ASE in hybrids and differential expression between generalists and molluscivores. *Trans*-regulatory divergence is determined by comparing the ratio of gene expression in parents with the ratio of alleleic expression in F_1 hybrids (Wittkopp et al. 2004). We identified expression divergence due to *trans*-regulation if genes did not show ASE in hybrids but were differentially expressed between generalists and molluscivores.

Results

Extensive misexpression in F_1 hybrid craniofacial tissues

We compared gene expression in craniofacial tissue from parental populations (McGirr and Martin 2018) with expression in hybrids and found extensive hybrid misexpression in 6,673

(51.4%) genes showing transgressive patterns of inheritance (Fig 1D, Fig. 2). There was an approximately equal number of genes showing overdominant and underdominant expression in hybrids, with 3,332 (25.7%) genes showing higher expression in hybrids relative to parental populations and 3,341 (25.7%) showing lower expression in hybrids relative to parental populations (Fig 1B, Fig 2). This equal number of genes showing higher and lower expression suggests that misexpression is not the result of a sequencing bias between lanes containing hybrid samples and parental samples, in which case we would expect to find an excess of genes expressed above or below parental levels. While there was a similar number of genes showing overdominant and underdominant inheritance, the magnitude of differential expression was higher for genes showing underdominant inheritance (Fig 2B; Wilcoxon rank sum test, $P < 1.0 \times 10^{-16}$).

Hybrid misexpression was the prevailing form of gene expression inheritance we found in craniofacial tissues. Misexpression was so widespread that more genes showed misexpression than conserved expression patterns, with only 6,226 (47.9%) showing no difference in expression between hybrids and parental populations. While many studies have reported the predominance of additive patterns of gene expression inheritance in F_1 hybrids (Hughes et al. 2006; Rottscheidt and Harr 2007; Davidson and Balakrishnan 2016), we only identified 36 (0.003%) additive genes for which expression in hybrids was intermediate between generalists and molluscivores but significantly different between generalists and molluscivores (Fig 2A, C). Similarly, we found few genes showing patterns of dominance, with 33 showing 'generalist-like' expression patterns and 21 showing 'molluscivore-like' patterns (Fig 2A, C).

Misexpressed genes are highly pleiotropic

We estimated pleiotropy for genes showing misexpression and conserved expression in hybrid craniofacial tissues based on their number of associated biological processes and protein-protein interactions (PPIs) known for zebrafish orthologs (see methods). We determined the number of biological process ontologies associated with 562 genes showing misexpression and 538 showing conserved expression. We also determined the number of PPIs annotated for 1,036 genes showing misexpression and 937 genes showing conserved expression. Counter to our predictions, we found that genes showing conserved expression in hybrids showed lower

pleiotropy than misexpressed genes across both of our measures of pleiotropy (Fig 3A, C; GLM; biological processes: $P = 0.04$; PPIs: $P = 1.03 \times 10^{-4}$).

Since our predictions about pleiotropy pertained most to genes shaping craniofacial divergence, we measured pleiotropy specifically for the genes we identified within the cranial skeleton development ontology described above (Table S3). We determined the number of biological processes ontologies associated with 41 cranial genes showing misexpression and 32 showing conserved expression. We also determined the number of PPIs annotated for 21 genes showing misexpression and 11 genes showing conserved expression. Interestingly, our analyses of cranial skeleton development genes mirrored results we found across all genes. Specifically, cranial genes showing conserved expression showed lower pleiotropy compared to cranial genes showing misexpression (Fig 3B, D; GLM; biological processes: $P = 0.0218$; PPIs: $P = 0.0109$).

Trans-regulatory variation underlies expression divergence between generalists and molluscivores

A gene that is differentially expressed between parental species will also show biased allelic expression at heterozygous sites in F_1 hybrids if expression divergence is due to *cis*-regulatory variation because both parental alleles are exposed to the same *trans*-regulatory environment (Wittkopp et al. 2004). Alternatively, a gene that is differentially expressed between parental species will not show biased allelic expression in hybrids if expression divergence is due to *trans*-regulatory divergence (Wittkopp et al. 2004). We identified 3,360 phased heterozygous sites that fell within 1,205 genes used for our differential expression analyses. We estimated allele specific expression (ASE) for these genes and paired these data with patterns of differential expression between parental species to identify genes controlled by *cis*-regulatory elements and *trans*-regulatory factors.

Out of the previously identified 120 genes differentially expressed between craniofacial tissue derived from generalists and molluscivores (Fig 1B; McGirr and Martin 2018), only five contained informative heterozygous sites amenable to allele specific expression analyses. This is a lower number of genes containing heterozygous variation than expected by chance (Fishers exact test, $P = 8.83 \times 10^{-8}$). The five genes tended to be shorter on average compared to the other

115 differentially expressed genes without heterozygous variation (t-test, $P = 0.0583$), meaning that the 115 genes were larger mutational targets and more likely to contain heterozygous variation based on mutation rate alone. This counterintuitive result might suggest that genes differentially expressed between generalist and molluscivore craniofacial tissues lack variation due to strong purifying selection.

Of the five genes containing heterozygous sites differentially expressed between generalists and molluscivores, none showed evidence of biased allelic expression, suggesting that expression divergence is explained by *trans*-regulatory variation (Fig 4, Fig 5). We did not find any evidence for expression divergence controlled by *cis*-acting variation, though we could only analyze a subset of genes differentially expressed between parental populations. Furthermore, we were unable to test for *cis* × *trans* interactions affecting expression patterns due to the unknown identity of maternal and paternal alleles. Interestingly, we only identified three genes that showed consistent ASE across all four hybrids, all of which were also misexpressed (Fig 4, Fig S2). *fam213b* and *vamp3* both showed ASE and higher expression in hybrids vs. parental species while *loc107086778* showed ASE and lower expression in hybrids (Fig S2).

Discussion

Molluscivores show extreme craniofacial divergence relative to their generalist sister species, exhibiting a novel maxillary protrusion and short robust jaws (Fig 1A; Lencer et al. 2016; Hernandez et al. 2018). Given the extreme craniofacial divergence observed between molluscivores and their generalist sister-species, we might expect to find genes expressed in hybrids outside the range of either parent species as a result of discordance between alternatively coadapted genes in networks shaping divergent craniofacial morphologies. However, genetic divergence between generalists and molluscivores is low, with only 79 SNPs fixed between species ($F_{st} = 0.08$, $D_{xy} = 0.000166$ (McGirr and Martin 2017; McGirr and Martin 2018)). Despite ongoing gene flow between species and low genetic divergence, we found rampant misexpression in hybrid craniofacial tissues, with more genes showing transgressive inheritance than conserved inheritance (Fig. 1B, C). Contrary to our predictions, genes showing misexpression showed higher pleiotropy than genes showing conserved inheritance (Fig 3). We also measured allele specific expression (ASE) for genes expressed in hybrids and parental

species and found evidence for *trans*-acting factors controlling regulatory divergence of five genes differentially expressed between generalists and molluscivores (Fig 4, Fig 5).

Misexpression is the prevailing mode of gene expression inheritance in hybrids

While most studies on hybrid misexpression search for regulatory divergence in 'speciation genes' associated with sterility and inviability (Malone and Michalak 2008; Renaut et al. 2009; Davidson and Balakrishnan 2016), our results highlight the importance of considering misexpression in the context of adaptive morphological traits. We found evidence of extensive misexpression in F_1 hybrid craniofacial tissues, with over half of genes expressed showing overdominance or underdominace.

It is unclear whether such extensive gene misexpression in hybrid craniofacial tissues might contribute to intrinsic post-zygotic isolation between these parental species. Hybrids exhibiting intermediate craniofacial phenotypes showed reduced survival and growth rates in the wild than hybrids resembling parental species (Martin and Wainwright 2013b; Martin 2016), but short-term experiments measuring hybrid fitness in the lab did not find the same pattern of reduced survival for hybrids with intermediate phenotypes (Martin and Wainwright 2013b). This was interpreted as evidence that complex fitness landscapes measured in field enclosures on San Salvador with multiple peaks corresponding to the generalist and molluscivore were due to competition and foraging ability in the wild (i.e. extrinsic reproductive isolation). However, additional analyses of these data suggest that absolute performance of hybrids may also play a role in their survival. The most transgressive hybrid phenotypes exhibited the lowest fitness, contrary to expectations from negative frequency-dependent selection (Martin 2016). It is still possible that intrinsic and extrinsic incompatibilities interact such that gene misexpression weakens performance more in the wild than in the lab. Hence, the hybrid misexpression we report here may be correlated with more transgressive hybrid phenotypes which exhibited lower survival rates in field enclosures (Martin 2016); although note that F_1 hybrids used in this study would all fall within an intermediate phenotypic position relative to parental trophic morphology whereas field experiments used F_2 and greater hybrid intercrosses and backcrosses.

We found far more misexpression in craniofacial tissues than a study of misexpression in whole-body tissue that measured gene expression in F_1 hybrids generated between benthic and limnetic lake whitefish (Renaut et al. 2009). Similar to generalists and molluscivores, these species show recent divergence times and exhibit derived trophic specializations (Renaut et al. 2009; Bernatchez 2004). However, Renaut et al. found only 9% of genes showing transgressive inheritance in F_1 hybrids. Generalist/molluscivore hybrids showed much more misexpression that was more similar to misexpression in backcrossed lake whitefish progeny (54% of genes misexpressed; Renaut et al. 2009), and other hybrids generated from species pairs separated by longer divergence times (Ranz et al. 2004; Landry et al. 2007). Finally, genes showing underdominance in hybrids showed a higher magnitude of differential expression compared to those showing overdominance (Fig. 2B), a pattern that has also been observed in crosses between lake whitefish (Renaut and Bernatchez 2011) and crosses between a generalist and specialist *Drosophila* species pair (McManus et al. 2010).

Pleiotropic constraint is not associated with conserved gene expression in hybrids

Negative pleiotropy is thought to constrain gene expression evolution because genes involved in fewer epistatic interactions may be simpler targets for expression divergence (Wagner and Zhang 2011; Papakostas et al. 2014; Chesmore et al. 2016). We identified genes showing transgressive inheritance in hybrids that may show misexpression due to mismatches between coadapted alleles in parental species. Hence, misexpression can implicate specific developmental gene networks shaping divergent craniofacial traits between generalists and molluscivores. These networks are known to be tightly integrated, where shifting gene expression can have cascading pleiotropic effects (Niederreither and Dollé 2008; Yamamoto et al. 2009). If adaptive changes in gene expression are expected to move along genetic lines of least resistance (Schluter 1996; Smith 2016), then we should expect adaptive regulatory variation to affect genes that are less constrained by pleiotropic interactions. Accordingly, genes showing misexpression should show lower pleiotropy than genes showing conserved expression in hybrids. Surprisingly, we found that genes showing conserved expression showed lower pleiotropy than misexpressed genes across both of our measures of pleiotropy (Fig. 3). Furthermore, this pattern was consistent when we restricted analyses to genes known to affect cranial skeleton development (Fig. 3B, C). This

result is in line with our previous finding that pleiotropy did not influence the probability of parallel evolution of gene expression between divergent trophic specialists (McGirr and Martin 2018). It is also consistent with empirical evidence and theoretical models of gene expression evolution that found pleiotropy constrains the variability of gene expression within species, but does not hinder divergence between species (Tulchinsky et al. 2014; Uebbing et al. 2016). Finally, the high pleiotropy of misexpressed genes may suggest misexpression affects many developmental processes that could result in reduced hybrid fitness.

Trans-regulatory effects on gene expression divergence

We combined differential expression analyses with ASE analyses to identify mechanisms of regulatory divergence between generalists and molluscivores. We previously showed that 120 genes were differentially expressed between generalists and molluscivore craniofacial tissues (McGirr and Martin 2018). Five of these genes contained informative heterozygous sites for ASE analyses, which was lower than the expected number of genes available for analyses based on the distribution of heterozygous sites across the rest of the transcriptome (Fishers exact test, $P = 8.83 \times 10^{-8}$). Perhaps this is due to strong purifying selection acting on genes showing differential expression between species, which would allow them to modulate expression of other downstream regulatory targets while maintaining the same molecular function to avoid adverse pleiotropic effects.

None of the five genes differentially expressed between generalists and molluscivores containing heterozygous sites showed ASE in hybrids, suggesting *trans*-regulatory variation underlying expression divergence (Fig. 4, 5). Our analyses likely underestimate the role of *cis*-variants contributing to expression divergence since we could not assign paternal and maternal alleles at heterozygous sites, precluding analyses of *cis* × *trans* interactions. We were also limited by the number of differentially expressed genes containing heterozygous sites. Despite these caveats, there are several reasons to believe *trans*-regulatory effects may dominate expression divergence between generalist and molluscivore craniofacial tissues.

Other studies untangling *cis*- and *trans*-regulatory mechanisms have found that *cis*-variants contribute more to interspecific divergence, while *trans*-variants contribute more to

intraspecific divergence (Prud'homme et al. 2007; Lemos et al. 2008; Signor and Nuzhdin 2018). Importantly, many of the studies supporting these principles examine interspecific hybrids generated by species pairs with much greater divergence times (Graze et al. 2009: *Drosophila melanogaster* and *D. simiulans* diverged 2.5 mya; Tirosh et al. 2009: *Saccharomyces cerevisiae* and *S. paradoxus* diverged 5 mya; Shi et al. 2012: *Arabidopsis thaliana* and *A. arenosa* diverged 5.3 mya). Given that generalists and molluscivores diverged only within the past 10,000 years and are known to hybridize in the wild, perhaps we should expect to see *trans* effects for the same reason *trans*-effects contribute more to intraspecific divergence. Namely, the larger mutational target of *trans*-regulatory factors results in the observed excess of intraspecific *trans*-effects (Wittkopp et al. 2008; Emerson et al. 2010; Suvorov et al. 2013).

Another reason we might expect *trans*-effects to influence expression divergence in this system is the lack of additive inheritance of gene expression levels we observed in hybrids. *Cis*-regulatory variation tends to have an additive influence on gene expression (Lemos et al. 2008; Tirosh et al. 2009; McManus et al. 2010). We found only 0.003% of genes showing additive inheritance in hybrids, where expression in hybrids was intermediate between generalists and molluscivores but significantly different between generalists and molluscivores (Fig 2A, C).

Conclusion

Pervasive misexpression in hybrid cranial tissues points to divergent evolution of developmental networks shaping novel traits in the molluscivore. It is unclear whether this sizable misexpression causes intrinsic incompatibilities in hybrids within this recent adaptive radiation. We find that genes showing misexpression are more pleiotropic than genes showing conserved expression, which may suggest misexpression affects many developmental processes that could result in reduced hybrid fitness. Our results suggest that examining misexpression in the context of the relevant adaptive tissues can paint a more complete picture of genetic incompatibilities that distinguish species.

Data Availability

All transcriptomic raw sequence reads are available as zipped fastq files on the NCBI BioProject database. Accession: PRJNA391309. Title: Craniofacial divergence in Caribbean Pupfishes.

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Author Contributions

JAM wrote the manuscript, extracted the RNA samples, and conducted all bioinformatic analyses. Both authors contributed to the conception and development of the ideas and revision of the manuscript.

Competing interests

We declare we have no competing interests.

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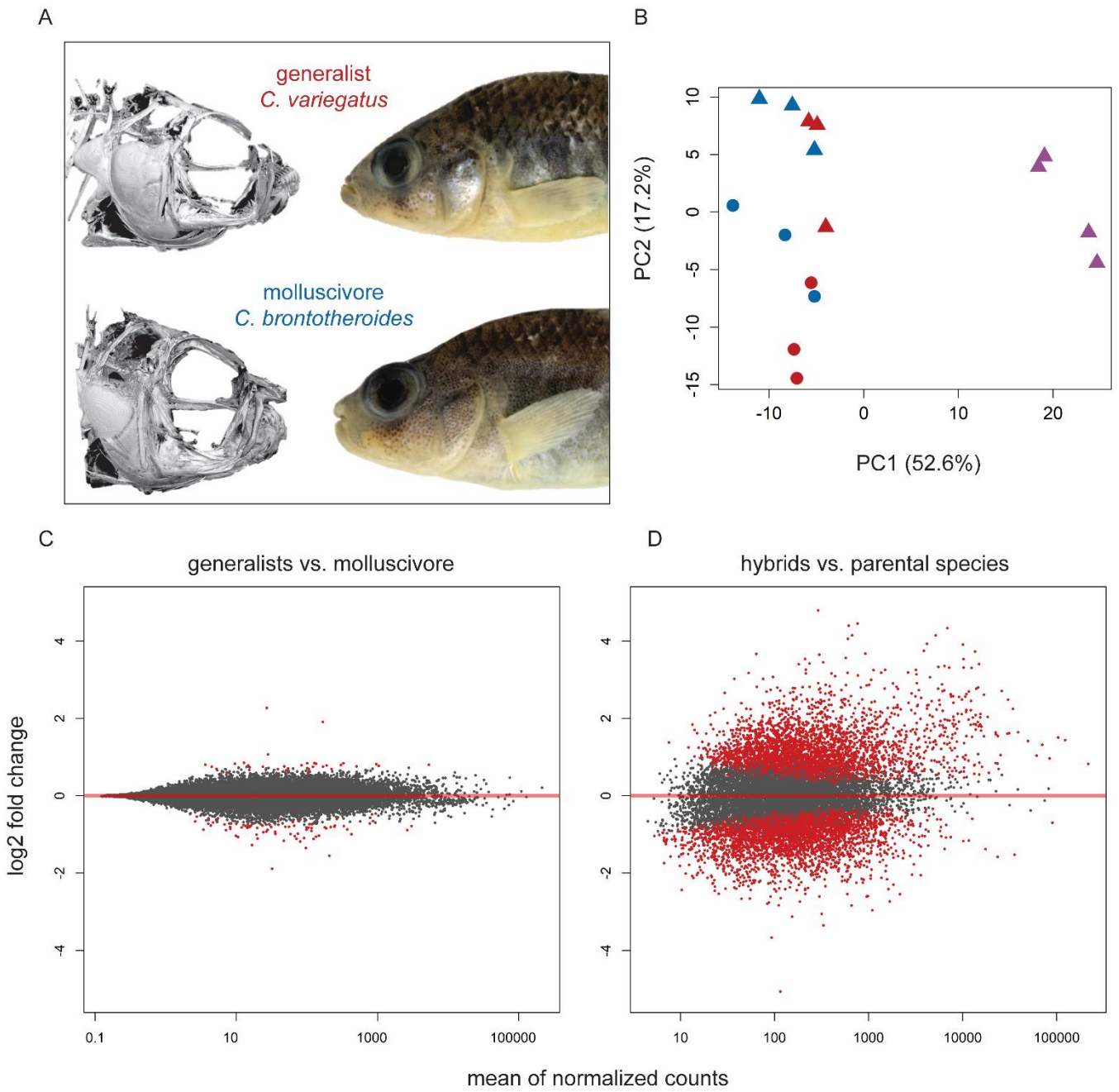
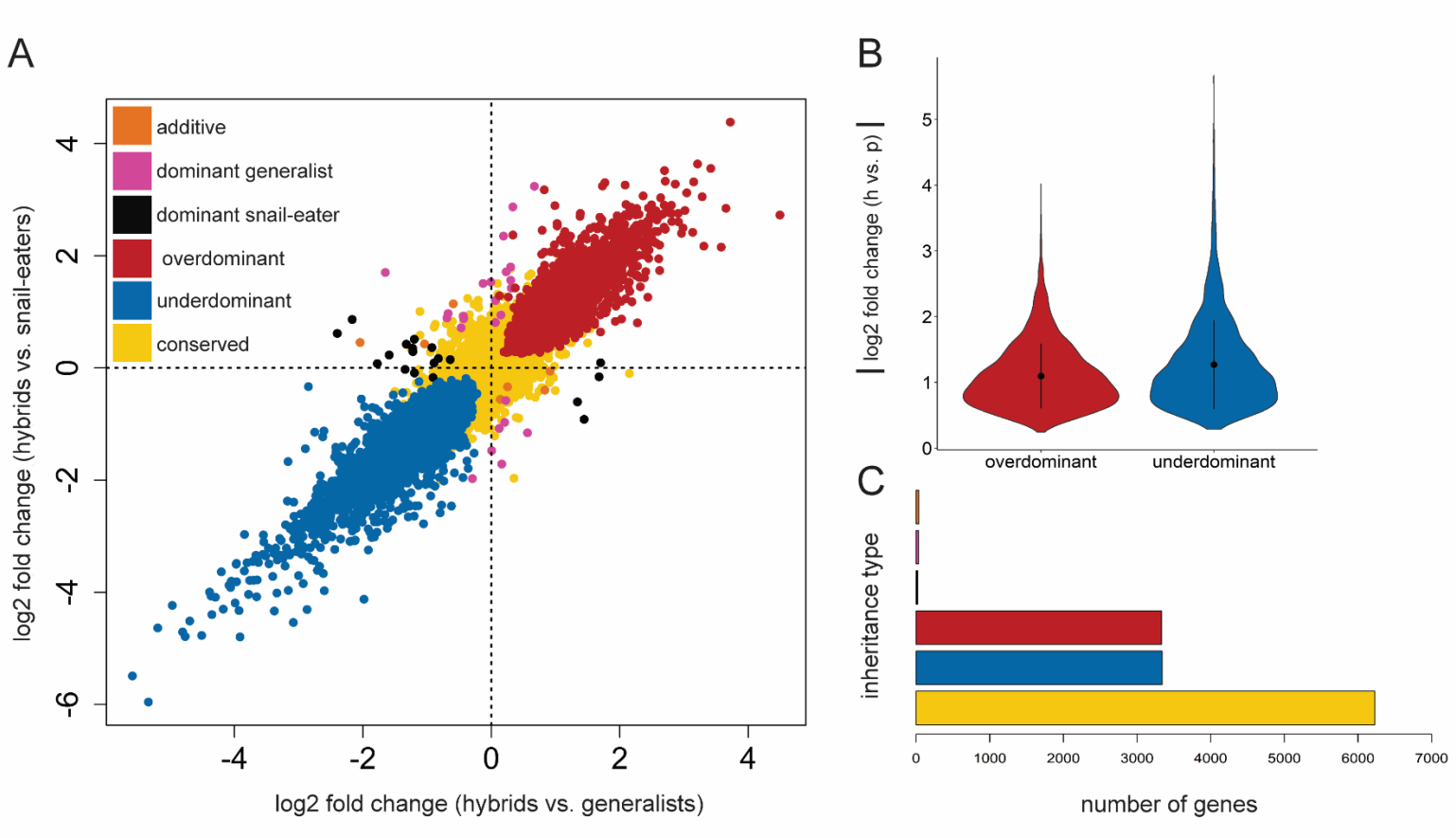


Fig 1. Extensive misexpression in F_1 hybrid craniofacial tissues. A) *Cyprinodon variegatus* (generalist, top) and *C. brontotheroides* (molluscivore, bottom). µCT scans of the cranial skeleton of each species modified from Hernandez et al. 2018. B) The first and second principal component axes accounting for a combined 69.8% of the total variation between generalist (red), molluscivore (blue), and hybrid (purple) samples across reads mapped to annotated features. Triangles represent samples from Little Lake and circles represent samples from Crescent Pond on San Salvador Island, Bahamas. Bottom panels show red points that represent differentially expressed genes between C) generalists vs. molluscivores and D) hybrids vs. parental species.



726 **Fig 2. Gene expression inheritance in hybrids.** A) Gene inheritance was characterized by
727 patterns of differential expression between hybrids vs. generalists and hybrids vs. molluscivores
728 across 12,989 genes. B) The magnitude of differential expression was higher for genes showing
729 underdominant inheritance than genes showing overdominant inheritance (Wilcoxon rank sum
730 test, $P < 1.0 \times 10^{-16}$). C) Misexpression was the prevailing mode of gene expression inheritance
731 in hybrids, with more genes showing transgressive inheritance (51.4%) than conserved
732 inheritance (47.9%).

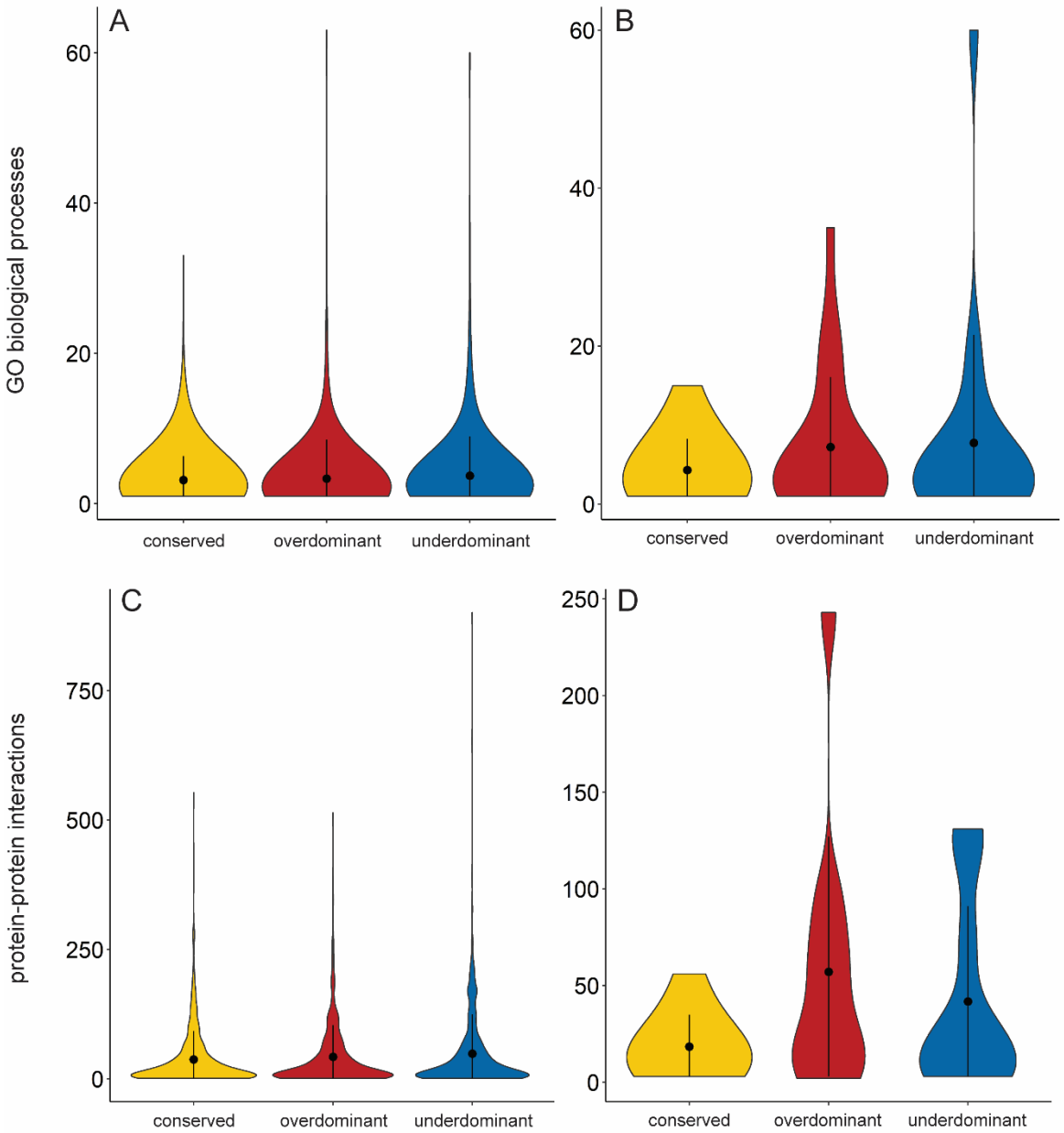


Fig. 3. Misexpressed genes are more pleiotropic than genes showing conserved expression patterns in hybrids. Top panels show the number of biological process ontologies associated with A) 562 zebrafish orthologs for genes showing misexpression and 538 showing conserved expression, and B) 41 cranial genes showing misexpression and 32 showing conserved expression. Bottom panels show the number of PPIs annotated for C) 1,036 zebrafish orthologs for genes showing misexpression and 937 showing conserved expression, and D) 21 cranial genes showing misexpression and 11 showing conserved expression. Orthologs for genes showing conserved expression in hybrids consistently showed lower pleiotropy than misexpressed genes in all comparisons (GLM; biological processes: all genes $P = 0.04$, cranial genes $P = 0.0218$; PPIs: all genes $P = 1.03 \times 10^{-4}$, cranial genes $P = 0.0218$). Cranial genes are those included in the zebrafish cranial skeleton development ontology (GO:1904888).

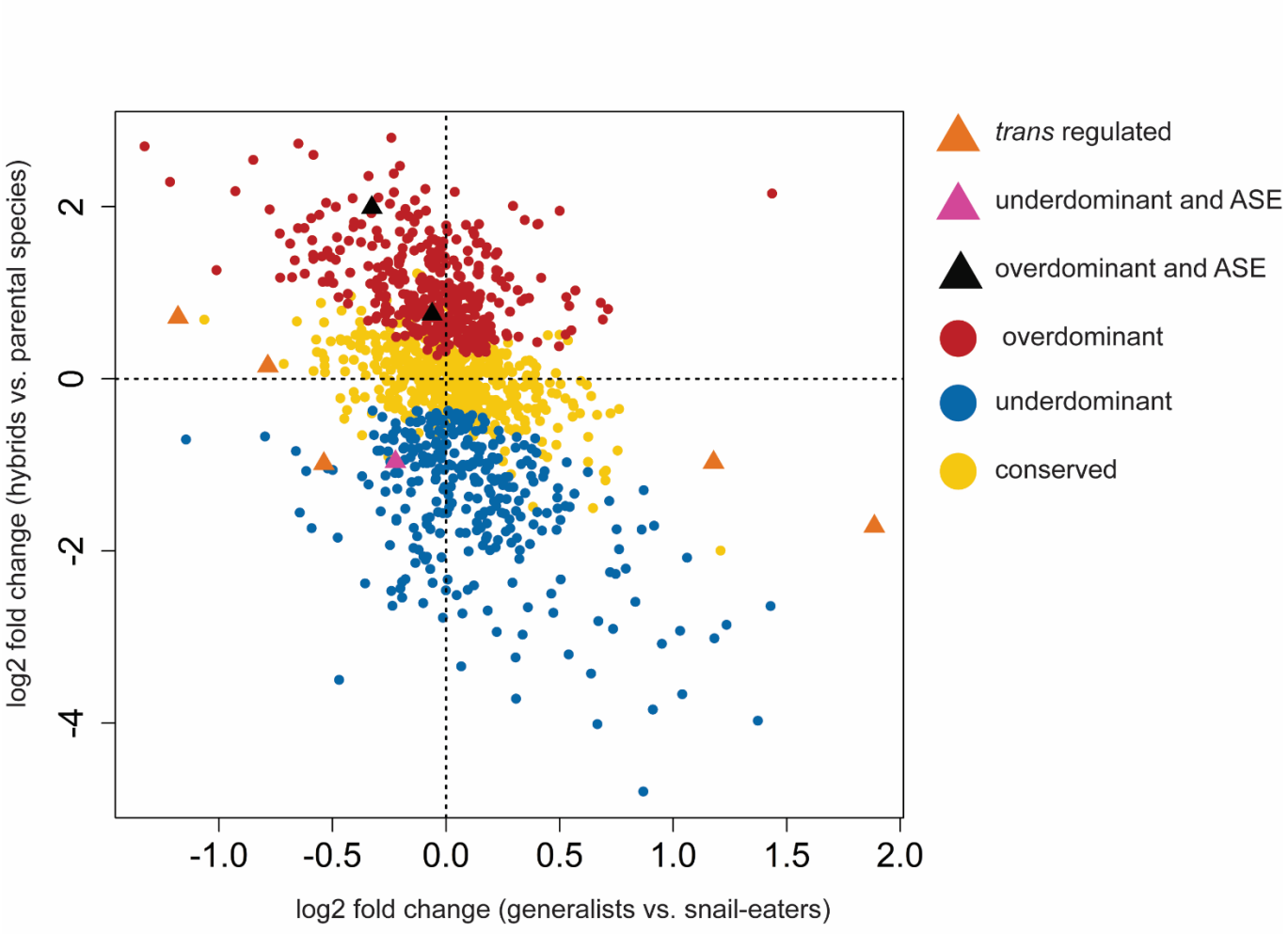


Fig. 4. Regulatory mechanisms of expression divergence between generalists and molluscivores. Allele specific expression measured across 1,205 genes containing heterozygous sites identified three genes that showed biased allelic expression in all hybrid samples (pink and black triangles). Orange triangles show genes that were differentially expressed between generalists and molluscivores but showed no biased allelic expression, suggesting that differential expression is controlled by *trans*-acting factors.

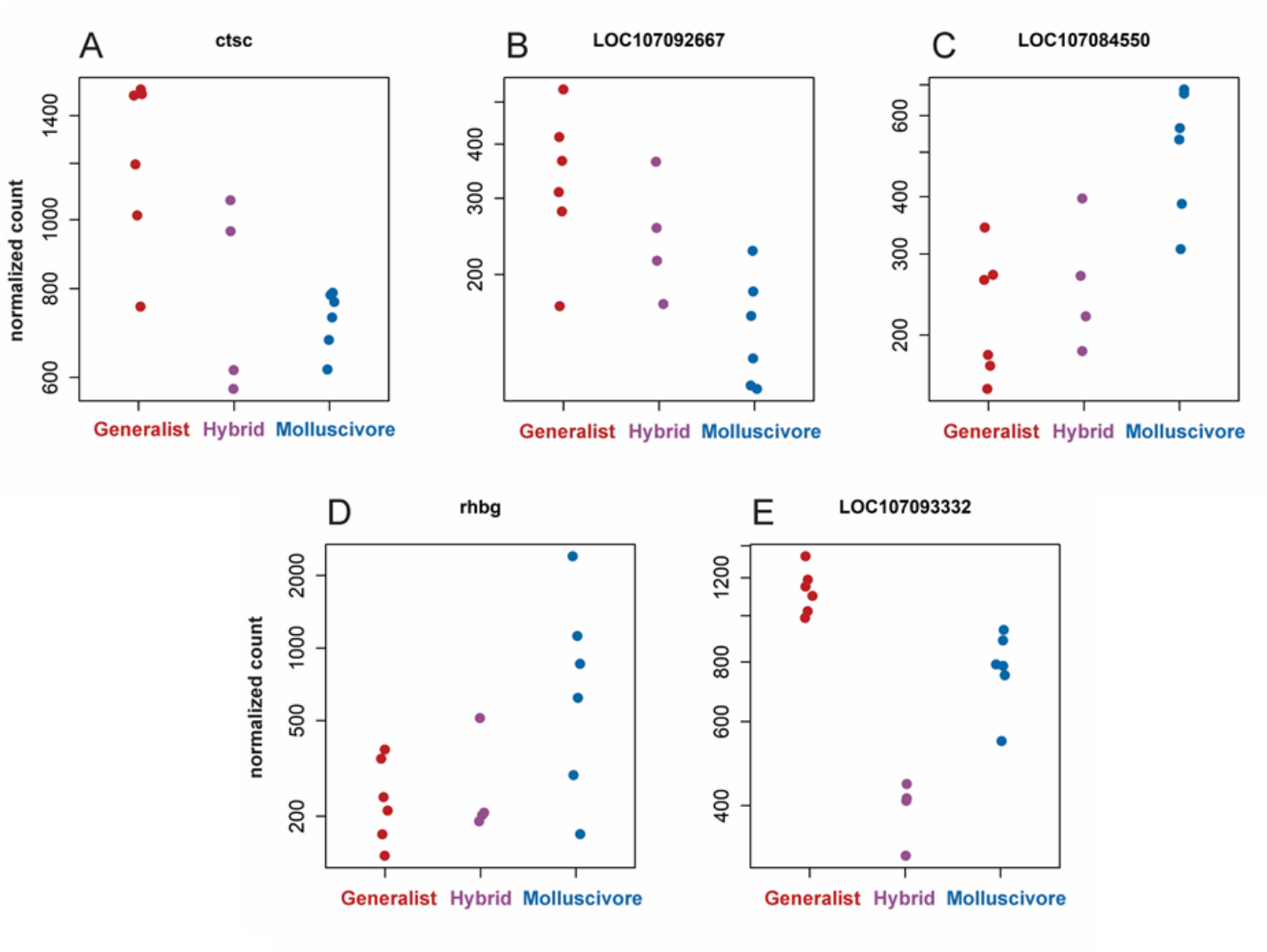


Fig. 5. A-E) Normalized read counts for genes differentially expressed between generalists (red) and molluscivores (blue) with no evidence of biased allelic expression suggesting expression divergence is explained by *trans*-regulatory variation. Hybrid read counts are shown in purple. Genes correspond to orange triangles in Fig. 4.