The transcriptional state and chromatin landscape of cichlid jaw shape variation across species and environments

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December 2, 2022

Abstract

Adaptive phenotypes are shaped by a combination of genetic and environmental forces, and while the literature is rich with studies focusing on either genetics or environment contributions, those that consider both are rare. Here we utilize the cichlid oral jaw apparatus to fill this knowledge-gap. First, we employed RNA-seq in bony and ligamentous tissues important for jaw opening to identify differentially expressed genes between species and across foraging environments. Our foraging treatments were designed to force animals to employ either suction or biting/scraping, which broadly mimic pelagic or benthic modes of feeding. We found a large number of differentially expressed genes between species, and while we identified relatively few differences between environments, species differences were far more pronounced when reared in the pelagic versus benthic environment. Further, these data carried the signature of genetic assimilation, and implicated cell cycle regulation in shaping the jaw across species and environments. Next, we repeated the foraging experiment and performed ATAC-seq procedures on nuclei harvested from the same tissues. Cross-referencing results from both analyses revealed subsets of genes that were both differentially expressed and differentially accessible in either the pelagic (n=15) or the benthic environment (n=11), as well as loci where differences were robust to foraging environment (n=13). All in all, these data provide novel insights into the epigenetic, genetic, and cellular bases of species- and environment-specific bone shapes, as well as the evolution of phenotypic plasticity in this iconic model system.

1 INTRODUCTION

A major ongoing challenge in biological research is to understand the origin and maintenance of biodiversity, with broad implications in conservation, ecology and evolutionary biology. Traditionally, these endeavors have involved characterizing the forces and mechanisms operating above the organismal level (e.g., selection, environmental change [Schluter 2000; Callaghan et al., 2004; Burns et al., 2009; Siepielski et al., 2014]) or within the organism (e.g., genetic and developmental mechanisms [Kawajiri et al., 2014; Hohenlohe, 2014; Margres et al., 2015]). Understanding the intersection of extrinsic and intrinsic forces (Mccairns & Bernatchez, 2012; van Heerwaarden & Sgrò, 2017; Laitinen & Nikoloski, 2019; Levis et al., 2020) holds significant potential to advance the field.

African cichlids are a hyperdiverse group of fishes that have long been used as an evolutionary model (Kocher, 2004; Seehausen, 2006), and have been especially useful in revealing both the genetic and environmental factors that contribute to biodiversity (McKaye et al., 1984; Sturmbauer & Meyer, 1992; Genner et al., 2004; Ding et al., 2015; Malinsky et al., 2018). In Africa, approximately 2000 cichlid species have arisen over the past ˜5 million years, which is unparalleled compared to the speciation rates of other vertebrates (Seehausen, 2006). Moreover, cichlid diversity is pronounced across several phenotypic axes, including coloration (Seehausen et al., 1999; Maan et al., 2006; Salzburger, 2009), activity levels (Lloyd et al., 2021), as well as reproductive and foraging behaviors (Balshine-Earn & Earn, 1998; Genner et al., 1999; Lopez-Fernandez

et al., 2014). Variation in feeding architecture, which relates to the foraging niche exploited by each species/population, is another critical axis of cichlid diversity (e.g., Cooper et al, 2010). Cichlid craniofacial variation is largely continuous, but there are also examples of extreme or discontinuous variants (reviewed by Powder and Albertson, 2016). In general terms, cichlids partition their foraging niche along a benthic-pelagic ecomorphological axis, with concomitant shifts in foraging anatomy (Young et al 2009; Cooper et al. 2010; Conith and Albertson, 2021). For instance, species inclined toward a benthic mode of feeding tend to have steeply descending facial profiles, small eyes positioned toward the top of their heads, and short, robust oral jaws with closely-spaced, multicuspid teeth optimal for biting and scraping (e.g., Figure 1A,B). On the opposite end of this spectrum, pelagic feeders tend to possess longer, streamlined heads, large eyes, and long, up-turned oral jaws with large, widely spaced teeth optimal for suction/ram feeding (e.g., Figure 1C,D) (Albertson et al., 2003; Cooper et al., 2010).

Significant efforts over the past 20 years have focused on characterizing the genetic basis of cichlid craniofacial variation (e.g., Albertson et al., 2005; Roberts et al., 2011; Powder et al., 2014; Hu & Albertson, 2017; Singh et al., 2017; DeLorenzo et al., 2022). In addition, cichlids have long been a model of phenotypic plasticity (Meyer 1987; Wimberger 1991; Huysseune 1995; Machado-Schiaffino et al., 2014; Schneider et al., 2014; Meuthen et al., 2018; Navon et al., 2020), which is defined as the ability of a single genotype to produce a range of phenotypes in response to environmental inputs. Plasticity is critical for organismal survival in an era of rapid environmental change (Willis et al., 2008; Sih et al., 2011; Gugger et al., 2015; Karasz et al., 2022; Morgan et al., 2022). It can also influence the direction and/or speed of future evolutionary change by exposing new phenotypic and genetic variants to natural selection (Ledon-Rettig et al., 2010 ProcB; McGuigan et al., 2011 evol; Landy et al., 2020 PNAS; Campbell et al., 2021). In spite of its importance across a range of biological disciplines, there are many outstanding questions about plasticity, including its genetic basis and evolutionary potential (Gibert 2017). Plasticity is well documented in cichlids across a range of morphological traits including full body, craniofacial, oral jaw and pharyngeal jaw shapes (Huysseune, 1995; Muschick et al., 2011; Gunter et al., 2013; Parsons et al., 2014; Navon et al., 2020). A notable theme that has come from these data is that closely related species can differ in either their magnitude or pattern of plasticity in response to the same stimulus (Parsons et al., 2014; Navon et al., 2020), suggesting that plasticity itself is an evolvable trait. If true, then plasticity must also have an explicit genetic basis (Kuttner et al., 2014; Lafuente et al., 2018; Diouf et al., 2020); however, understanding plasticity at this level has proven challenging (Gibert 2017).

Previous efforts in our lab have sought to describe the genetic basis of plasticity, and have described roles for Wnt (Parsons et al., 2014) and Hh (Hu & Albertson 2017; Navon et al., 2020) signaling, respectively. In addition, QTL analyses in cichlids have demonstrated the critical importance of the environment in determining the genotype-phenotype (G-P) relationship. Specifically, the genetic basis of variation in multiple hard and soft tissue traits was shown to depend, almost entirely, on the foraging environment in which the animals were reared (Parsons et al., 2016; Zogbaum 2021). Such genetic mapping studies led to the discovery of *crocc2* as an environmentally-dependent regulator of jaw shape (Gilbert & Tetrault et al., 2021). *Ciliary rootlet coiled-coil 2* (*crocc2*) encodes a protein that is a major structural component of the primary cilium's rootlet (Yang et al., 2002). Primary cilia are important mechanosensors that help cells sense and respond to environmental stimuli, but roles of the rootlet in mechanosensing are less clear (Styczynska-Soczka & Jarman, 2015). Notably, this gene was only implicated in regulating cichlid jaw shape in the mechanically demanding benthic/biting environment (Parsons et al 2016; Gilbert & Tetrault et al., 2021), and functional analyses in zebrafish showed that mutations in *crocc2* led to degeneration of cilia, decreased mechanosensing abilities, dysmorphic bone shapes, and mis-regulation of gene networks in bone tissue (Gilbert & Tetrault et al., 2021). Together, this incipient literature has implicated a small handful of genes that contribute to mechanosensitive signal transduction pathways (e.g., Hh) and structural components of the cell (e.g., rootlets) in the evolution and plasticity of cichlid bone shape. Here we seek to advance this research program by taking a genome-wide approach.

In particular, to address the question of genetic and epigenetic control of plasticity in the cichlid feeding apparatus, we utilize two complementary methods of assessing transcriptional output: RNA-seq to analyze

gene expression, and ATAC-seq to assess chromatin accessibility. We focused on an important functional complex - i.e., the interopercle-retroarticular (IOP-RA) complex - which (1) is part of the opercle 4-bar linkage chain, (2) helps to drive lower jaw depression, (3) is comprised of hard and soft tissues, (4) varies among Malawi cichlids in a manner that predicts foraging mode/habitat, and (5) has been shown to be plastic in previous research (Figure 1; Hu and Albertson 2014; 2017; Navon et al 2021). Our goals are to identify genes that are both differentially expressed (DE) and differentially accessible (DA) between species and environments.

2 MATERIALS AND METHODS

2.1 Fish Husbandry

Juvenile-stage cichlids were purchased and housed in 40-gallon glass aquaria at ˜28°C on a 14hr light/10hr dark cycle. Each aquarium was part of a recirculating system, with automated daily water changes and chemical dosing to ensure consistent water quality. Juvenile-stages were chosen to mitigate the confounding effects of dominance. It is also the period of development when all bony elements have formed but animals are still actively growing (Fujimura and Okada, 2007; 2008a; 2008b). Cichlid husbandry follows a protocol approved by the institutional animal care and use committee at the University of Massachusetts.

2.2 Experimental design

To determine how alternative feeding regimes affected gene expression and the *cis* -regulatory system, we used a benthic diet or pelagic diet to impose a power or speed demand, respectively, on the oral jaw. This experimental design was used in two cichlid species, *Tropheops sp.* "red cheek" (TRC) and *Maylandia zebra* (MZ) (Figure 1A-D) to compare species differences in plasticity. Diet treatments for RNA-seq ran for 17 days, and 28 days for ATAC-seq (Figure 1E). The combination of approaches allowed us to examine both differential expression and differential chromatin accessibility between species and environments. That the experiments were performed at two different time-points following the onset of foraging challenges, allowed us to identify loci with effects over extended periods of time.

2.3 Foraging challenges

We separated each species into two diet treatments that would impose different functional demands on the oral jaw. Each 40-gallon tank held 5-10 individuals of a single species that were provided either a benthic diet or pelagic diet. For benthic feeders, we mixed ground high-quality cichlid flake food and freeze dried brine shrimp into 1.5% food-grade agar to create a food "paste" that was spread onto store bought lava rocks. We allowed the rocks to dry overnight and 2 rocks were placed into each benthic tank each day. Pelagic feeders were given ground up flake food and live brine shrimp daily. Animals were given 1 week to train on each diet before the start of the experiment (Figure 1E).

2.4 RNA-seq

We terminated the diet treatment at 17 days and each individual was sacrificed. The interopercle, interopercle-mandibular ligament, and retroarticular (i.e., IOP-RA complex, Figure 1) was dissected from each fish for RNA-seq (left-side) or qPCR (right-side). It is to be noted that this complex is a mix of tissue types (e.g bone, ligament, epithelial tissue). For RNA-seq, 6 samples from each treatment and species were stored in Trizol (Invitrogen) at -80°C, homogenized using a Next Advance Bullet Blender and 5 UFO beads each, and processed using the phenol-chloroform method of RNA extraction, but did not undergo cDNA conversion. We standardized each sample to 500ng total RNA in 50uL and produced libraries using the TruSeq Stranded mRNA Library Prep Kit (Illumina). Any remaining RNA not used for RNA-seq was stored at -80°C. Libraries were sequenced at the University of Massachusetts Medical School Deep Sequencing Core with a HiSeq 4000 with 50 x 50 paired end reads.

Raw reads from RNA-Seq were assessed using FastQC (Andrews, 2010) and ends were trimmed accordingly using cutadapt (Martin, 2011). Cleaned reads were -mapped against the Maylandia zebra genome version

UMD2a (Yates et al., 2020) with Bowtie2 (Langmead and Salzberg, 2012) and a matrix of read counts was generated from the alignments with HT-Seq-count (Anders et al., 2015).

We used edgeR (Chen et al., 2014; Robinson et al., 2010) to identify differentially expressed genes between treatments, as well as those that showed an additive effect between species and environment. Results were groundtruthed by visually comparing normalized counts (counts-per-million; cpm) among treatments. Gene ontology (GO) terms were assigned using biomaRt (Durinck et al., 2005, 2009) via the biomartr package (Drost and Paszkowski, 2017) and enrichment analysis was conducted via topGO (Alexa and Rahnenfuhrer) in the R environment (R Core Team, 2021) using the weight01 algorithm and Fisher's exact test.

Tissues from individuals not used for RNA-seq, were stored in Trizol at -80°C, and homogenized as previously described. We followed the phenol-chloroform RNA extraction method and converted RNA to cDNA for gene expression validation purposes. These samples were standardized to an RNA concentration of 70ng/uL.

qPCR was used to measure gene expression of genes found to be differentially expressed and/or differentially accessible from the RNA-seq and ATAC-seq datasets, and those related to bone development/homeostasis using the comparative CT method. qPCR primer sequences are listed in the supplementary table (Table S1).

2.5 ATAC-seq

We designed an ATAC-seq protocol optimized for bony/ligamentous fish tissues (adapted from Buenrostro et al., 2013; Corces et al., 2017). The ATAC-seq diet treatment was terminated 28 days after foraging treatment began (Figure 1E). Similar to the RNA-seq experiment, each animal had the IOP-RA complex from the left side of the face removed after being euthanized. Briefly, each sample was placed in 3% collagenase II in 5% FBS/DMEM for cell collection for 2 hours. To ensure we collected cells of the appropriate size, we filtered the cells through a 70um strainer. Cell quality and count was confirmed by inverted light microscopy. We collected up to 500,000 cells for each sample. Cells were then lysed to isolate nuclei and underwent a transposition reaction to cut chromatin, and the resulting DNA fragments were purified using a Qiagen MinElute Cleanup Kit. We constructed libraries from the transposed DNA and performed double-sided bead purification to remove large (>1000bp) and small (e.g primer dimer) DNA fragments. The detailed protocol is attached in the supplementary information. Libraries were sequenced in the same manner as RNA-seq libraries.

Raw reads were again aligned against the Maylandia zebra genome using bowtie2, and data were converted to appropriate formats for downstream analyses using samtools (Li et al., 2009) and bedops (Neph et al., 2012), with parallelization enabled by gnu parallel (Tange, 2018). Peaks were called twice using Genrich (https://github.com/jsh58/Genrich): once where we combined replicates from each treatment to provide more power for peak calling for occupancy analysis and once where peaks were called for each replicate individually to improve statistical power for downstream differential accessibility analysis. In both instances, flags were set to filter mitochondrial reads and PCR duplicates before peak calling.

Occupancy and differential accessibility were assessed using DiffBind (Stark and Brown), with the latter analysis calling the edgeR algorithm. Peaks that were enriched for occupancy in one treatment or differentially accessible between any two treatments were annotated by intersecting the genomic coordinates of the peaks with the Maylandia zebra gtf file using bedtools (Quinlan and Hall, 2010), with the requirement that 30% of the peak must overlap with the annotated feature. Peaks identified from the occupancy analysis were then filtered down to those that were enriched in a single treatment only, and examined for enrichment of GO terms using biomaRt (Drost and Paszkowski, 2017) and topGO (Alexa and Rahnenfuhrer) with the latter invoking the classic algorithm and Fisher's exact test.

3 RESULTS & DISCUSSION

3.1 Extensive species differences in gene expression

The cichlid genome has over 25000 protein-coding genes (Conte et al., 2019). Of these, 17525 were expressed in focal tissues, and 5318 were differentially expressed (DE) between our two species, across both foraging conditions, with 2667 upregulated in MZ (relative to TRC - red in Figure 2A) and 2651 upregulated in

TRC (relative to MZ - blue in Figure 2A) (Table 1). Cluster analysis using the top 500 most differentially expressed genes (DEGs) illustrates a clear separation of species (Figure 2B), but differences between foraging environments are less apparent. While MZ shows some separation by environment, TRC does not (more on this below).

Gene ontology (GO) analyses were performed on DEGs, using the annotated MZ genome (ensembl.org), to determine what biological processes were enriched (Figure 2C). Whereas processes related to cell cycle regulation were among the most enriched in MZ, a diversity of other processes were enriched in TRC. These data suggest that there may be divergent modes of bone growth operating in these species at the time when tissues were collected.

3.2 Differential gene expression is rare between foraging environments within species

Craniofacial plasticity is well documented in cichlids (Meyer 1987; Wimberger 1991; van Snick Gray & Stauffer, 2004; Schartau et al., 2009; Navon et al., 2020), and, to an extent, skeletal plasticity has been associated with changes in gene expression (Parsons et al., 2014; Parsons et al., 2016; Navon et al., 2020). However, most work on the genetics of craniofacial plasticity in cichlids has focused on the lower pharyngeal jaw (Gunter et al., 2013; Schneider et al., 2014). Our work complements this body of literature by focusing on skeletal and soft-tissue elements critical for lower oral jaw depression (i.e., IOP-RA complex, Figure 1). While 38 DEGs were detected between foraging environments in MZ, none were detected for TRC (Table 1, Figure 3A,B), suggesting that MZ is more plastic than TRC, and/or plasticity arises earlier in MZ compared to TRC. Of the nearly forty DEGs within MZ, 25 were upregulated in the pelagic environment, whereas 13 were upregulated in the benthic environment (Table 1, Figure 3B), which is consistent with previous data showing greater rates of bone deposition in MZ exposed to a pelagic environment (Navon et al 2020).

Within MZ, genes upregulated in the pelagic environment (relative to the benthic environment) seemed to be largely associated with proliferation, which was reflected by the GO analysis (Figure 3C). For example, *ccna2* is a cyclin that activates *cdk2* and promotes cell cycle progression through both G1/S and G2/M phases (Pagano et al., 1992), and *cks1b* slows the progression of G1/S and can block entry to M phase (Westbrook et al 2007). In addition, *cdc20*regulates metaphase-anaphase transition during mitosis via activation of APC, which targets proteins for degradation (Visintin et al., 1997; Yu 2002). Finally, *cdca5* , also known as sororin, plays an important role in cell proliferation (Fu et al., 2020), and more specifically in the binding of Cohesin to chromatin during cell division (Rankin et al 2005; Schmitz et al. 2007). Data presented here strongly suggests that cell proliferation is critical in mediating skeletal plasticity in this species.

For MZ in the benthic environment, the only GO term enriched for upregulated genes (relative to pelagic) was stress response. In addition, a diversity of other processes are implicated by the other genes, including skeletal muscle changes in response to stimuli (e.g.,*arrdc3b* , Gordon et al., 2019; *myoglobin,* Beyer et al., 1984), and osteoblast proliferation via regulation of *cyclins*(e.g., *per2* , Fu et al., 2005). Our previous work suggested that benthic foraging might be a non-preferred environment for MZ, at least in terms of environmentally-stimulated bone growth (Navon et al., 2020). Here, this assertion is supported by the observation that genes upregulated in the pelagic environment all seemed to contribute to the same biological process – e.g., cell proliferation – with known roles in bone formation/growth (Capecchi et al., 2018; Shekhar et al., 2019; Gao et al., 2020; Du et al., 2021). Alternatively, genes upregulated in the benthic environment were involved in a diversity of processes, including stress response.

Craniofacial plasticity has been noted in both MZ and TRC (Parsons et al 2014; Navon et al., 2020), and so the lack of more extensive DEGs between foraging treatments, especially in TRC, was somewhat surprising. Previous studies have used time points measured in months (Schneider et al., 2014) or years (Gunter et al., 2013) to examine DEGs between foraging environments in the cichlid lower pharyngeal jaw, and so it is possible that we did not allow enough time for a plastic response to manifest. However, we have previously demonstrated a measurable plastic response in bone matrix deposition after 5 weeks (Navon et al., 2020), which should be underlain by an earlier transcriptional response. Indeed, mechanical load has been shown to induce gene expression changes in bone cells in a matter of hours (Raab-Cullen et al., 1994; Mantila Roosa et

al,. 2011; Govey et al., 2015; Kelly et al., 2016). It is therefore also possible that a more robust plastic response in gene expression might occur earlier than the time point sampled here. In addition, FDR is a stringent metric, and it is possible that biologically relevant changes in expression have occurred but are not detected by standard pipelines. In this regard, examining genes with high fold changes, but FDR-values >0.05, might prove fruitful. For example, a transcript that was upregulated in benthic TRC (logFC = 5.44) corresponds to *receptor transporting protein 3*(*rtp3*), which has been linked to human femoral cortical thickness and buckling ratio, as well as hip fractures (Zhao et al., 2010), and another upregulated benthic gene (logFC = 4.83), *poly(ADP-ribose) polymerase 14* (*parp14*), has been shown to regulate cell-cycle progression via Cyclin D1 (O'Connor et al., 2021). Thus, viable candidates for craniofacial bone plasticity may be found just under the threshold set by RNA-seq protocols.

Finally, we stress that a relatively low number of DEGs within species does not preclude more general roles for the environment in determining species-specific bone shapes. As a next step we therefore assessed the effect of foraging environments on DE between species.

3.3 The pelagic environment drives species-specific differences in gene expression and reveals signatures of genetic assimilation.

We have shown previously that foraging conditions can have a marked impact on the genotype-phenotype map. Specifically, quantitative trait loci (QTL) for the same trait map to largely distinct regions of the genome when animals are reared under alternate benthic/pelagic foraging conditions (Parson et al., 2016; Zogbaum et al., 2021). We therefore examined expression differences between species within each environment, and documented a marked imbalance in DEGs. Specifically, when only considering animals exposed to pelagic conditions, we found over 3500 DEGs between species, whereas fewer than 1000 DEGs were detected between species when only comparing animals reared under benthic conditions (Table 1, Figure 4A-C). Additionally, when comparing environment-specific DEGs to the total dataset (i.e., combining both environments), we found that over 2500 genes from pelagic animals were represented in the global comparison, whereas only 155 genes from benthic animals overlapped between datasets (Table 1, Figure 4C). These data underscore the importance of environmental context in determining the genetic basis of species-specific bone shapes (Parsons et al 2016; Zogbaum et al, 2021). More specifically, they suggest that the pelagic foraging environment is driving species differences in gene expression within the IOP-RA functional complex.

This trend is drawn out when comparing genes from an additive model (S+E), whereby DEGs were detected at the level of both species and foraging environment (Table S2; Figure 5A). When illustrated in a heatmap, these data support the assertion that species differences in gene expression are driven by the pelagic environment, and reveal patterns consistent with either genetic accommodation or assimilation. Genetic assimilation is a mechanism by which plasticity is lost over evolutionary time as genetic variation that facilitated plasticity in an ancestral population becomes fixed as descendent populations adapt to a specific environment (reviewed by Pigliucci et al., 2006). If we assume that plasticity is ancestral, evidence for genetic assimilation is apparent in several gene clusters (denoted by pink dots, Figure 5A), whereby TRC expression levels are indistinguishable between foraging environments and match those of benthic MZ. Consistent with previous data many of the DEGs identified by this model contribute to cell cycle regulation – e.g., *cdc20* , *cdca5* , *cdca8* ,*ccne2* , *ccnb1* , *ccnb2* , *ccnf* . A list of all the DEGs in this model can be found in Table S2.

Alternative to genetic assimilation is genetic accommodation, or an increase in genetic plasticity over evolutionary time. We cannot rule out that this is the case, as it is possible that plasticity has been enhanced beyond the ancestral condition in MZ. Regardless, the main conclusion to be drawn from these data is that the evolution of plasticity in this system may be traced to divergent patterns of gene expression associated with cell cycle regulation.

We next performed GO analyses for DEGs between species in each foraging environment. When considering animals reared in the pelagic foraging environment, GO analysis revealed a diversity of biological processes; however, those associated with cell division were among the most enriched in MZ, whereas translation and cell differentiation were among the most enriched processes in TRC (Figure 4D). For animals reared in

the benthic environment, comparatively fewer biological processes were enriched in general, consistent with fewer DEGs being identified. Similar to pelagic fishes, this analysis found enrichment of cell cycle genes in MZ, and cell differentiation in TRC (Figure 4E). While a greater number of DEGs, contributing to a larger number of biological processes, underlie species-specific differences in the pelagic environment, there are notable consistencies between environments. Specifically, an increase in cell number seems to be important for skeletal growth in MZ, whereas cell differentiation may shape growth in TRC at the time points when tissues were collected.

Unsurprisingly, enriched GO terms for the additive model are similar to those for MZ in the pelagic environment, and include cell cycle, cell division, and chromosome segregation (Figure 5B). In addition, this analysis found enrichment of cytoskeleton organization, which is critical to many cellular functions relevant to bone formation and plasticity, including mechanotransduction (Gunst & Zhang, 2008), and primary cilia formation (Mirvis et al., 2018), which we and others have found to be necessary for load-induced bone formation (Chen et al., 2016; Moore et al., 2019; Gilbert & Tetrault et al., 2021).

3.4 Species-specific differences in chromatin accessibility are influenced by foraging conditions

Areas of the genome that contain open chromatin are more accessible to transcriptional machinery and therefore able to increase gene expression, whereas closed chromatin sites are less accessible for transcription. The differences in accessibility (e.g., between species or environments) are considered differentially accessible (DA), and may be due to either genetic (e.g., deletion of a TF binding site) or epigenetic (e.g., methylation changes) processes. When comparing species across both environments, we identified 10770 areas of accessible chromatin, and of these 297 were DA. Note that many genes have contain more than one accessible chromatin peak (e.g., Figure 6B,D). The number of DA loci was therefore considerably less than the number DE loci, which may reflect differences in the timing and/or nature of each experiment. In addition, we did not observe a marked bias in one environment versus the other in terms of the number of differentially accessible genes (DAGs), with 114 DAGs identified in pelagic fishes and 157 in benthic fishes (Table 1). These data suggest that species differences in DA are not being driven by one environment at the time when tissues were collected.

The overlap between RNA-seq and ATAC-seq datasets implicates loci acting over extended periods (i.e., at both timepoints) following the onset of foraging trials (Figure 6A, Table S3). In all, we identified 15 genes that were both DE and DA in fishes reared in the pelagic foraging environment, and 11 from the benthic environment. We also identified 13 genes that were DE and DA in both environmental conditions, which suggest that their expression is robust to differences in the environment The direction of DE and DA across all genes was generally consistent (Table S3). In particular, out of 39 loci, the polarity of DE and DA was similar in 34. Differences in the other 5 could be due to DA being associated with the binding of a repressive transcription factor. Alternatively, given that RNA-seq and ATAC-seq experiments were performed at different time points, it is also possible that expression of these factors may oscillate over time.

A few of the genes in these overlapping datasets have been implicated in craniofacial development, including *KIAA0586* (also known as*talpid3*), which is essential for primary cilia formation and Hedgehog (Hh) signaling (Schock et al., 2016). The identification of*KIAA0586* is especially notable given previous work from our lab, which has demonstrated important roles for genes associated with the primary cilium-Hedgehog molecular mechanism in species-specific shaping and plasticity of craniofacial bones in cichlids and zebrafish (Hu & Albertson, 2014; Navon et al., 2020; Gilbert et al., 2021; Zogbaum et al., 2021). While *KIAA0586* is well-studied in the context of early craniofacial patterning (reviewer by, Schock et al., 2016), roles at latter stages, including growth and plasticity, have not been explored. Taken together, multiple independent experiments in the cichlid system support the thesis that the primary cilium-Hedgehog "signal transduction machinery" is an important and evolvable mechanism for shaping the craniofacial skeleton. As opposed to*KIAA0586* , other genes in this dataset are largely new to the field of craniofacial biology, but implicate biological processes important in bone patterning, formation, growth and homeostasis, including chromatin remodeling (e.g *actr6* [Yoshida et al., 2010]), cell signaling (e.g *asb5* [Yoshioka et al., 2006],*etv4* [Mao et al., 2009]), and cell growth (e.g *impdh1*[Chang et al., 2015]).

Determining whether or not these factors are DA due to genetic or epigenetic factors would be a fruitful line of future study. Resequencing a panel of cichlids around DA peaks would allow us to assess whether any indels or SNPs might underlie differences noted here. In addition, the co-occurrence of DA peaks and CpG islands would suggest that differential DNA methylation might be driving differences in expression. For example, the DA peak associated with *KIAA0586*expression is in intron 7-8 (Table S3), which is large and contains several predicted CpG islands, although none overlap with the DA peak (Figure 6C).

3.5 Validation of select candidate genes by qPCR

We validated a subset of genes from this analysis with qPCR, focusing on those that overlapped in the RNA-seq/ATAC-seq datasets (Figure S1A-E). Trend across transcript counts (i.e., counts per million, cpm) and qPCR expression were generally equivalent, but we note that qPCR picked up significant differences in gene expression that did not meet the threshold for RNA-seq significance. For example, *actr6,* which plays important roles in heterochromatin formation in yeast,*Drosophila* and vertebrates (Ohfuchi et al., 2006), was DE expressed between benthic and pelagic TRC according to qPCR, but not by cpm (Figure S1A). In addition, *gnmt* , a methyltransferase involved in the methionine pathway, which is linked to bone density (Wang et al., 2011; Wang et al., 2011; Ables et al., 2012; Vijayan et al., 2014), exhibits an expression pattern that is similar to cpm, but it is also plasticity expressed among Maylandia reared in different environments (Figure S1D). We also validated the expression of *cdc20* , a gene that is involved in cell-cycle regulation (Visintin et al., 1997; Yu 2002), and was significantly DE between benthic and pelagic MZ. Expression of this gene via qPCR did not quite reach significance at the 0.05 alpha level, but exhibited a lot of variation across samples, and showed a similar pattern that was trending toward significance (p=0.17, Figure S1E).

4 CONCLUSIONS

A number of general themes emerge from these data. First, they provide clear support for the hypothesis that foraging environment influences the genotype-phenotype map for craniofacial skeletal traits (Parsons et al., 2016; Navon et al., 2021; Zogbaum et al., 2021). More specifically, our data suggest that pelagic foraging "drives" species- and environment-specific DE. This may seem counterintuitive as diets that involve large/hard prey items are generally considered to be the more mechanically demanding compared to small/soft food (Muschick et al., 2011; Gunter et al., 2013; Hulsey et al., 2020). However, Navon et al (2020) showed that in MZ bone matrix was deposited at a fast rate under pelagic foraging conditions, and speculated that suction feeding imposes mechanical load on the feeding apparatus as animals repeatedly open and protrude their jaws. Our data support this assertion, and thus we consider the foraging treatments utilized here to challenge the feeding apparatus in two distinct ways (compared to a "standard" flaked food diet); our benthic treatment was designed to impose high amplitude but low frequency loading onto the feeding apparatus as animals scrapped food from rocks, whereas our pelagic treatment translated to higher frequency but lower amplitude loading as animals repeatedly protruded their jaws to gather small food items.

Our data also detected evidence for genetic assimilation. In particular, when considering loci that were DE between species + environments, patterns in MZ benthic fish resembled those across TRC. *Tropheops*species, including *sp.* "red cheek", are generally found in a benthic environment (Ribbink et al., 1983), and may have lost a degree of plasticity as they evolved to specialize on benthic food items. MZ on the other hand are true generalists in the sense that they routinely foraging from both the benthic and pelagic zones (Ribbink et al., 1983). While plasticity has been noted in TRC (Parsons et al., 2014; Navon et al., 2020), our data suggest that MZ may be more plastic than TRC in that they mount a more pronounced transcriptional response, at least at the time point analyzed in this study.

Cell cycle regulation consistently appeared in GO analyses, describing species differences, as well as plasticity within MZ. This implicates cell proliferation as an important biological mechanism of species- and environment-specific bone growth in cichlids. This observation is notable as our previous work has implicated Hedgehog signaling in the evolution and plasticity of the cichlid jaw, including the IOP-RA complex (Hu & Albertson, 2014; Parsons et al., 2016; Navon et al., 2020). While canonical members of the Hedgehog signaling pathway were not significantly DE or DA in this dataset (although *KIAA0586*regulates the signal,

Schock et al., 2016), cell proliferation is well-known to be regulated by this pathway (St. Jacques et al., 1999; Tiet et al., 2006; Sun & Deng, 2007; Zaman et al., 2019), providing a potential cellular mechanism through which variation in Hedgehog signaling leads to differences in bone shape among and within cichlid species.

Finally, with these large overlapping genome-wide datasets, we were able to narrow down thousands of DEGs to roughly two dozen that were both DE and DA. Given that each experiment was conducted at a different time point, this reduced dataset points to loci whose expression is important for species divergence over extended periods of time. Among these were genes that were both sensitive and robust to the environment. Notably, nearly all of these genes are new to the field of bone biology, and while some encode known effectors of well studied signaling pathways (e.g., interleukin/Wnt, Talpid/Hh) and cell behaviors (e.g., Casp6/apoptosis, Impdh1b/cell-cycle), others implicate largely novel mechanisms (e.g., Gnmt/methionine cycle). Thus, this work establishes a robust foundation for future studies into how genotype and the environment combine to influence bone formation, remodeling, and evolution.

ACKNOWLEDGEMENTS

We would like to thank current and former members of the Albertson Lab for helpful comments and suggestions on various aspects of this project. This project was funded by an NIH grant awarded to R.C.A (DE026446).

DATA ACCESSIBILITY

All data are available on GitHub at https://github.com/tetra22e/Genomics2022

BENEFITS-SHARING

Benefits from this research accrue from the sharing of our data and results via open access publishing, and on public databases as described above.

AUTHOR CONTRIBUTIONS

R.C.A. and E.M.T. designed the experiments and wrote the manuscript. E.R.T. and C.M. designed the ATAC-seq protocol and executed all sequencing experiments. E.R.T. and B.A. performed the qPCR validation. E.R.T. and J.S. analyzed the RNA- and ATAC-seq data. R.C.A., E.R.T., J.S., and C.M. interpreted the results. J.S. and C.M. contributed to editing the manuscript.

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TABLE 1: Number of genes that are DE or DA from RNA-seq and ATAC-seq datasets, respectively. In each comparison/model, the total numbers are provided, followed by the numbers that are upregulated in a particular species or environment.

RNA-seq (DE)	MZ:TRC	MZB:TRCB	MZP:TRCP	MZB:MZP	TRCB:TRCP	S+E
Total	5318	984	3761	38	0	128
MZ	2667	549	1927	N/A	N/A	N/A
TRC	2651	435	1834	N/A	N/A	N/A
Benthic	N/A	N/A	N/A	13	0	27
Pelagic	N/A	N/A	N/A	25	0	101
ATAC-seq (DA)	**MZ:TRC**	**MZB:TRCB**	**MZP:TRCP**	**MZB:MZP**	**TRCB:TRCP**	
Total	297	157	114	0	0	
MZ	202	118	60	N/A	N/A	
TRC	95	39	54	N/A	N/A	
Benthic	N/A	N/A	N/A	0	0	
Pelagic	N/A	N/A	N/A	0	0	

FIGURES

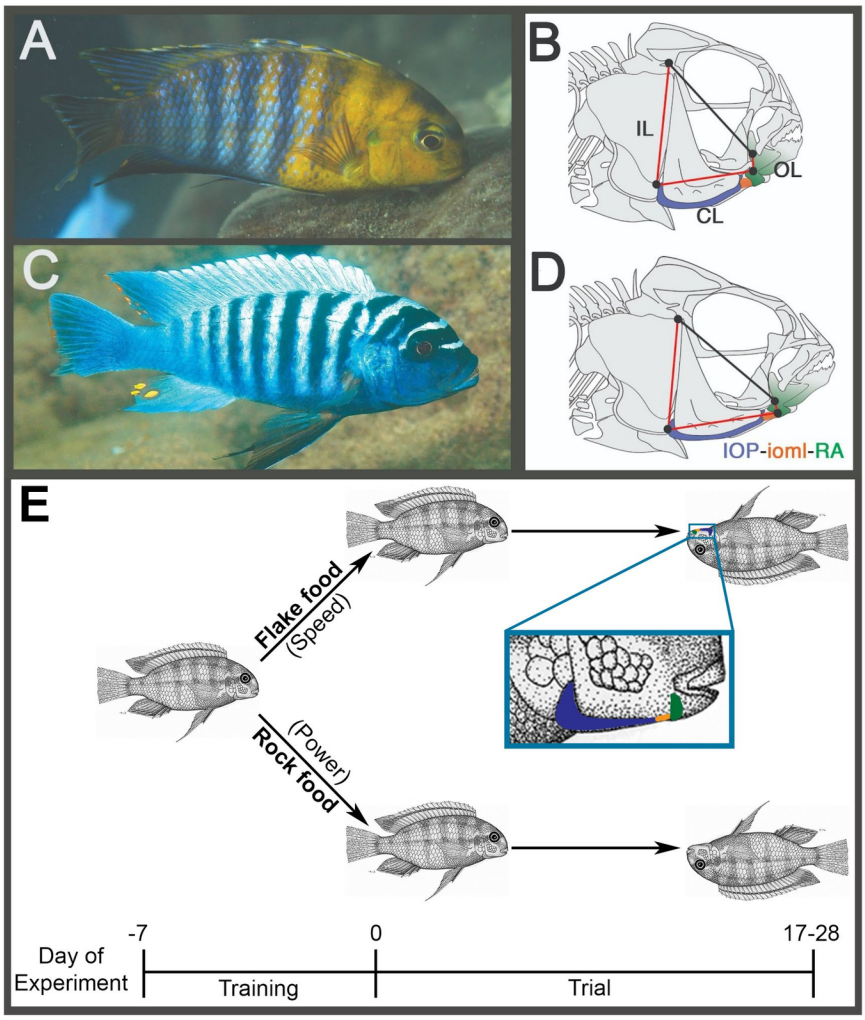

FIGURE 1: African cichlids from Lake Malawi display differences in foraging anatomy. (A) *Tropheops sp* . "red cheek" (TRC) has a more downturned oral jaw apparatus, and a steeply sloping craniofacial profile, adapted for benthic foraging via scraping/biting/nipping. (B) A schematic of the opercle 4-bar linkage chain, which is critical for lower jaw depression, is shown for TRC. (C) *Maylandia zebra* (MZ) is characterized by a more upturned oral jaw, better suited for pelagic feeding via fast jaw rotation. (D) A schematic of the opercle 4-bar linkage chain is shown for MZ. Relative to TRC, MZ possesses a longer coupler link (CL) and shorter output link (OL). (A, C) Images courtesy of Ad Koning at Cichlid Press. (B, D) Red bars indicate movable linkages - input link (IL), CL, and OL - while the black bar represents the fixed link. Blue depicts the interopercle (IOP) bone, and green shows the retroarticular process (RA) of the lower jaw, while orange is the interopercle-mandibular (ioml) ligament that connects the two bones. Throughout the text we refer to this as the IOP-RA functional complex. (E) Experimental schematic in which we fed cichlids either flake or rock food for 17 days (RNA-seq) or 28 days (ATAC-seq), with a 1 week training period. Inset shows an external view of the IOP-RA complex dissected for all experiments.

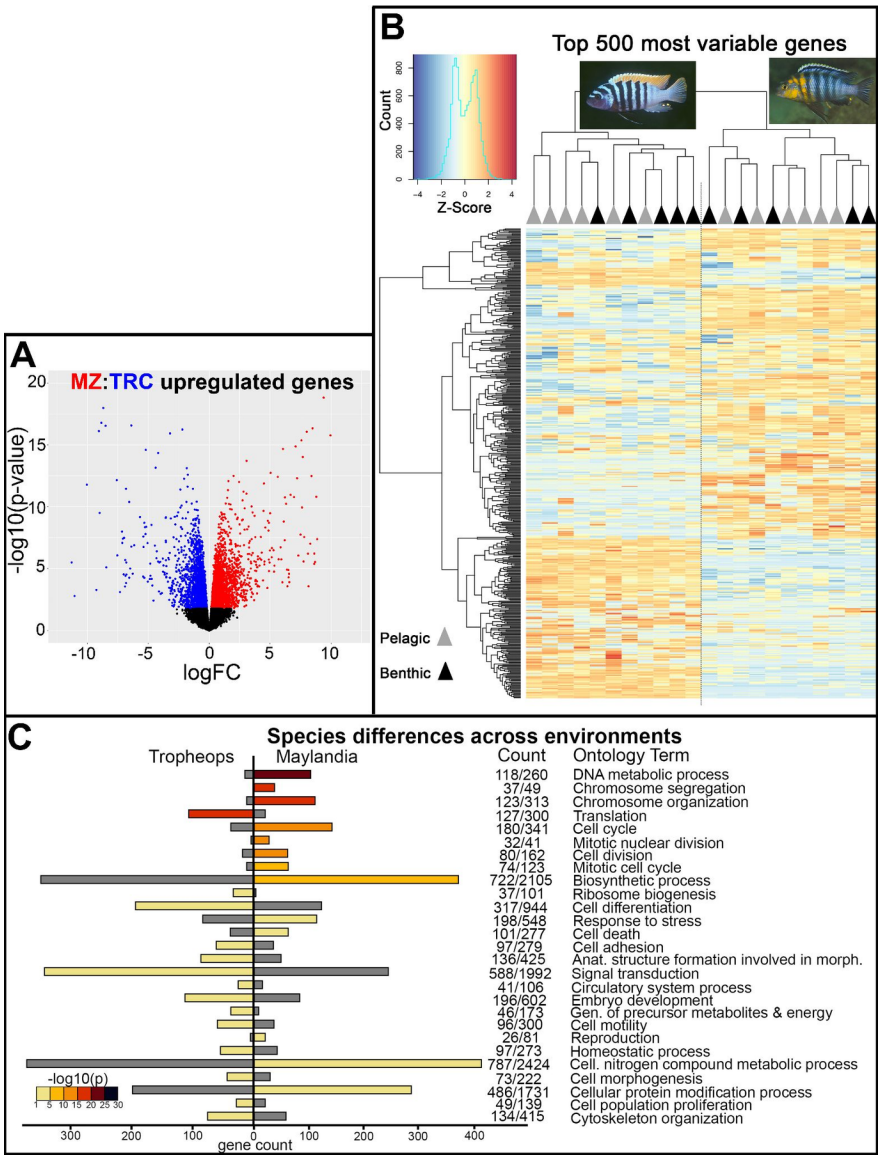

FIGURE 2: Differential expression between species is more robust than between foraging environments. (A) Volcano plot of the pairwise comparison between MZ and TRC across environments, showing a large number of DEGs (n=5318) with roughly equal numbers of significantly upregulated genes between species (n=2667 MZ; n=2651 TRC). Given the nature of the comparison, genes considered upregulated in MZ are downregulated in TRC, and vice versa. Red indicates upregulated genes for MZ, blue depicts genes upregulated in TRC, while black represents genes that do not meet the significance threshold of <0.05 FDR. (B) Heatmap of the top 500 most variable genes in the RNA-seq dataset is shown. Individuals from the pelagic foraging treatment are labeled with gray triangles, while benthic individuals are labeled with black triangles. Species cluster together, but there is less obvious structuring by foraging environment, although MZ segregate by environment more so than TRC. Photographs courtesy of Ad Koning at Cichlid Press. (C) Enriched GO terms associated with genes upregulated in the MZ:TRC comparison across environments. Colors are representative of the -log10(p-value). Gray bars indicate no significance. Gene counts are given as a total for both species along with the corresponding GO term.

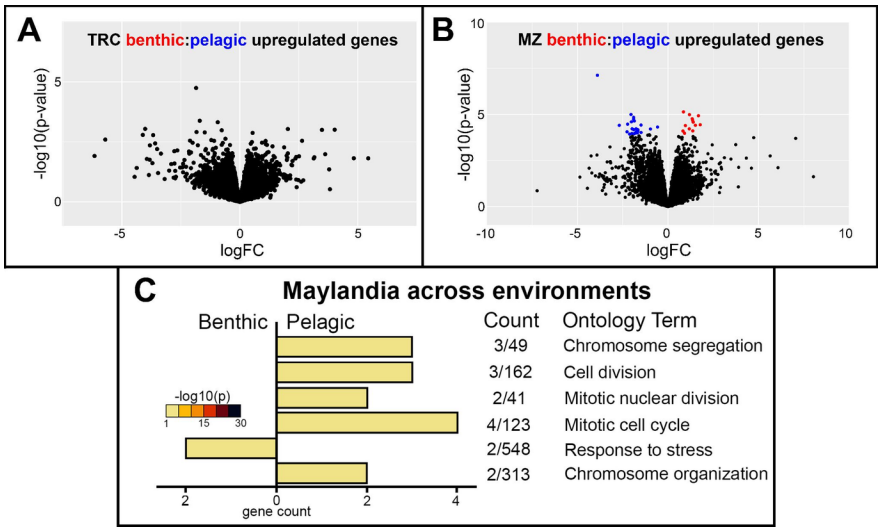

FIGURE 3: Little differential expression is detected between foraging environments. (A) Within TRC, the volcano plot shows no DEGs between environments. (B) Within MZ, a small number of DEGs (n=38) were detected between environments, with more upregulated in the pelagic (n=25; blue) versus the benthic (n=13; red) environment. Black dots represent genes that are not significantly DE at FDR <0.05. (C) Within MZ, more GO terms were returned for animals exposed to pelagic versus benthic environments. Cell-cycle regulation features prominently in the pelagic environment.

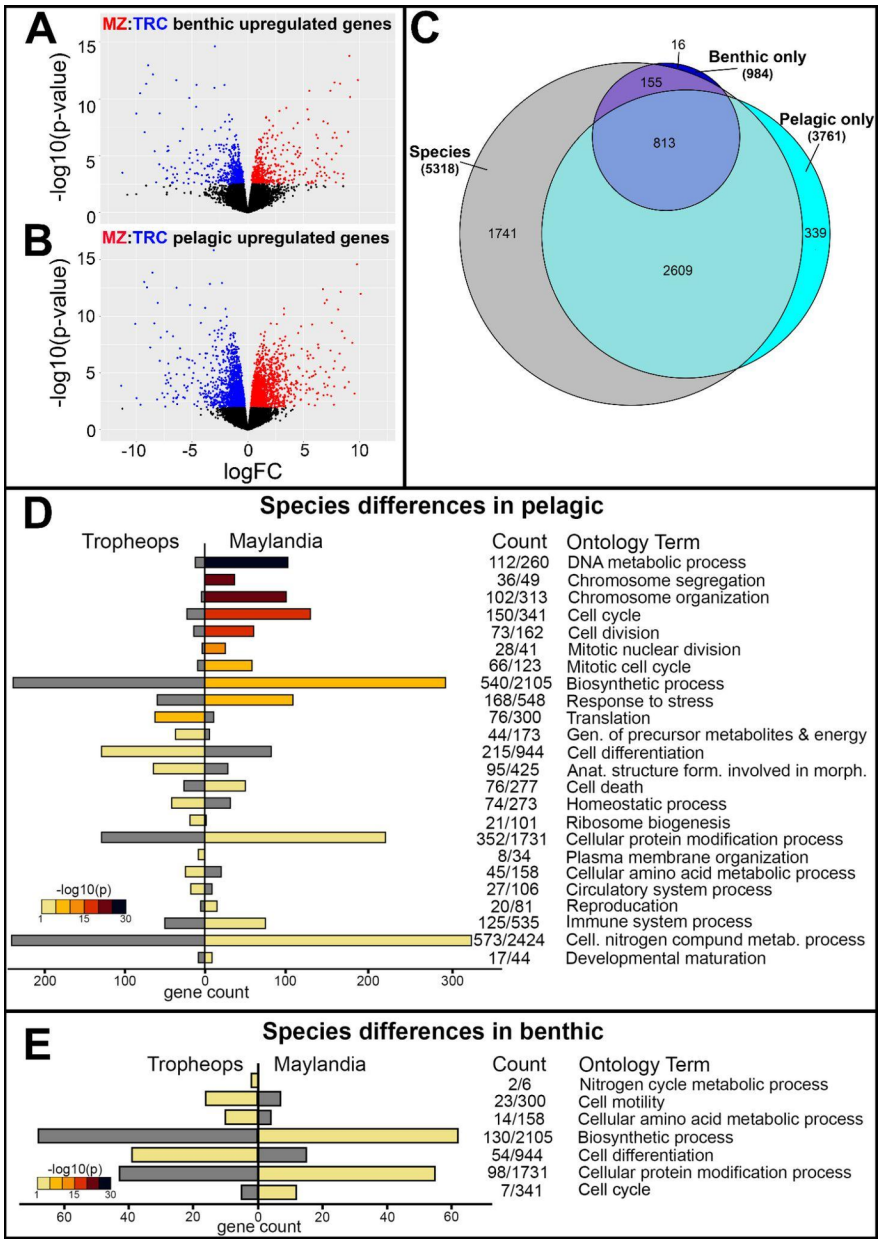

FIGURE 4: The pelagic environment drives differences in expression between species and implicates cell cycle regulation as a mode of increased bone deposition. (A) Volcano plot depicting DE between species in the benthic environment (n=984 total; n=549 MZ in red; n=435 TRC in blue). (B) Nearly four times the number of DEGs are detected in the pelagic environment (n=3761 total; n=1927 MZ in red; n=1834 TRC in blue). (C) The venn diagram shows that not only are there more DEGs in the pelagic versus benthic environment, but that most (813/984 = 83%) of the DEGs detected in the benthic environment are also DE in the pelagic environment. Alternatively, only 22% (813/3761) DEGs detected in the pelagic environment are also DE in benthic fish. (D, E) Enriched GO terms are shown for MZ versus TRC in the pelagic (D) and benthic (E) environments. Colors are representative of the -log10(p-value). Gray bars indicate no significance. Gene counts are given as a total for both species along with the corresponding GO term. Many terms enriched for in MZ in both environments are associated with cell cycle (e.g., cell

cycle, cell division, etc).

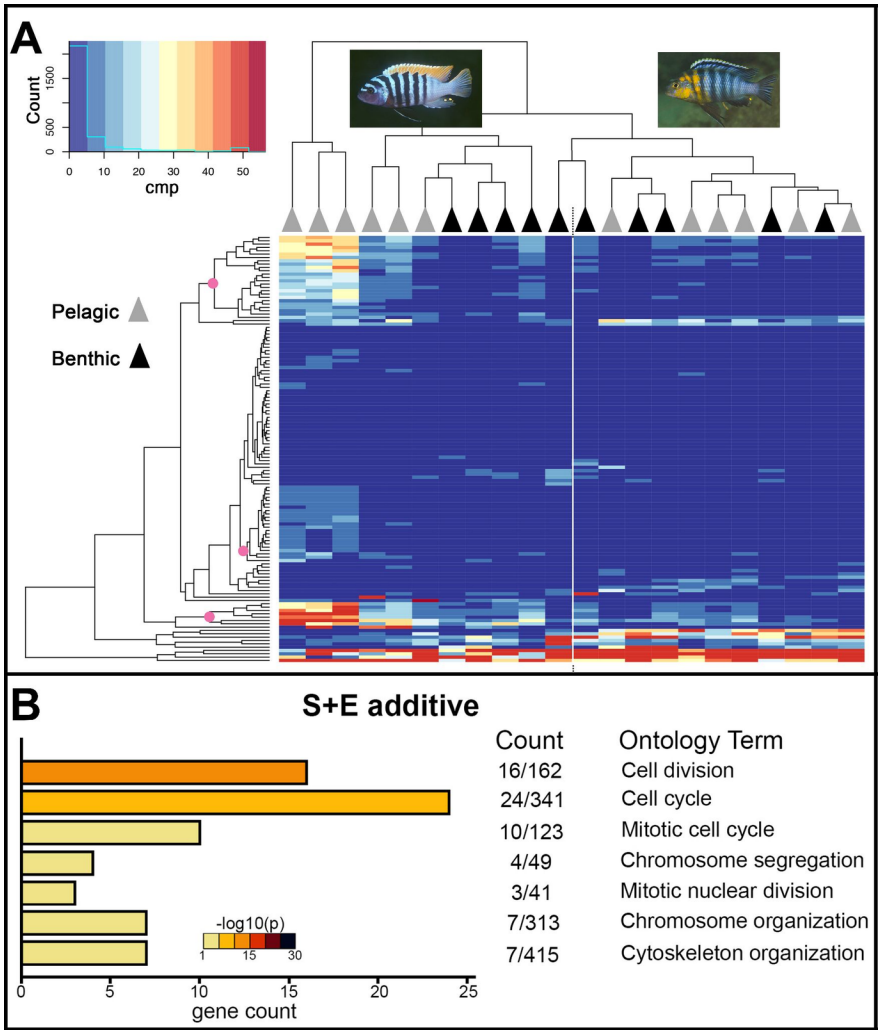

FIGURE 5: Differential expression across species plus environment reveals signatures of genetic assimilation and further supports a role for the cell cycle. (A) We constructed an additive model to identify genes that were differentially expressed between species and environment (S+E), which identified 128 DEGs. With the exception of a single MZ individual, species clustered together. Clustering by environment was observed in MZ but not TRC. Assuming that plasticity represents the ancestral condition, this analysis also provides evidence for genetic assimilation, whereby pelagic MZ exhibited relatively high gene expression compared to benthic MZ, which generally resemble TRC in terms of cpm counts. Pink dots on the cladogram to the left of the heatmap denote gene clusters that exemplify this pattern. We note that 3 MZ exhibited especially robust expression levels. (B) Enriched GO terms associated with genes in the S+E model. Colors are representative of the -log10(p-value). Gray bars indicate no significance. The nature of the additive model precludes us from having an up- vs down-regulated analysis of genes, because it takes into account both species and environment at the same time. These GO terms are similar to previous analyses in returning processes involved in cell cycle regulation.

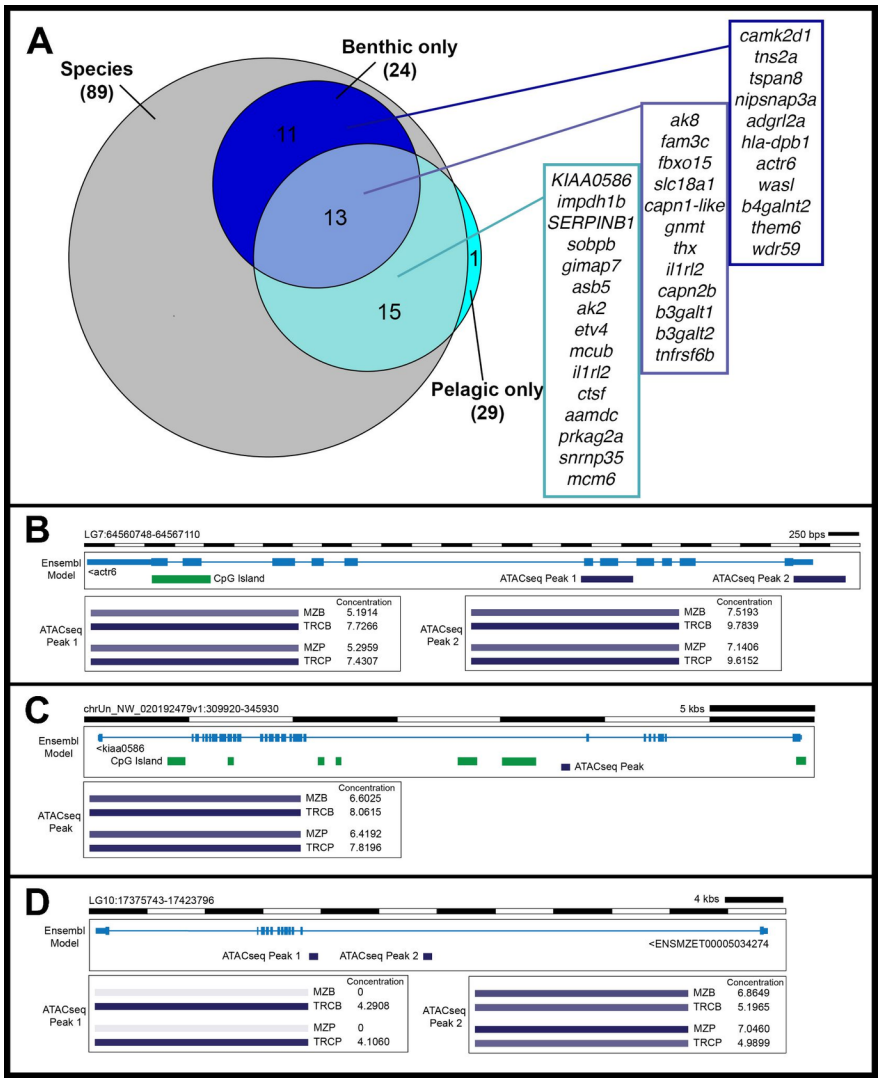

FIGURE 6: The overlap of RNA-seq and ATAC-seq datasets narrows the list of candidate genes. (A) In total, 89 genes were identified that were both differentially expressed (DE) and differentially accessible (DA) between species. Of these, 15 overlapped with genes identified in the pelagic dataset (light blue), 11 overlapped with genes from the benthic dataset (dark blue), and 13 were identified in both foraging environments. These overlapping datasets identified a relatively small subset of genes where expression differences may be due to differences in the *cis*- regulatory region. Boxes to the right of the venn diagram display the specific genes from each area of overlap. (B-D) Diagrams of genes are shown (each panel has its own scale bar), as well as the location of ATAC-seq peaks based on DA analyses, and CpG islands (from the UCSC genome browser CpG island track based on at least 50% GC content, >200 bp, and >0.6 ratio of observed number of CG dinucleotides). Shown are representative data from the benthic dataset (B, *actr6* on LG7), the pelagic dataset (C, *kiaa0586* on an unlinked contig), and a gene that was significantly DE and DA in both environments (D, *capn1-like* on LG10). Beneath each gene model, ATAC-seq peaks are colored based on concentrations, with lighter colors indicating lower concentrations and darker colors indicating higher concentrations.