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 extracted mRNA from eight day old whole-larvae tissue and from craniofacial tissues dissected from 17-20 day old larvae to compare gene expression between a total of seven F_1 hybrids and 24 individuals from parental species populations. We found 3.9% of genes differentially expressed between generalists and molluscivores in whole-larvae tissues and 0.6% of genes differentially expressed in craniofacial tissue. We found that only 2.1% of genes were misexpressed in whole-larvae hybrids at 8 dpf whereas 19.1% of genes were misexpressed in hybrid craniofacial tissue at 17-20 dpf, after correcting for potential sequencing biases. We also measured allele specific expression across 15,429 phased heterozygous sites to identify regulatory mechanisms underlying differential expression between generalists and molluscivores. Together, our results highlight the importance of considering misexpression as an early indicator of genetic incompatibilities in the context of rapidly diverged morphology and suggests that wide-spread compensatory regulatory divergence drives hybrid misexpression in developing tissues that give rise to novel craniofacial traits.

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 developing tissues that give rise to adaptive traits (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 both parental species (Michalak and Noor 2004; Ranz et al. 2004; Haerty and Singh 2006; Rockman and Kruglyak 2006; Malone and Michalak 2008; Renaut et al. 2009). This pattern of expression causes Dobzhansky-Muller incompatibilities (DMIs) if incompatible alleles in hybrids cause misexpression that results in reduced hybrid fitness and thus increased 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 findings from these studies with analyses of hybrid misexpression in tissues that give rise to 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 for 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). Finally, *cis* and *trans* regulatory variants can compensate for one another if stabilizing selection favors an optimal level of gene expression. Hybrid misexpression is expected when different compensatory variants have accumulated in diverging lineages. (Denver et al. 2005; Landry et al. 2005; Bedford and Hartl 2009; Goncalves et al. 2012).

Here we investigate F_1 hybrids from crosses between two closely related species of *Cyprinodon* pupfishes to understand regulatory mechanisms that led to the evolution of novel craniofacial adaptations in this group (Fig 1A). *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 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 consumes mainly algae and detritus and is hereafter referred to as the 'generalist.' Almost all

other Caribbean pupfish species are generalists, 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 F_1 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 (genome-wide average 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 also aim to characterize misexpression in a developing tissue that gives rise to novel craniofacial phenotypes within a young species pair with ongoing gene flow. We dissected craniofacial tissue from 17-20 day old F_1 hybrids and extracted total mRNA to quantify gene expression levels. We also extracted whole-larvae mRNA from 8 day old generalists, molluscivores, and their hybrids. We found misexpression in hybrids at both stages, with more extensive misexpression in hybrid craniofacial tissues. Finally, we quantified allele specific expression (ASE) across exome-wide phased heterozygous sites to uncover mechanisms of regulatory divergence and found evidence for *cis* and *trans*-variants controlling patterns of differential expression between generalists and molluscivores, and compensatory variation influencing patterns of hybrid misexpression.

Materials and Methods

Study system and sample collection

Our methods for raising larvae and extracting RNA were identical to previously outlined methods (McGirr and Martin 2018). We collected fishes for breeding from three hypersaline lakes on San Salvador Island, Bahamas (Little Lake, Osprey Lake, and Crescent Pond) using a hand net or seine net between 2011 and 2017. These fishes 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 lab bred larvae were raised exclusively on newly hatched brine shrimp after hatching and before euthanasia. Individuals were euthanized in an overdose of buffered MS-222 and stored in RNA later (Ambion, Inc.) at - 20°C for up to 18 months. We used RNeasy Mini Kits (Qiagen catalog #74104) to extract RNA from all samples.

We previously generated 24 transcriptomes belonging to generalists and molluscivores collected at two early developmental stages: 8-10 days post fertilization and 17-20 dpf (McGirr and Martin 2018). RNA was extracted from whole-larvae tissue at 8-10 dpf. We dissected all 17-20 dpf samples to extract RNA from anterior craniofacial tissues containing the dentary, angular, articular, maxilla, premaxilla, palatine, and associated craniofacial connective tissues (Fig. S1). Dissections were performed using fine-tipped tweezers washed with RNase AWAY (Molecular BioProducts). These 24 samples were generated by breeding populations of lab-raised fishes that resulted from either one or two generations of full-sib breeding between wild caught fishes from Little Lake and Crescent Pond on San Salvador Island, Bahamas (Table 1). 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.

Here we analyze an additional 19 transcriptomes from generalists, molluscivores, and their hybrids (Table 1). First, we crossed a generalist female with a molluscivore male to generate four F_1 hybrids that were collected at 17-20 dpf and extracted RNA from dissected craniofacial tissues. A lab-reared female generalist was used to generate hybrids that was derived from wild caught generalists from Little Lake following one generation of full-sib mating. A lab-reared male molluscivore was used to generate hybrids that was derived from wild caught molluscivores from Little Lake following two generations of full-sib mating.

We performed separate crosses to collect larvae at exactly 8 dpf (190-194 hours after fertilization rather than 8-10 days). We crossed a generalist female with a molluscivore male to generate three F_1 hybrids for whole-larvae RNA extractions. The parents of these hybrids were wild-caught from Osprey Lake. Finally, we extracted whole-larvae RNA from six generalists and six molluscivores collected at 8 dpf. These samples were generated from wild-caught individuals from Osprey Lake and Crescent Pond. In total, we analyzed transcriptomes from 43 individuals that involved four separate rounds of sequencing (Table 1 and S1).

RNA sequencing and alignment

The previously reported 24 transcriptomes were sequenced at the High Throughput Genomic Sequencing Facility at UNC Chapel Hill in April 2017 (McGirr and Martin 2018). The 24 libraries were prepared at the facility using the KAPA stranded mRNA-seq kit (KAPA Biosystems 2016) followed by sequencing on one lane of Illumina 150 paired-end Hiseq4000 (Table 1 and 2).

19 additional transcriptomes were sequenced at The Vincent J. Coates Genomics Sequencing Laboratory at the University of California, Berkeley. All 19 libraries were prepared at the facility using the Illumina stranded Truseq RNA kit (Illumina RS-122-2001) and all sequencing was performed on Illumina 150 paired-end Hiseq4000. Four libraries for RNA extracted from 17-20 dpf hybrid craniofacial tissues were pooled on a single lane and sequenced in June 2017. 15 libraries for whole-larvae RNA samples collected at exactly 8 dpf were pooled across one and three lanes and sequenced in May (n = 9) and July (n = 6) 2018, respectively (Table 1 and S1).

We filtered all raw reads using Trim Galore (v. 4.4, Babraham Bioinformatics) to remove Illumina adaptors and low-quality reads (mean Phred score < 20) and mapped filtered reads to the scaffolds of the *Cyprinodon* reference genome (NCBI, *C. variegatus* annotation release 100, total sequence length = 1,035,184,475; number of scaffolds = 9259, scaffold N50 = 835,301; contig N50 = 20,803; (Lencer et al. 2017)) using the RNA-seq aligner STAR with default parameters (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

199 counts across 24,952 previously annotated features (Lencer et al. 2017) with an average coverage
200 depth of 136 reads (Table S2 and S3).We assessed mapping and count quality using MultiQC
201 (Ewels et al. 2016). We previously showed that there was no difference between generalists and
202 molluscivores in the proportion of reads that map to annotated features of the *Cyprinodon*
203 reference genome (McGirr and Martin 2018). Similarly, here we found no difference in the
204 proportion of reads mapping to features between generalists, molluscivores, and hybrids (Fig.
205 S2; ANOVA, $P = 0.6$), but we did find that fewer reads mapped to features in 17-20 dpf samples
206 than 8 dpf samples (ANOVA, $P = 2.38 \times 10^{-10}$).

207 Since we analyzed RNA from 43 individuals that were sequenced across four different
208 dates and their libraries were prepared with either KAPA or TruSeq stranded mRNA-seq kits, we
209 tested whether a significant amount of between-sample variance in read counts was explained by
210 sequencing date or library preparation kit. We fit linear models (using the lm() function in R) to
211 determine whether normalized counts across individuals were influenced by 1) the number of
212 PCR duplicate reads produced during sequence amplification, 2) the uniformity of coverage
213 across a transcript, or 3) the depth of coverage across GC-rich transcripts. All of these measures
214 could have been influenced by different library preparation methods (Alberti et al. 2014; Van
215 Dijk et al. 2014; KAPA Biosystems 2016). We quantified the number of duplicate reads and the
216 median percent GC content of mapped reads for each sample using RSeQC (Wang et al. 2012).
217 We also used RSeQC to estimate transcript integrity numbers (TINs) which is a measure of
218 potential *in vitro* RNA degradation within a sample. TIN is calculated by analyzing the
219 uniformity of coverage across transcripts. (Wang et al. 2012, 2016). We performed ANOVA to
220 determine whether the proportion of duplicate reads, GC content of reads, TINs, the number of
221 normalized read counts, number of raw read counts, or number of raw fastq reads differed
222 between samples grouped by library preparation method and by sequencing date.

223

224 ***Differential expression analyses and hybrid inheritance of expression patterns***

225 We performed differential expression analyses with DESeq2 (v. 3.5 (Love et al. 2014)). This
226 program fits negative binomial generalized linear models for each gene across samples to test the
227 null hypothesis that the fold change in gene expression between two groups is zero. DESeq2 uses
228 an empirical Bayes shrinkage method to determine gene dispersion parameters, which models

within-group variability in gene expression, and logarithmic fold changes in gene expression. DESeq2 normalizes raw read counts by calculating a geometric mean of counts for each gene across samples, dividing individual gene counts by this mean, and using the median of these ratios as a size factor for each sample. These sample-specific size factors account for differences in library size and sequencing depth between samples. Gene features showing less than 10 normalized counts in every sample in each comparison were discarded from analyses. Differential expression between groups was determined with Wald tests 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). We also used DESeq2 to perform clustering and principal component analyses (Fig. S3).

We conducted pairwise comparisons to identify genes differentially expressed between hybrids vs. parental species, hybrids vs. generalists, hybrids vs. molluscivores, and generalists vs. molluscivores. “Parental species” refers to generalists and molluscivores derived from the same populations as the parents of the hybrid samples. We did not sequence any of the parents crossed to generate hybrids. We defined genes as misexpressed in hybrids if they were significantly differentially expressed between hybrids and the parental species samples. First, we compared whole-larvae gene expression between samples collected at 8 dpf (six generalists, six molluscivores, and three hybrids). All of the 8 dpf samples were sequenced at the Vincent J. Coates Genomic Sequencing Laboratory, University of California Berkeley (VJCGSL UCB) and their libraries were all prepared using the TruSeq stranded mRNA-seq kit. Second, we compared craniofacial tissue gene expression between samples collected at 17-20 dpf (six generalists, six molluscivores, and four hybrids). The generalist and molluscivore samples were sequenced at the High-Throughout Sequencing Facility, University of North Carolina Chapel Hill (HTSF UNC) and their libraries were prepared using the KAPA stranded mRNA-seq kit, while the hybrids were sequenced at the VJCGSL UCB and their libraries were prepared using the TruSeq kit. In order to understand how sequencing at different facilities and using different library prep methods affected the proportion of genes misexpressed between hybrids and parental species at 17-20 dpf, we performed a third set of comparisons between hybrids collected at 8 dpf (sequenced at VJCGSL UCB with TruSeq) and generalists and molluscivores from a previous study collected at 8-10 dpf (sequenced at HTSF UNC with KAPA; (McGirr and Martin 2018)). We measured how many genes were differentially expressed between 8 dpf hybrids vs. 8-10 dpf

parental species than there were differentially expressed between 8 dpf hybrids vs. 8 dpf parental species. This allowed us to estimate an upper-limit on the proportion of genes falsely identified as differentially expressed between 17-20 dpf hybrids and 17-20 dpf parental species due to samples being sequenced at different facilities with different library preparation kits.

To determine whether genes showed additive, dominant, or transgressive patterns of inheritance, we quantified differences in gene expression between hybrids vs. parental species and compared them to genes differentially expressed between generalists vs. molluscivores (Fig. 2). Hybrid inheritance was considered additive if hybrid gene expression was intermediate between generalists and molluscivores with significant differential expression between generalists and molluscivores, respectively. Inheritance was dominant if hybrid expression was significantly different from one parent species but not the other. Genes showing misexpression in hybrids showed transgressive inheritance, meaning hybrid gene expression was significantly higher (overdominant) or lower (underdominant) than both parental species.

Gene ontology enrichment analyses

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 differentially expressed between species and misexpressed in hybrids 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.

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 within proximal non-coding DNA affected expression levels, and *trans*-regulatory variation in cases where genetic variation in factors bound to *cis*-regulatory elements affected expression levels. We also identified genes

showing compensatory regulatory divergence, where *cis* and *trans* regulatory variants interact resulting in similar levels of gene expression between species but biased allelic expression in hybrids (Wittkopp et al. 2004; Landry et al. 2005; McManus et al. 2010).

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 across all filtered reads mapped to annotated features for 17-20 dpf samples and 8 dpf samples 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 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 15,429 phased heterozygous sites across 32 individuals with sequencing coverage ≥ 20× that fell within 3,974 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). We measured ASE across 3,974 genes expressed in parental species and hybrids. A gene was considered to show ASE in hybrids if a phased heterozygous allele within that gene showed consistent biased expression in all hybrid samples (17-20 dpf n = 4; 8 dpf n = 3) and did not show ASE in any of the parental samples (n = 12 for both developmental stages).

We combined information from differential expression analyses and ASE analyses to determine mechanisms of regulatory divergence between species (Table 2). A gene showing allele specific expression in F_1 hybrids that is differentially expressed between parental species is expected to result from *cis*-regulatory divergence (Wittkopp et al. 2004). We identified expression divergence due to *cis*-regulation if genes showed significant differential expression between generalists and molluscivores, significant ASE in hybrids, and did not show ASE in generalists or molluscivores. *Trans*-regulatory divergence can be determined by comparing the ratio of gene expression in parents with the ratio of allelic 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. Gene expression controlled by compensatory variation in parental species is often associated with misexpression in their hybrids (Landry et al. 2005, 2007; Goncalves et al. 2012). We identified gene expression controlled by compensatory regulatory variation if a gene did not show differential expression between generalists and molluscivores, showed significant ASE in hybrids, and did not show ASE in generalists or molluscivores.

Results

Differential expression between generalists and molluscivores

We previously found 1,014 genes differentially expressed in whole-larvae tissue between six generalists and six molluscivores collected at 8-10 dpf (McGirr and Martin 2018). Here we compared gene expression in whole-larvae tissue collected at exactly 8 dpf (190-194 hours after fertilization rather than 8-10 dpf) between six generalists and six molluscivores. We found 700 out of 17,723 (3.9%) genes differentially expressed between species (Fig 1C). 235 of the 700 genes were annotated as zebrafish orthologs and used for gene ontology enrichment analyses. Encouragingly, the only significantly overrepresented ontology was skeletal system morphogenesis (GO:0048705) which matched 11 differentially expressed genes (Table S4).

We previously found 120 genes differentially expressed in craniofacial tissue between species at 17-20 dpf (McGirr and Martin 2018). Here we reexamined gene expression in those same individuals using a more conservative threshold for genes to be included in differential

346 expression analyses (where a gene must show >= 10 normalized counts in every sample included
347 in the comparison). As expected, we found fewer genes differentially expressed using this more
348 conservative threshold (81 out of 13,901 (0.6%); Fig 1E). These 81 genes did not show
349 enrichment for any biological process ontologies.

350

351 ***Hybrid misexpression in whole-larvae tissue***

352 We compared gene expression in whole-larvae tissue collected at 8 dpf from generalist and
353 molluscivore populations (n = 12) with expression in their F_1 hybrids (n = 3) and found that 370
354 out of 17,705 genes (2.1%) were misexpressed in hybrids (Fig. 1D). Slightly more genes showed
355 underdominant inheritance (209; 1.2%) than overdominant inheritance (154; 0.89%; Fig. 3A and
356 C). The magnitude of differential expression was higher for genes showing underdominant
357 inheritance than overdominant inheritance (Fig. S4; Wilcoxon rank sum test, $P = 8.5 \times 10^{-5}$). Of
358 the 370 genes showing misexpression, 138 were annotated as zebrafish orthologs used for gene
359 ontology enrichment analyses. The only significantly overrepresented term was cellular lipid
360 metabolic process (GO:0044255).

361 The majority of genes showed conserved levels of expression with no significant
362 difference between hybrids and parental species (84.9%). In line with other hybrid expression
363 studies (Hughes et al. 2006; Rottscheidt and Harr 2007; Davidson and Balakrishnan 2016), most
364 genes that did not show conserved inheritance showed additive inheritance (399; 2.3%). We
365 found some genes showing evidence for dominance, with 89 (0.51%) showing 'generalist-like'
366 expression patterns and 168 (0.97%) showing 'molluscivore-like' patterns of inheritance (Fig 3A
367 and C).

368

369 ***Hybrid misexpression in craniofacial tissue***

370 We compared gene expression in craniofacial tissue collected at 17-20 dpf from generalist and
371 molluscivore populations (n = 12) with expression in their F_1 hybrids (n = 4) and found extensive
372 hybrid misexpression. More than half of genes (6,590 out of 12,769 (51.6%)) were differentially
373 expressed in hybrids compared to parental species expression (Fig 1F). There was an

374 approximately equal number of genes showing overdominant and underdominant expression in
375 hybrids, with 3,299 (25.83%) genes showing higher expression in hybrids relative to parental
376 species and 3,291 (25.77%) showing lower expression in hybrids (Fig 1F, Fig 3B and D). While
377 there was a similar number of genes showing over- and underdominance, the magnitude of
378 differential expression was higher for genes showing underdominance (Fig. S4; Wilcoxon rank
379 sum test, $P < 2.2 \times 10^{-16}$). Of the 6,590 genes showing misexpression, 2,876 were annotated as
380 zebrafish orthologs used for gene ontology enrichment analyses. Misexpressed genes were
381 enriched for 210 ontologies, including embryonic cranial skeleton morphogenesis (GO:0048701;
382 Table S5 and S6).

383

384 ***Hybrid misexpression is influenced by library preparation and sequencing conditions***

385 All of the 8 dpf samples were sequenced at the same facility using the same library preparation
386 kit. However, the 17-20 dpf generalist and molluscivore samples were sequenced at a different
387 facility than the 17-20 dpf hybrid samples and used a different library preparation kit. We took
388 two approaches toward understanding how sequencing at different facilities and using different
389 library kits may have affected the proportion of genes misexpressed between hybrids and
390 parental species at 17-20 dpf.

391 First, we performed another differential expression comparison between whole-larvae
392 hybrids collected at 8 dpf and whole-larvae parental species that we collected for a previous
393 study between 8-10 dpf (McGirr and Martin 2018). The 8 dpf hybrids were sequenced at the
394 same facility with the same library kit as the 17-20 dpf hybrids, while the 8-10 dpf parental
395 species were sequenced at the same facility with the same library kit as the 17-20 dpf parental
396 species. This design mirrored the comparison we used to estimate 17-20 dpf hybrid craniofacial
397 misexpression, but at an earlier developmental stage (Fig. S5). Whereas comparisons between 8
398 dpf hybrids and parental species sequenced under the same conditions revealed 370 genes (2.1%)
399 misexpressed, comparisons between hybrids and parental species sequenced at different
400 sequencing centers with different library preparation kits suggested that 997 (6%) genes were
401 misexpressed – a 37% increase (Fig. S5). This presents a major caveat to our findings, but does
402 not suggest that all genes showing hybrid misexpression in 17-20 dpf craniofacial tissues are
403 false-positives. Using this estimate of bias to correct for different library preparation methods for

404 our 17-20 dpf samples, we estimate that 19.1% genes were misexpressed in hybrid craniofacial
405 tissue rather than 51.6%.

406 We also investigated whether a significant amount of between-sample variance in read
407 counts was explained by library preparation method or sequencing date. For each sample we
408 quantified the number of normalized read counts, raw read counts, and raw fastq reads. We also
409 estimated the proportion of duplicate reads out of total mapped reads, the median percent GC
410 content across mapped reads, and the uniformity of coverage across mapped reads (median
411 transcript integrity numbers (TINs)). All of these measures could be influenced by different
412 library preparation methods (Alberti et al. 2014; Van Dijk et al. 2014; KAPA Biosystems 2016).
413 Library preparation method was not associated with differences in the number of normalized
414 read counts or median TINs (Fig. 4 A and B; Welch two sample t-test, $P > 0.05$). When we
415 grouped samples by sequencing date rather than library preparation method, we found that the
416 17-20 dpf hybrid craniofacial samples (sequenced 6/17) did not show any difference in median
417 GC content, raw read counts, or raw fastq reads compared to samples sequenced on different
418 dates (Fig S6). However, these samples did show lower proportions of duplicate reads, fewer
419 normalized read counts, and lower TINs compared to samples sequenced on all other dates (Fig.
420 4C-E; ANOVA; $P < 0.01$). TINs quantify the uniformity of coverage across transcripts and are
421 informative as a measure of *in vitro* RNA degradation, which likely suggests that hybrid
422 craniofacial samples experienced more degradation than other samples prior to sequencing.
423 Indeed, lower TIN was significantly correlated with a lower number of normalized counts across
424 samples (Fig. 4F; linear regression; $P = 2.0 \times 10^{-5}$). Given that hybrid craniofacial samples
425 showed lower TINs and lower normalized counts (Fig. 4C and D), we expected to see a bias
426 toward underexpressed genes in hybrids relative to parental species. Instead, we found
427 approximately the same number of genes overexpressed in hybrids (25.83%) as there were genes
428 underexpressed (25.77%; Fig. 1F and 3B).

429 Overall, we found that our estimate of the proportion of genes misexpressed in 17-20 dpf
430 hybrid craniofacial tissue (51.6%) may be biased due to differences in the number of duplicate
431 reads produced by two different library preparation methods (Fig. 4E). We quantified this bias by
432 measuring hybrid misexpression between samples collected at an earlier developmental stage
433 and found that 19.1% of genes were misexpressed in 17-20 dpf hybrid craniofacial tissues after

434 correcting for library preparation biases (Fig. S5). We found that 17-20 dpf hybrid craniofacial
435 tissues likely experienced more *in vitro* RNA degradation than other samples, but this did not
436 produce a bias toward more genes showing underdominant expression in hybrids (Fig. 3B).

437

438 ***Compensatory variation underlies misexpression in hybrids***

439 A gene that is differentially expressed between parental species is expected to also show biased
440 allelic expression at heterozygous sites in F_1 hybrids if expression divergence is due to *cis*-
441 regulatory variation because both parental alleles are exposed to the same *trans*-regulatory
442 environment (Wittkopp et al. 2004). Alternatively, a gene that is differentially expressed between
443 parental species is not expected to show biased allelic expression in hybrids if expression
444 divergence is due to *trans*-regulatory divergence (Wittkopp et al. 2004). If a gene shows similar
445 gene expression levels between parental species but shows biased allelic expression only in
446 hybrids, it may be regulated by compensatory variation, and such genes are likely to be
447 misexpressed in hybrids (Landry et al. 2005). We identified 15,429 phased heterozygous sites
448 across all 8 dpf and 17-20 dpf individuals with sequencing coverage $\geq 20\times$ that fell within 2,761
449 (8 dpf) and 1,911 (17-20 dpf) genes used for differential expression analyses. We estimated
450 allele specific expression (ASE) for these genes and paired these data with patterns of differential
451 expression between parental species to identify genes controlled by *cis*-regulatory elements,
452 *trans*-regulatory factors, and *cis* × *trans* compensatory variation (Table 2).

453 Out of the 700 genes differentially expressed between generalists and molluscivores at 8
454 dpf, 139 contained informative heterozygous sites amenable to allele specific expression
455 analyses. We also measured ASE across sites within 2,770 genes that showed no difference in
456 expression between generalists and molluscivores at 8 dpf. We found more gene expression
457 divergence between species controlled by *trans* regulation (125, 4.3%) than *cis* regulation (14,
458 0.48%; Fig. 5A and C). The magnitude of expression divergence was weaker for genes
459 controlled by *trans* regulation compared to *cis* regulation (Wilcoxon rank sum test, $P < 1.6 \times 10^{-11}$). We found 157 genes (5.4%) that were likely regulated by compensatory mechanisms, which
461 showed ASE only in hybrids and were not differentially expressed between generalists and
462 molluscivores. Of these, nine genes (0.33%) also showed misexpression in hybrids (Fig. 5A and
463 C).

Out of the 81 genes differentially expressed in craniofacial tissue between generalists and molluscivores at 17-20 dpf, 18 contained informative heterozygous sites amenable to allele specific expression analyses. We also measured ASE across sites within 2,387 genes that showed no difference in expression between generalists and molluscivores at 17-20 dpf. We found 10 genes (0.41%) controlled by *trans* regulation and 8 genes (0.33%) controlled by *cis* regulation (Fig. 5B and D). Strikingly, we found 1080 genes (44.81%) that were likely regulated by compensatory mechanisms. In support of this wide-spread compensatory regulation, 581 of these 1080 genes (53.8%) also showed misexpression in hybrids (Fig. 5B and D).

We found many more genes showing ASE in 17-20 dpf hybrid craniofacial tissue than any other samples (Fig. 6A; ANOVA, $P = 2.81 \times 10^{-5}$). Since misexpression is expected in hybrids when gene expression is controlled by compensatory variation between parental species (Landry et al. 2005; Bedford and Hartl 2009), the high number of genes showing compensatory regulation and high number of genes showing ASE in hybrids supports the validity of extensive misexpression in 17-20 dpf hybrid craniofacial tissue. We likely overestimated the amount of misexpression in this tissue because hybrids were sequenced using a different library preparation kit than parental species (see above). However, ASE was estimated by examining allelic ratios in individual samples and should not suffer from this bias. 17-20 dpf hybrid craniofacial tissue was sequenced at the same facility using the same library preparation kit as all of the 8 dpf samples (Table 1 and S1), yet we only found a high number of genes showing ASE in the 17-20 dpf hybrids (Fig 6A).

We tested whether this pattern might be due to higher rates of *in vitro* degradation in hybrid samples (reflected by low TINs), which could increase variance in the abundance of reads at heterozygous sites and bias ASE estimates. Lower TIN was correlated with higher ASE (Fig. 6D; linear regression; $P = 9.04 \times 10^{-14}$). This correlation persisted when 17-20 dpf hybrid craniofacial samples were excluded from the model (Fig. 6E; linear regression; $P = 0.034$). However, the proportion of genes showing ASE was much higher in 17-20 dpf hybrid craniofacial samples than predicted by the latter linear model. Even the lowest TIN for a 17-20 dpf hybrid sample (32.68) predicted a much lower range of genes showing ASE (8.2% -14.1%) compared to the observed range (32.8% - 51.6%). We also estimated ASE again with a higher coverage threshold (>=100 counts supporting each heterozygous allele) to reduce the chances of

494 increased variance affecting binomial tests and still found that hybrid craniofacial samples
495 showed more ASE than other samples (Fig. 6B; ANOVA, $P = 3.85 \times 10^{-4}$).

496

497 **Discussion**

498 Molluscivores show extreme craniofacial divergence relative to their generalist sister species,
499 exhibiting a novel maxillary protrusion and short robust jaws (Fig 1A; Martin and Wainwright
500 2013a; Hernandez et al. 2018). Given the extreme craniofacial divergence observed between
501 molluscivores and their generalist sister-species, we might expect to find genes expressed in
502 hybrids outside the range of either parent species as a result of discordance between alternatively
503 coadapted genes in networks shaping divergent craniofacial morphologies. However, genetic
504 divergence between generalists and molluscivores is low, with only 79 SNPs fixed between
505 species (genome-wide average $F_{st} = 0.08$, $D_{xy} = 0.000166$ (McGirr and Martin 2017; McGirr and
506 Martin 2018)). Despite this low genetic divergence and ongoing gene flow between species, we
507 found misexpression in hybrids at two developmental stages and tissue types. We also measured
508 allele specific expression (ASE) for genes expressed in hybrids and parental species and found
509 evidence for compensatory divergence influencing hybrid misexpression at both developmental
510 stages.

511

512 ***Hybrid misexpression during juvenile development***

513 While many studies on hybrid misexpression search for regulatory divergence in 'speciation
514 genes' associated with sterility and inviability (Malone and Michalak 2008; Renaut et al. 2009;
515 Davidson and Balakrishnan 2016), our results highlight the importance of considering
516 misexpression over multiple early developmental stages and in the context of adaptive
517 morphological traits. We found evidence of slight misexpression in 8 dpf whole-larvae hybrid
518 tissues (2.1% of genes) and extensive misexpression in 17-20 dpf hybrid craniofacial tissues
519 (19.1% of genes after correcting for bias due to library preparation method).

520 There are several reasons why we might expect to find a higher proportion of genes
521 misexpressed in 17-20 dpf hybrid craniofacial tissues relative to 8 dpf whole-larvae tissues. The

522 molluscivore shows exceptional rates of morphological diversification, particularly in
523 craniofacial traits (Martin and Wainwright 2011). Perhaps 17-20 dpf is a crucial developmental
524 window when gene networks shaping these traits become extensively misregulated in hybrids. It
525 is just after this stage that the relative length of the premaxilla, maxilla, palatine, and lower jaw
526 tend to increase more for generalists than molluscivores (Lencer et al. 2016). It is also possible
527 that regulatory changes are compounded throughout development and have cascading effects,
528 resulting in higher rates of misexpression in later stages. Finally, some of the increased
529 misexpression in hybrid craniofacial tissue can likely be attributed to our sampling design. We
530 found that hybrid craniofacial samples showed lower TINs and lower normalized counts (Fig. 4A
531 and D), suggesting that these samples may have experienced more *in vitro* RNA degradation
532 than other samples (Wang et al. 2016). However, under this scenario, we would expect to see a
533 bias toward lower gene expression in hybrids relative to parental species. Alternatively, we
534 found approximately the same number of genes overexpressed in hybrids (25.83%) as there were
535 genes underexpressed (25.77%), suggesting that many genes were overexpressed in hybrids
536 despite potential RNA degradation.

537 We found roughly twice the amount of misexpression in hybrid craniofacial tissues
538 compared to a study of misexpression in whole-body tissue that measured gene expression in F_1
539 hybrids generated between benthic and limnetic lake whitefish (Renaut et al. 2009). These
540 populations also diverged within the past 10 kya and occupy different habitats within lakes
541 (Renaut et al. 2009; Bernatchez 2004). We also found that genes showing underdominance in
542 hybrids showed a higher magnitude of differential expression compared to those showing
543 overdominance in 8 dpf and 17-20 dpf tissues (Fig. S4), a pattern that has also been observed in
544 whitefish (Renaut and Bernatchez 2011) and a generalist/specialist *Drosophila* species pair
545 (McManus et al. 2010).

546

547 ***The consequences of hybrid misexpression***

548 It is unclear whether such extensive gene misexpression in hybrid craniofacial tissues might
549 contribute to intrinsic post-zygotic isolation between generalists and molluscivores. F_2 hybrids
550 exhibiting intermediate and transgressive craniofacial phenotypes showed reduced survival and
551 growth rates in the wild relative to F_2 hybrids resembling parental species (Martin and

552 Wainwright 2013b; Martin 2016), but short-term experiments measuring F_2 hybrid survival in
553 the lab did not find any evidence of reduced survival for hybrids with intermediate phenotypes
554 (Martin and Wainwright 2013b). This was interpreted as evidence that complex fitness
555 landscapes measured in field enclosures on San Salvador with multiple peaks corresponding to
556 the generalist and molluscivore phenotypes were due to competition and foraging ability in the
557 wild (i.e. extrinsic reproductive isolation). However, additional analyses of these data suggest
558 that absolute performance of hybrids may also play a role in their survival. The most
559 transgressive hybrid phenotypes exhibited the lowest fitness, contrary to expectations from
560 negative frequency-dependent disruptive selection (Martin 2016). It is still possible that intrinsic
561 and extrinsic incompatibilities interact such that gene misexpression weakens performance more
562 in the wild than in the lab. However, note that F_1 hybrids used in this study would fall within an
563 intermediate phenotypic position relative to parental trophic morphology whereas field
564 experiments used F_2 and later generation hybrid intercrosses and backcrosses.

565

566 ***Hybrid misexpression is controlled by compensatory divergence***

567 When an optimal level of gene expression is favored by stabilizing selection, compensatory
568 variation can accumulate between species and cause misexpression in hybrids (Landry et al.
569 2005; Bedford and Hartl 2009). We combined results from differential expression analyses with
570 allele specific expression (ASE) results to identify mechanisms of regulatory divergence between
571 generalists and molluscivores. In 8 dpf whole-larvae tissue, we found 4.3% of genes controlled
572 by *trans* regulation, 0.48% controlled by *cis* regulation, and 5.4% controlled by compensatory
573 regulation (Fig. 5B). The low amount of genes controlled by compensatory regulation was
574 reflected by the low amount of genes misexpressed in 8 dpf hybrids. In 17-20 dpf hybrid
575 craniofacial tissues, we found 0.41% of genes controlled by *trans* regulation, 0.33% controlled
576 by *cis* regulation, and 44.81% controlled by compensatory regulation (Fig. 5B). The large
577 number of genes we found controlled by compensatory regulation is consistent with the
578 extensive misexpression observed in hybrid craniofacial tissue, and the majority of genes
579 showing signs of compensatory regulation were also misexpressed in hybrids (53.8%). This
580 independent line of evidence supporting misexpression in 17-20 dpf hybrid craniofacial tissue
581 was not biased by differences in library preparation methods because allele specific expression

582 was estimated by measuring allelic ratios in individual samples. 17-20 dpf hybrid craniofacial
583 tissue was sequenced at the same facility using the same library preparation kit as the 8 dpf
584 samples, yet we only found a high number of genes showing ASE in the 17-20 dpf hybrids (Fig.
585 6). These results are also in line with studies finding widespread compensatory evolution in other
586 systems with greater divergence times between species (Landry et al. 2005, 2007; Takahasi et al.
587 2011; Goncalves et al. 2012; Bell et al. 2013; Mack and Nachman 2017; but also see Fraser
588 2018).

589 We did not expect to find more genes controlled by *trans* regulation than *cis* regulation at
590 either developmental stage. Other studies untangling *cis*- and *trans*-regulatory mechanisms have
591 found that *cis*-variants contribute more to interspecific divergence, while *trans*-variants
592 contribute more to intraspecific divergence (Prud'homme et al. 2007; Lemos et al. 2008; Signor
593 and Nuzhdin 2018). Importantly though, many of the studies supporting this pattern examined
594 interspecific hybrids generated by species pairs with much greater divergence times (Graze et al.
595 2009: *Drosophila melanogaster* and *D. simulans* diverged 2.5 mya; Tirosh et al. 2009:
596 *Saccharomyces cerevisiae* and *S. paradoxus* diverged 5 mya; Shi et al. 2012: *Arabidopsis*
597 *thaliana* and *A. arenosa* diverged 5.3 mya). Given that generalists and molluscivores diverged
598 only within the past 10,000 years and are known to hybridize in the wild, perhaps we should
599 expect to see more *trans* effects for the same reason *trans*-effects contribute more to intraspecific
600 divergence. Namely, the larger mutational target of *trans*-regulatory factors results in the
601 observed excess of intraspecific *trans*-effects (Wittkopp et al. 2008; Emerson et al. 2010;
602 Suvorov et al. 2013).

603

604 ***Conclusion***

605 We found more misexpression in F_1 hybrid cranial tissues relative to whole-larvae hybrid tissues
606 sampled at an earlier developmental stage. This points to divergent evolution of developmental
607 networks shaping novel traits in the molluscivore. It is unclear whether such rampant
608 misexpression causes intrinsic incompatibilities in hybrids within this recent adaptive radiation.
609 Investigating mechanisms regulating gene expression between generalists and molluscivores that
610 result in hybrid misexpression will shed light on whether the variants shaping novel traits may
611 also contribute to reproductive isolation. Examining misexpression across multiple early

612 developmental stages in the context of developing tissues that give rise to adaptive traits can
613 paint a more complete picture of genetic incompatibilities that distinguish species.

614

615 **Data Availability**

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

618

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628

629 **Author Contributions**

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

633

634 **Competing interests**

635 We declare we have no competing interests.

636

637

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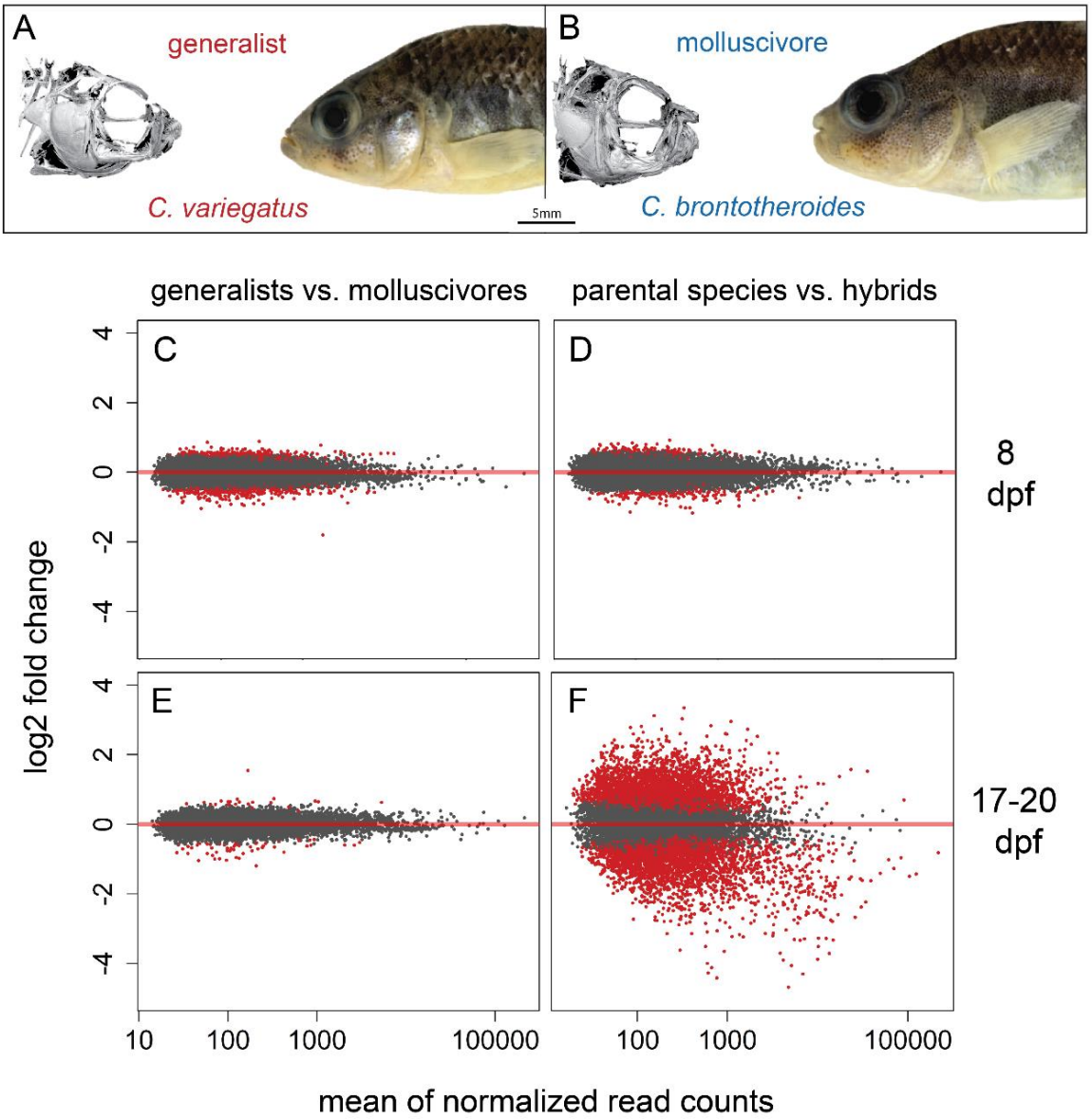
827 **Table 1.** Sampling design for mRNA sequencing. Parental fishes crossed to produce larvae for
828 sequencing were either wild-caught (F_0) or lab-raised over *n* generations (indicated by F_n).

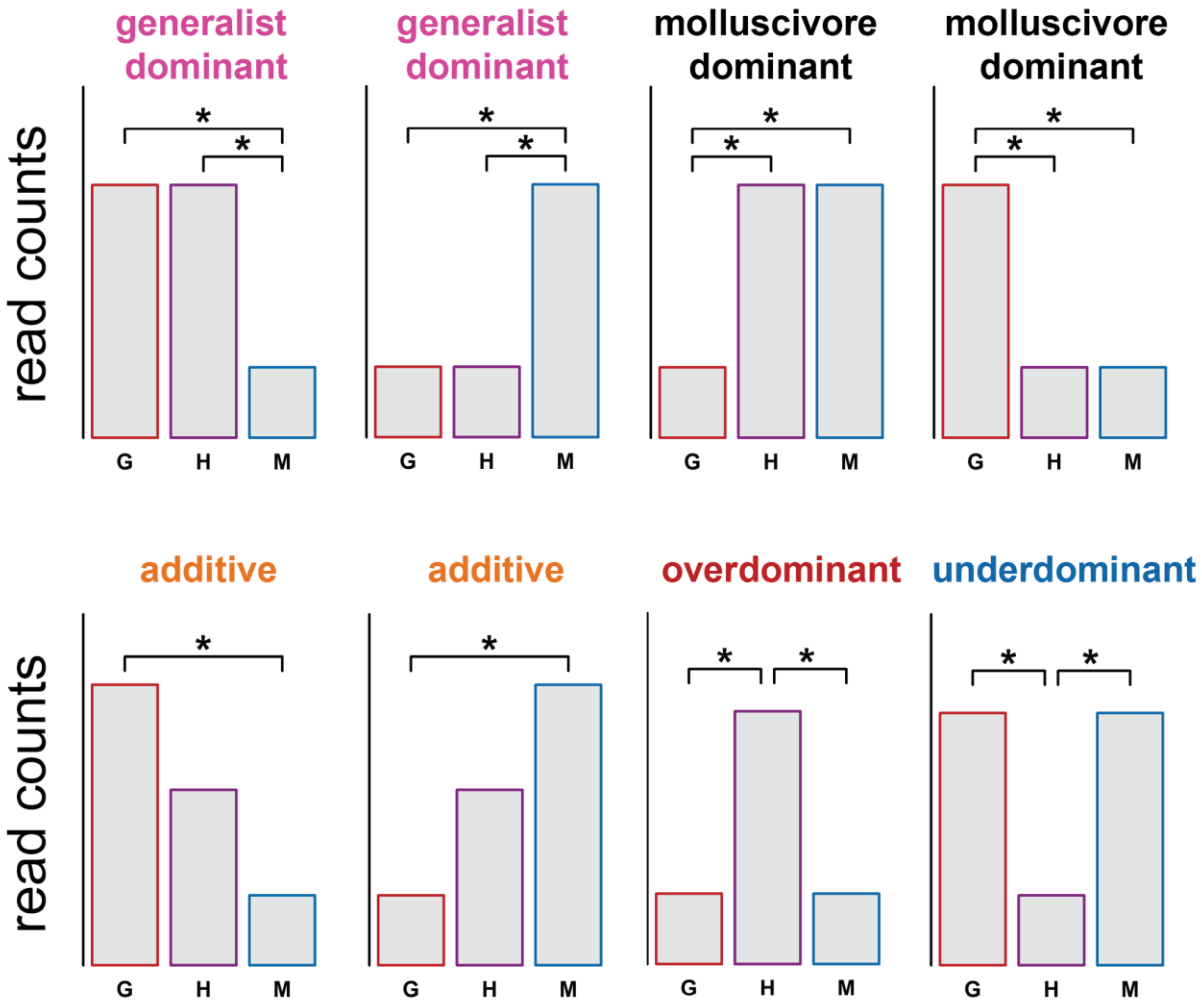
mothers	fathers	offspring sampled	lake population	sequencing round	stage (dpf)
F_0 generalist	F_0 molluscivore	3 hybrids	osprey lake	4	8
F_0 generalists	F_0 generalists	3 generalists	osprey lake	3	8
F_0 molluscivores	F_0 molluscivores	3 molluscivores	osprey lake	3	8
F_0 generalists	F_0 generalists	3 generalists	crescent pond	3	8
F_0 molluscivores	F_0 molluscivores	3 molluscivore	crescent pond	4	8
F_1 generalists	F_1 generalists	3 generalists	little lake	1	8-10
F_2 molluscivores	F_2 molluscivores	3 molluscivores	little lake	1	8-10
F_2 generalists	F_2 generalists	3 generalists	crescent pond	1	8-10
F_2 molluscivores	F_2 molluscivores	3 molluscivores	crescent pond	1	8-10
F_2 generalist	F_3 molluscivore	4 hybrids	little lake	2	17-20
F_1 generalists	F_1 generalists	3 generalists	little lake	1	17-20
F_2 molluscivores	F_2 molluscivores	3 molluscivores	little lake	1	17-20
F_2 generalists	F_2 generalists	3 generalists	crescent pond	1	17-20
F_2 molluscivores	F_2 molluscivores	3 molluscivores	crescent pond	1	17-20

829 **Table 2.** Classification of regulatory mechanisms underlying a gene's expression pattern in
830 hybrids and parental species. 'Yes' or 'no' indicates a significant or non-significant statistical
831 test outlined in the methods (DE = differentially expressed, ASE = allele specific expression).

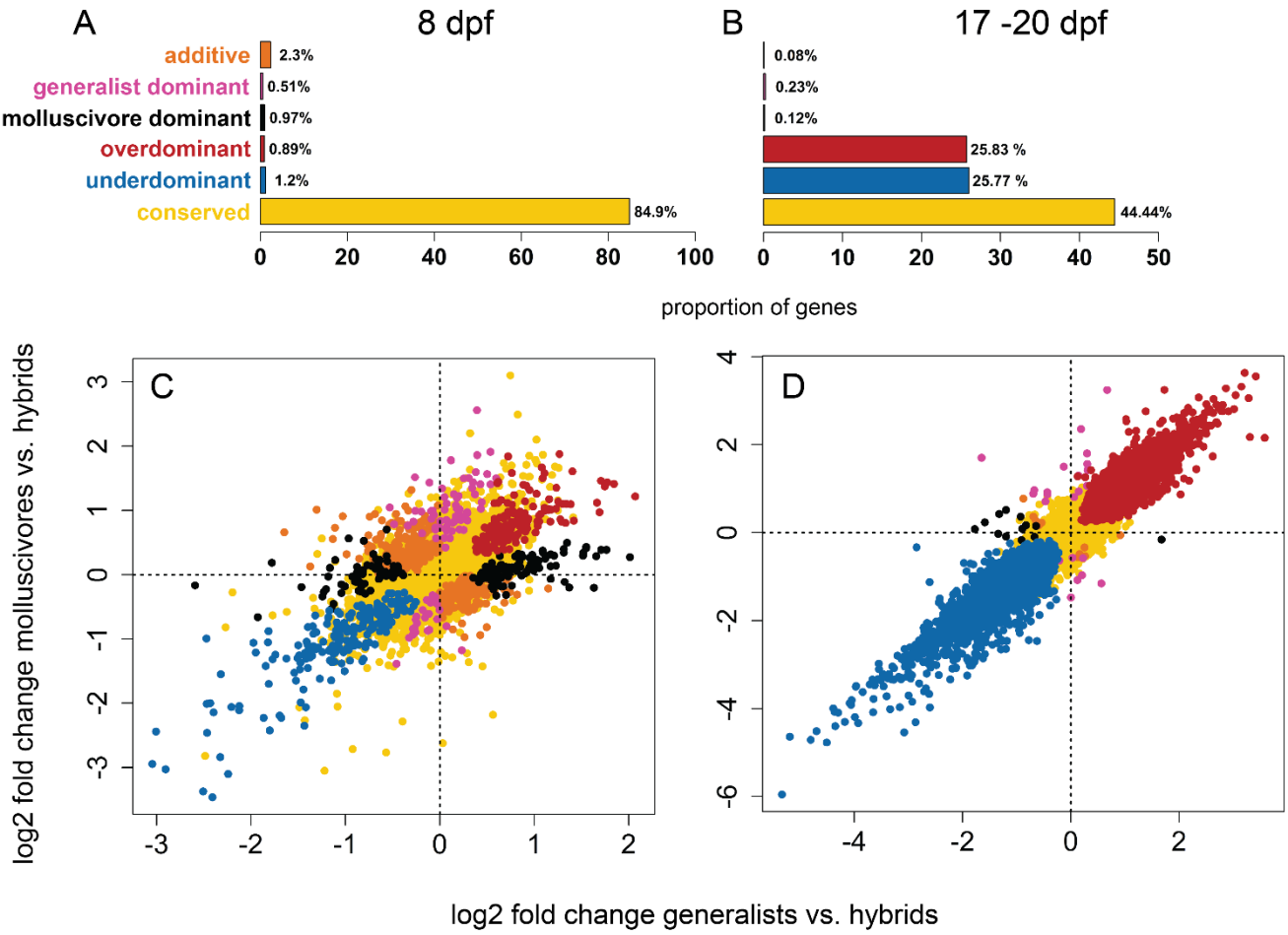
regulatory mechanism	DE between parental species	ASE in hybrids only
cis	yes	yes
trans	yes	no
compensatory	no	yes
conserved	no	no



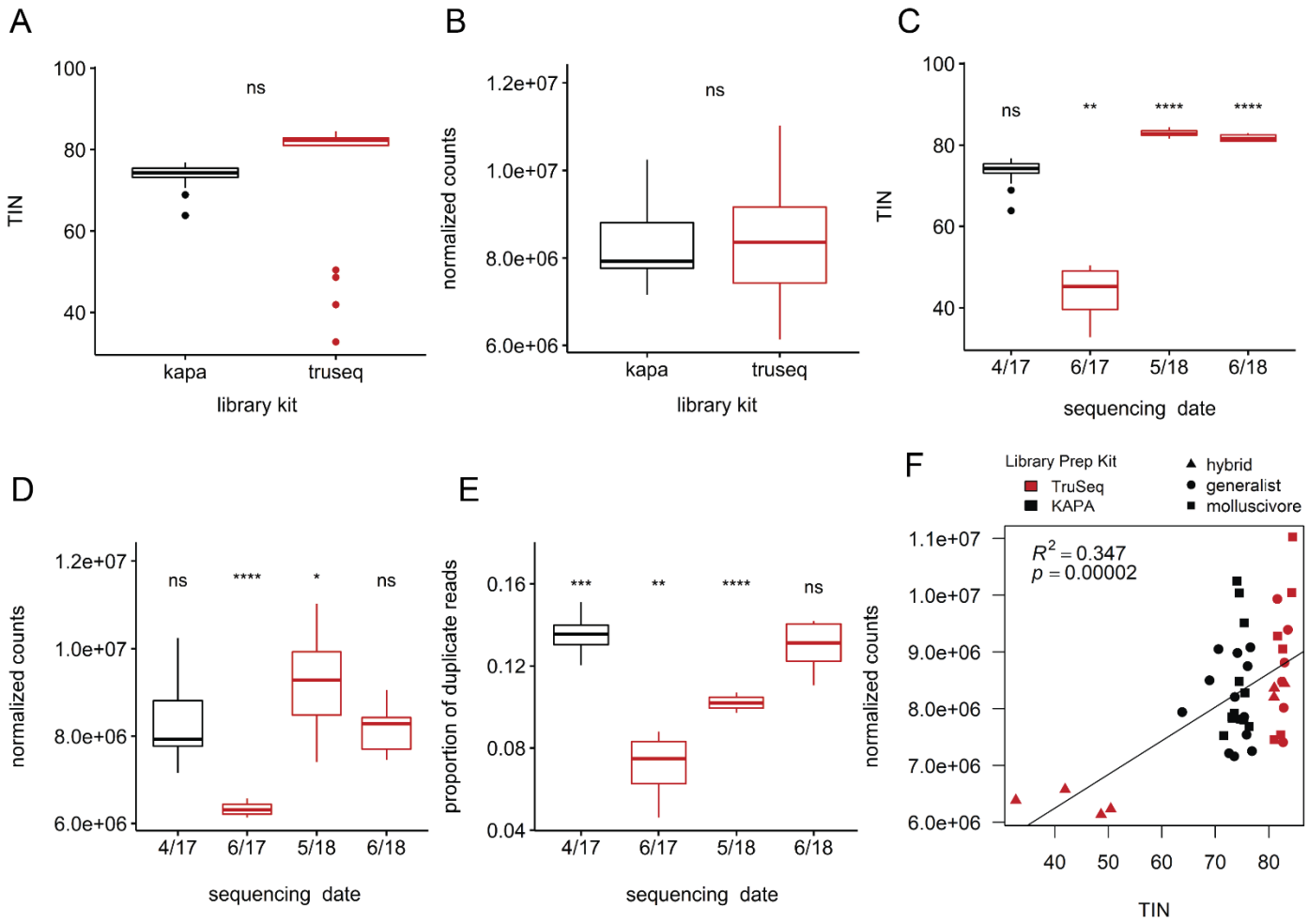
832 **Fig 1. Extensive misexpression in F_1 hybrid craniofacial tissues**. A) *Cyprinodon variegatus* –
833 the generalist. B) *C. brontotheroides* – the molluscivore (μCT scans of the cranial skeleton of
834 each species modified from Hernandez et al. 2018). Variation in gene expression between
835 generalists vs. molluscivores at 8 dpf, D) parental species vs. hybrids at 8 dpf, E) generalists vs.
836 molluscivores at 17-20 dpf, and F) parental species vs. hybrids at 17-20 dpf. Red points indicate
837 genes detected as differentially expressed at 5% false discovery rate with Benjamini-Hochberg
838 multiple testing adjustment. Grey points indicate genes showing no significant difference in
839 expression between groups. Red line indicates a $\log_2$ fold change of zero between groups. Points
840 above/below the line are upregulated/downregulated in molluscivores relative to generalists (C
841 and E) or hybrids relative to parental species (D and F).



842 **Fig 2. Classifying gene expression inheritance in hybrids.** Schematic showing how gene
843 expression inheritance in hybrids was classified. Asterisks indicate significant differential
844 expression between groups. G = generalists, H = hybrids, M = molluscivores.



845 **Fig 3. Gene expression inheritance in hybrids.** The proportion of A) 17,705 and B) 12,769
846 genes showing each class of hybrid gene expression in heritance. Log_2 fold changes in gene
847 expression between molluscivores vs. hybrids on the y-axis and between generalists vs. hybrids
848 on the x-axis for C) 8 dpf whole-larvae samples and D) 17-20 dpf craniofacial samples.



849 **Fig 4. Effects of sequencing facility and library preparation kit.** Boxplots show samples
850 grouped by library preparation method (A and B) or by the date they were sequenced (C-E) and
851 whether samples were prepared using Truseq stranded mRNA library kits (red) or KAPA
852 stranded mRNA library kits (black). There was no difference in A) median transcript integrity
853 numbers (TIN) or B) number of normalized counts between groups prepared with different
854 library kits (Welch two sample t-test, $P > 0.05$). 17-20 dpf hybrid craniofacial samples
855 (sequenced 6/17) showed significantly lower C) TIN, D) normalized read counts, and D)
856 proportion of duplicate reads compared to samples sequenced on other dates (ANOVA; $P <$
857 0.0001 = ****, *** = 0.001, ** = 0.01, * = 0.05). F) Lower TIN was correlated with lower
858 normalized read count.

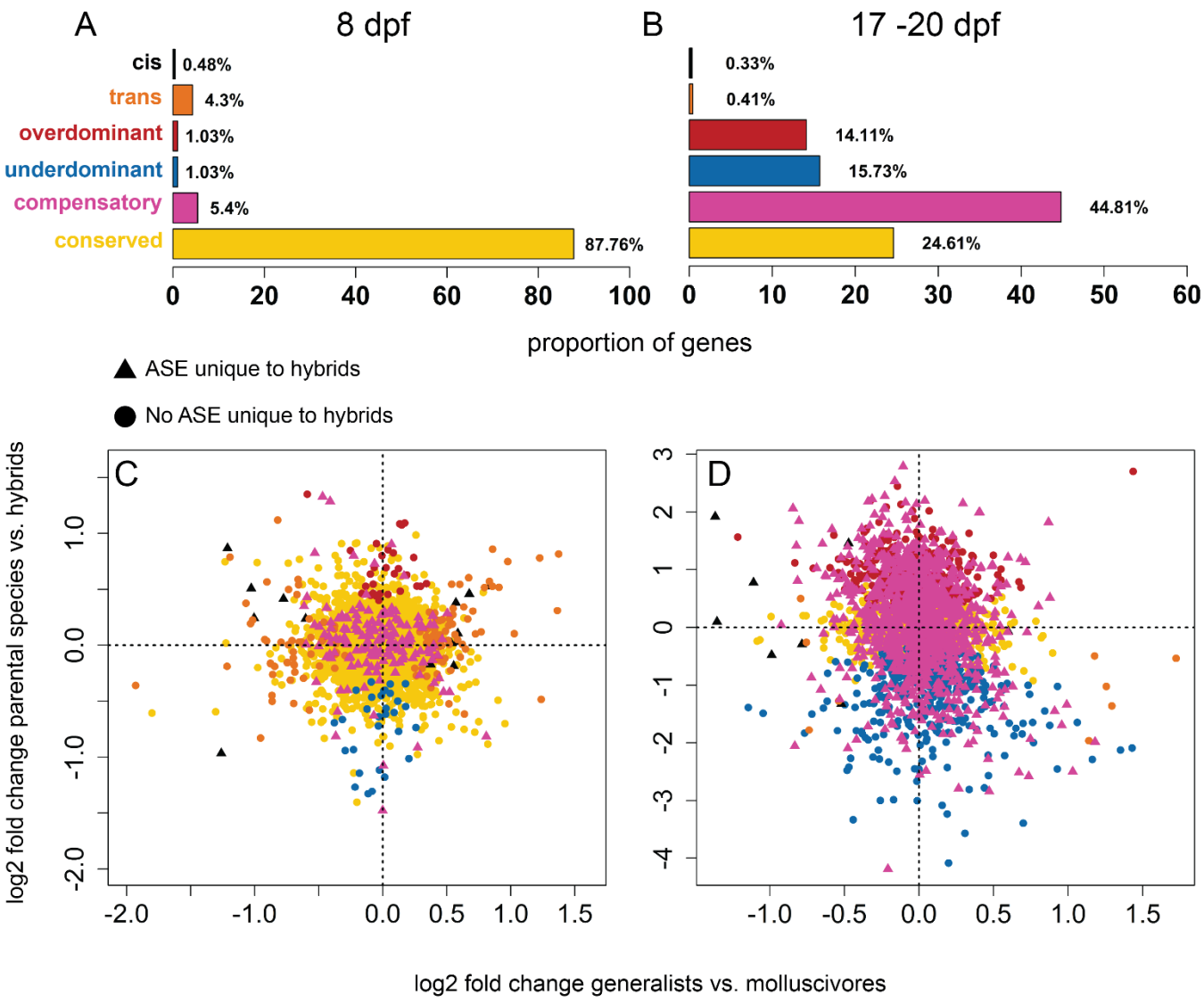


Fig 5. Regulatory mechanisms of expression divergence between generalists and molluscivores. The proportion of genes showing each class of regulatory mechanism across A) 2,909 and B) 2,403 genes containing phased heterozygous sites used for allele specific expression (ASE) analyses. Log_2 fold changes in gene expression between parental species vs. hybrids on the y-axis and between generalists vs. molluscivores on the x-axis for C) 8 dpf whole-larvae samples and D) 17-20 dpf craniofacial samples. Triangle points indicate genes showing significant ASE in all hybrids that did not show ASE in generalists or molluscivores. We found many more genes showing ASE and misexpression in hybrids at 17-20 dpf compared to 8 dpf whole-larvae hybrids.

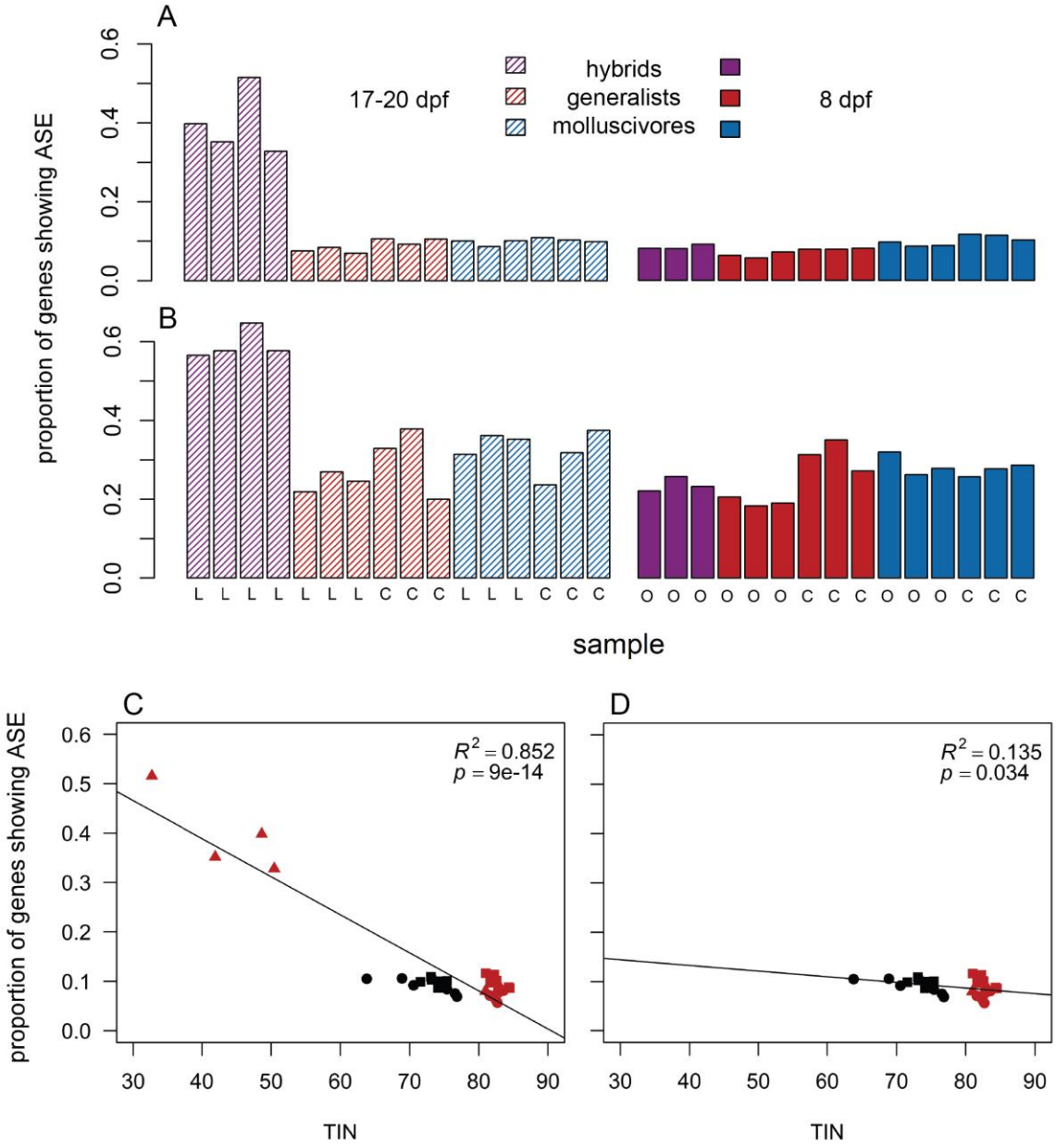


Fig 6. Hybrid craniofacial tissues show high levels of allele specific expression. 17-20 dpf hybrid craniofacial samples (striped purple bars) showed a higher proportion of genes showing significant allele specific expression compared to all other samples using a coverage threshold of A) ≥ 10× reads supporting each heterozygous allele (ANOVA, $P = 2.81 \times 10^{-5}$) and B) ≥ 100× reads supporting each allele (ANOVA, $P = 3.85 \times 10^{-4}$). 8 dpf = solid, 17-20 dpf = striped; hybrids = purple, generalists = red, molluscivores = blue; L = Little Lake, C = Crescent Pond, O = Osprey Lake. C) TIN was significantly negatively correlated with ASE (linear regression; $P = 9.04 \times 10^{-14}$). D) This correlation persisted when 17-20 dpf hybrid craniofacial samples were excluded from the linear model (linear regression; $P = 0.034$). However, the observed proportion of genes showing ASE was much higher in 17-20 dpf hybrid craniofacial samples than predicted by the linear model in D.