INVESTIGATING THE GENETIC BASIS OF BONE PLIABILITY IN VARIOUS CICHLID SPECIES

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Abstract

Phenotypic plasticity, an important source of biodiversity, is the ability for an organism with a single genotype to produce myriad phenotypes in response to different environmental stimuli. The genetic mechanisms involved in phenotypic plasticity and their ability to cause evolutionary change remains poorly understood. Here, we experiment with the cichlid feeding apparatus with a focus on the lower jaw, a widely used model to understand rapid evolutionary divergence and phenotypic plasticity. We first demonstrate that changes in the cis-regulatory landscape of DNA can explain differential gene expression of some genes. This finding expands our understanding of the types of molecular mechanisms involved in phenotypic plasticity. We provide further evidence, via qPCR validation, for dynamic expression of a subset of these bone-marker genes over time, thus highlighting the fact that timing is important in plasticity studies. Finally, we show that morphological changes to the feeding apparatus in response to alternate feeding environments arise after the transcriptional response, and are localized to specific structures involved in foraging; the exact same structures that were used for our genetic studies. Evolutionarily, fish facial anatomy is homologous to human facial anatomy.
Load-induced developmental plasticity is known to occur in humans and is the reason why dental interventions, such braces, are effective therapies. It is also the reason why impact exercise is an effective treatment for various degenerative bone diseases. These data further the understanding of phenotypic plasticity in fishes and thereby provide a more general understanding of bone health and diseases.

Introduction

Phenotypic plasticity is the ability for a specific genotype to portray a myriad of phenotypes in varying environments. In the field of evolutionary biology, investigations into phenotypic plasticity have offered monumental insights into evolutionary theory. For example, phenotypic plasticity can be an original source of biodiversity as environmental change can influence an organism's phenotype through the expression of different sets of genes (Navon et al, 2020). If adaptive, this expression can become fixed (i.e., genetic assimilation; Gibert, 2017). In this way, plasticity can shape biodiversity by biasing the direction of evolution (e.g., flexible stem evolution; West-Eberhard, 2003).

Biodiversity is necessary for the health and maintenance of any ecosystem. Adaptive radiations, while necessary for biodiversity, are closely associated with phenotypic plasticity. An adaptive radiation is described as the rapid evolution of multiple species in various niches from a single ancestor (Schluter, 2000). New species have the ability to develop phenotypes that are specifically favorable in their environment, while diverging from other species. A major source of variation in any ecological niche generally relates to foraging environment and as a result lead to the appearance of phenotypes concerning foraging preference and/or method.

Fishes from the family *Cichlidae*, particularly African species, are excellent models to examine due to their diversity and rapid speciation. In one of the fastest speciation events ever, approximately 2000 different, but evolutionarily related, species of cichlid have evolved over the last 5-6 million years in the African rift-valley (Seehausen, 2006). Among all cichlid species, one of the major phenotypic points of diversity is the craniofacial/jaw profile and those structures involved in foraging. Based on each species' preferred feeding style, a benthic-pelagic exists that represents the different craniofacial morphologies that coincide with different foraging environments. Some species represent the pelagic-specialist end of the spectrum with jaws

optimized for suction feeding and appear to be more long/narrow. On the contrary, *Labeotropheus fuelleborni* are benthic specialists whose jaws are specialized for power/biting and appear to be more wide and short. In summary, cichlid jaws are plastic; their size and shape can change based on the environment (Schneider and Meyer, 2016). Thus, cichlids and more specifically, their jaws, are an effective model to study plasticity. Plasticity is thought to influence the direction of evolutionary change and consequently, cichlid jaws are also a model to study the evolution of the feeding apparatus.

In this work, the goal is to investigate the genetic framework behind bone plasticity in response to different mechanical loads imposed on the cichlid feeding apparatus over time. Different mechanical loads simulate foraging habitats that cichlids would face in the wild: benthic (biting and scraping from rocks) and pelagic (suction feeding from the water column). From this project, grounded in theory, the Albertson lab hopes to better understand how bone plasticity affects evolutionary change. Specifically, we predict that by identifying traces of flexible-stem evolution or genetic assimilation we can implicate more genes involved in the plastic response of bone and evolution of the feeding apparatus. At the molecular level, we will examine whether the environment is affecting the *cis*-regulatory landscape of DNA and how any resulting changes in gene expression will affect the organism's phenotype. By studying the genetic basis underlying bone pliability in fishes, we can make more accurate conclusions regarding species divergence and possibly the genetic causes of human bone disorders or diseases.

Review of Literature

Plasticity and its Ecological and Evolutionary Consequences

Phenotypic plasticity enables an organism to better adapt its phenotype to changes in its environment (Parsons et al., 2016). The different phenotypes that can arise from different environmental causes tend to result in individuals that have characteristics specialized for their immediate environment (Schneider et al., 2014). Inevitably, the physical environment maintains some sort of relationship with an organism's genetic environment and the environment can cause phenotypic variation (Parsons et al., 2016). Environmental cues may act on "mechanically responsive" genes that help give rise to plasticity in traits (Schneider et al., 2014). Schneider et al. also identified that many plastic genes are functionally related and co-expressed. Phenotypic plasticity is also representative of the variation that exists in a population and allows organisms to phenotypically adapt to any changes in their environment. In other words, phenotypic plasticity should influence fitness (Parsons et al., 2016). This would then infer that plasticity itself or the ability to be plastic is a trait acted on by natural selection. The "Baldwin Effect" refers to plasticity enabling populations to thrive in niches that an organism isn't normally used to occupying (Parsons et al., 2016).

An explanation for how a favorable and plastic trait becomes fixed in the genome is then required. Genetic assimilation is a prediction stating that changes in phenotype can become encoded in genetic material (Parsons et al., 2016). While individuals can be plastic, it is plasticity at the population level that influences the direction of evolutionary change or trajectory. This phenomenon is known as the flexible stem hypothesis, whereby developmental plasticity in ancestral populations can influence evolution by exposing novel genetic variation, which may become fixed if populations adapt to a novel environment (West-Eberhard, 2003).

Roles for Hedgehog Signaling in Bone Development and Plasticity

The hedgehog (HH) signaling pathway plays a crucial role in animal development as it controls cell proliferation, differentiation, and survival and is highly conserved across species (Ben et al., 2011). HH signaling requires the primary cilium which is a major mechanosensor in the eukaryotic cell, thus the primary cilium is often referred to as the "HH signal transduction machine" (Goetz et al., 2009). Primary cilia structure is seen on a variety of different cell types including bone progenitors. Mice lacking primary cilia on bone cells are unable to remodel bones in response to different mechanical loads (Moore et al., 2018). *Ptch1* is an important regulatory membrane protein in the HH pathway (Parsons et al., 2016). Previous studies in our lab indicate that genetic variation and differential expression of *ptch1* mRNA is associated with differences in craniofacial geometry and influences the tradeoff between speed/power (jaw rotation). In particular, *ptch1* is differentially expressed in different species of cichlid occupying different ecological niches (Navon et al., 2020). Differential expression of *ptch1* affects the rate of bone deposition which can be modulated by environmental input. HH also regulates bone plasticity (Navon et al., 2020). Specifically, experimental down regulation of HH signaling resulted in global reduction of plasticity in all measured craniofacial bones, whereas up-regulation of HH signaling resulted in gain of plasticity in the interopercle bone (IOP), which is critical for feeding.

Further studies into cichlid craniofacial architecture and HH signaling have proved to be useful for this project. Gill arches are involved in many aspects of foraging including capturing, filtering, and processing prey (Zogbaum et al., 2021). In between the anterior and posterior oral jaw are branchial arches which house gill rakers (GRs). GRs are a focus of this study due to their involvement in moving food toward the pharyngeal jaw aspects (Zogbaum et al., 2021). By

breeding purebred cichlids with their own F_3 hybrids, Zogbaum et al. found that in different environments, representing the benthic-pelagic ecomorphological axis, differences in the foraging apparatus are dependent on the environmental differences. The genetic coding responsible for differences in GR anatomy between foraging environments is also distinguishable; those genes responsible for GR number and size are distinct depending on the feeding environment in which animals are exposed to (Zogbaum et al., 2021). For GR number linked to many gill arches, the *ptch1* locus was implicated (Zogbaum et al., 2021). Zogbaum et al. dove further into the role of HH signaling in GRs and found that HH negatively regulates overall GR number and is required for plasticity of GRs.

Ciliogenesis is the process by which the microscopic outgrowths on a cell's surface form. Of the many different functions of cilia, sensing chemical and mechanical changes in the local environment is among the most important and necessary. In zebrafish and mice, HH signaling has been implicated in intraflagellar transport which points to a connection between primary cilia in HH signaling (Ben et al., 2011). For HH signaling to be relevant to cilia, cilia need to develop correctly from the beginning. The *talpid3* (*ta3*) gene is a ciliary related gene (Li et al., 2017). In chicken, the *ta3* mutation has a coiled-coil domain crucial for primary ciliogenesis, and the *ta3* gene is highly conserved from sea anemones to humans (Ben et al., 2011). The mutation tends to cause abnormal patterning of limbs and other structures that are the result of HH signaling (Davey et al., 2006). When mapping the *ta3* locus, KIAA0586 was discovered as well as a frameshift mutation in its ortholog (Davey et al., 2006). KIAA0586 gene product is a novel protein that localizes to the cytoplasm most likely involved in the regulation of GliActivator and GliRepressor (Davey et al., 2006). Ben et al. identified a zebrafish *ta3* locus and used a zinc-finger nuclease-mediated targeted mutagenesis to better understand the role for this gene in

development. Elimination of *ta3* function causes defects in neural tube, somites, limbs, and craniofacial features (Ben et al., 2011). This is very similar to the effects of elimination of *ta3* function in chick; the loss of expression of the ventral marker *nkx2.2* mimics the chick mutant phenotype and represents the loss of HH signaling (Ben et al., 2011). The phenotypes of maternal and zygotic mutant *ta3* in zebrafish was similar to chick *ta3* mutants: absence of primary cilia, non-normal HH signaling, and GFP-tagged Gli2a localized to basal bodies even when primary cilia were absent (Ben et al., 2011).

Li et al. set out to examine how downstream effectors in the HH pathway, Gli2 and Gli3, are activated in primary cilia. Gli2 normally acts as a transcriptional activator whereas Gli3 is a repressor (Li et al., 2017). It was found that Gli2 and Gli3 are inhibited via phosphorylation by protein kinase A (PKA); their phosphorylation causes ubiquitination and subsequent degradation (Li et al., 2017). Li et al. found that HH signaling inhibits phosphorylation and subsequent degradation of the Gli2 and Gli3 proteins and converts $Gli2^{FL}$ and $Gli3^{FL}$ into transcriptional activators. The *ta3* mutation resulted in reduced processing and phosphorylation of Gli2 and Gli3 and $Gli2^{FL}$ and $Gli3^{FL}$ were inactive (Li et al., 2017). This is perplexing as the decrease in phosphorylation is associated with $Gli2^{FL}$ and $Gli3^{FL}$ activation and as a result, $Gli2^{FL}$ and $Gli3^{FL}$ activity should be augmented (Niewiadomski et al., 2014). The *ta3* mutation reduces HH signaling due to $Gli2^{FL}$ and $Gli3^{FL}$ inactivity but their activation is dependent not only on dephosphorylation, but other mechanisms like desumoylation and acetylation (Li et al., 2017). Davey et al. found similar results. In some aspects of the developing embryo, the *ta3* phenotype is ligand independent and it is necessary for the proper function of Gli repressor (Gli3) and activor (Gli2) for HH signaling in vertebrates (Davey et al., 2006).

Bone structure is affected by genetic changes but physical forces imposed on the feeding apparatus precede genetic changes. When bones are presented with mechanical forces, they adapt by depositing more bone matrix which tends to occur at the periosteal surface (Moore et al., 2018). Moore et al. hypothesized that periosteal osteochondroprogenitors (OCPs) sense mechanical load through their primary cilia and differentiate into osteoblasts. It was found that in mice lacking OCPs, bone deposition and formation was reduced (Moore et al., 2018). In vitro, OCPs were found to sense changes in their immediate environment and showed signs of osteoblast differentiation through changes in osteogenic markers; this same response was not present when primary cilia were knocked down (Moore et al., 2018). How HH signaling may influence this process is unknown, but is a broad goal of the Albertson Lab.

Other Studies on Cichlids and Their Use as a Model Organism

In previous research on cichlids in Lakes Malawi, Tanganyika, and Victoria, fish in each of these lakes were found to differ on an "ecomorphological axis" concerning their oral jaw length and craniofacial profile (Parsons et al., 2016). Cichlid fishes, East African cichlids in particular, are preferred model organisms to study due to their relatively recent adaptive radiation and their diverse/plastic feeding mechanism (Zogbaum et al., 2020). It was found that phenotypic variation in foraging mode induced in a lab-setting mirrored the ancestral cichlid species (Parsons et al., 2016). This would provide evidence that the cichlid jaw itself is a flexible stem. The flexible stem hypothesis predicts that evolution of ancestral populations should line up with patterns of phenotypic divergence. Two closely related cichlid species, *Labeotropheus fuelleborni* and *Tropheops* 'red cheek', exhibited differing levels of plasticity in their craniofacial profile when different foraging modes presented to the two species (Parsons et al., 2016). When

Parsons et al. examined the genetic components underlying phenotypic plasticity in *Labeotropheus fuelleborni* (LF) and *Tropheops* 'red cheek' (TRC) they implicated a plastic response in the overall skull geometry of the fish (Parsons et al., 2016). This mirrored variation in ancestral Malawi cichlids gives rise to the notion that the cichlid jaw is a flexible stem. Many morphological structures in the craniofacial skeleton are sensitive to the environment. 21 quantitative trait loci (QTL) are unique to environment/foraging type and only 1 QTL was shared between environments/foraging type (Parsons et al., 2016). Of the traits acting across environments, alternate alleles at a locus were either upregulated or downregulated as a result of environment/foraging type (Parsons et al., 2016). Plasticity is not only a result of loci that are environmental-specific but also loci acting across environments (Parsons et al., 2016). Parsons et al. was also the first study to implicate *ptch1* in cichlid plasticity.

Another major justification for the use of cichlids in plasticity studies because they meet many of the criteria of a model organism (Albertson and Pauers, 2019). One major criterion is evolutionary success. Evolutionary success is a blanket term that encompasses a slew of definitions. Success generally relates to some degree of diversity within a population of individuals (Collier, 1998). Having myriad genotypes, cellular structure, functional relationships, or species within a taxonomic group all are key players when considering diversity and success. (Albertson and Pauers, 2019). From their adaptive radiation, cichlids evolved to fill distinct niches like piscivore and herbivore but also further specialized within niches (i.e. herbivore cichlids will exhibit algal scrapers, biters, and pickers; Albertson and Pauers, 2019). Albertson and Pauers study two distinct species of cichlid, specialized *Labeotropheus* and diverse *Tropheops*. Due to their differences, one would expect that the two species would exhibit very different levels of morphological disparity. Albertson and Pauers find that through studying the

geometric morphometrics of the cichlid feeding apparatus, *Labeotropheus* and *Tropheops* show similar levels of morphological disparity. This finding suggests that being specialized (versus diverse) has not been a limiting factor for craniofacial variability (Albertson and Pauers, 2019). It was also found that species having greater ecological distribution increases morphological disparity when compared to species that have limited distribution (Albertson and Pauers, 2019).

Adaptive Radiations and Evolutionary Change

The cichlid adaptive radiation occurred in the African lakes of Malawi, Tanganyika, and Victoria. The rate of speciation in these lakes occurred, in part, due to their size and ecological variability (Seehausen, 2006). The rate of speciation is directly correlated to the availability of different niches; as niches become filled, speciation decreases (Seehausen, 2006). New adaptive radiations and the beginnings of older ones exhibit high rates of speciation whereas when time passes, speciation significantly declines (Seehausen, 2006). Further, the number of cichlid species in a lake is correlated to the size of the lake; more species in larger lakes. This supports a widespread prediction that a species' diversity is the result of environmental diversity (Seehausen, 2006). Adaptive radiations, while necessary for biodiversity, are closely associated with phenotypic plasticity. An adaptive radiation is described as the rapid evolution of multiple species in various niches from a single ancestor (Schluter, 2000). Thus, ecological diversity is required for radiations; Darwin's finches are the epitome of adaptive radiations and how they occur (Tebbich et al, 2010). Adaptive radiations occur when a population evolves in a way that allows some individuals to explore a new ecological niche and over time, results in either "continuous" or "discontinuous" phenotypic variation (Parsons et al., 2014). Genetic architecture interacts with the environment during ontogeny and affects phenotypic variation; new and

possibly advantageous phenotypic variation is encoded into the genome (Parsons et al., 2014). Tebbich et al. posits that phenotypic variation is accompanied with some degree of learning but eventually the phenotypic variation transforms into morphological adaptations. Morphological adaptations accompanied with changes in gene expression can lead to speciation and radiation.

Adaptive radiation is often associated with the term "parallel evolution." Parallel evolution refers to the independent evolution of similar phenotypes in many related evolutionary lineages whereas nonparallel evolution is "partial" parallel evolution (Manousaki et al., 2013). Manousaki et al. set out to understand the relationship between environmental differences, trophic traits, and gene expression with parallel evolution in two Great lakes and two crater lakes in Nicaragua. Studying lips (thick and thin) in *Amphilophus citrinellus*, it was found that foraging traits (i.e. lip size and head/body shape) show signs of parallel evolution (Manousaki et al., 2013). Further, in separate but similar foraging environments, jaw size and shape showed evidence of nonparallel evolution; each lake in the study has its own evolutionary trajectory (Manousaki et al., 2013). Using transcriptomics, Manousaki et al. discovered six genes (apolipoprotein D, a glycoprotein precursor, LIM domain protein, calpain-9, a GTPase, and one hypothetical protein) that are associated with the parallel evolution of thick-lipped phenotypes. Genes that were differentially expressed are associated with a particular lake and specialized phenotype (Manousaki et al., 2013).

Diverse foraging environments should generally cause an increase in plasticity while stable environments cause a reduction in plasticity (or an increase in specialization) due to evolutionary costs. Increased specialization would subsequently lead to decreased plasticity as there is a tradeoff between the level of specialization and the ability to be plastic (Parsons et al., 2014). If an individual develops a novel trait it may come at the cost of the ability to respond to

new environmental changes. Parsons et al. investigated the genetic basis of eco-morphologically novel traits, if these traits affect phenotypic plasticity and if there is an evolutionary tradeoff occurring. A focus on *Wnt* signaling led this investigation as the pathway is involved in craniofacial patterning, development, and variation and is responsible for distinguishing cichlids with different jaw shapes (Parsons et al., 2014). Downstream players like *B-catenin* and *lef1* were proved to be active during the ontogeny of the skull where expression was especially strong in LF (Parsons et al., 2014). A similar pattern of expression was seen in zebrafish proving that *Wnt* signaling is conserved across species. Upregulation of Wnt signaling can cause novel craniofacial morphology in cichlids as bone deposition and ossification are directly affected (Parsons et al., 2014). Further, extreme morphological phenotypes are more likely when *Wnt* signaling is increased globally (Parsons et al., 2014). Parsons et al. then went on to prove that novel craniofacial morphologies are able to withstand changes in the environment at the expense of the capacity for evolutionary change (i.e. more specialization = less plasticity)(Parsons et al., 2014). Extreme specialization of LF in their preferred environment suggests a tradeoff with variation of the craniofacial profile (Parsons et al., 2014).

All in all, this literature shows that plasticity is important for ecology and evolution. It also shows that the molecular regulation of plasticity is not well understood but that HH signaling and cilia are important for plasticity. Finally, the literature proves that cichlids are a useful model to study plasticity, its genetic basis, and evolutionary consequences. In this thesis, I hope to elucidate the mechanisms and genes that cause phenotypic plasticity over time in different cichlid species and foraging environments.

Methods

Fish Husbandry and Experimental Design

Work with animals was approved and occurred under IACUC protocol #2018-0094. Initially, cichlids were split into diet treatments and trained in 40-gal glass aquaria for seven days on standard flake food sprinkled into the water column or pasted onto lava rocks. Individuals were subsequently split into 5-10 membered groups each occupying their own 40-gal tank. Food composition and amount was held consistent across treatments; high-quality algae flaked food (purchased from Worldwide Aquatics, Inc.) was ground and either sprinkled into the water column (pelagic) or mixed with 1.5% food-grade agar solution and ground freeze-dried brine shrimp then pasted onto lava rocks (benthic). For benthic foragers, rocks were dried overnight after the paste was added and two rocks were placed into each benthic tank daily. Pelagic foragers were given ground flake food and live brine shrimp daily.

At one, two, four, and eight weeks benthic and pelagic groups from various cichlid species were sacrificed and dissected. Cichlid species that were examined in this experiment were representative of the particular foraging mode that they specialize in: benthic, pelagic, or generalist. We quantified differences in gene expression from genes implicated in the plastic response over eight weeks. Candidate genes implicated in the plastic bone response were obtained from previous RNAseq and ATACseq experiments. We quantified differences in gene expression over eight weeks because our previous research shows that plastic bone responses from environmentally sensitive genes will be evident, especially over time. Time points were constructed at one, two, four, and eight weeks where groups of fish reared in a benthic or pelagic environment were sacrificed and their gene expression of the retroarticular, interopericle, and interopercle ligament was examined.

RNA Extraction, Reverse Transcription PCR (RT-PCR), and Quantitative PCR (qPCR)

The retroarticular process, interopercle, and associated cartilage were collected from individuals reared in either a pelagic or benthic environment. The tissues were placed in trizol and stored at -80℃. While trizol aided in tissue degradation, the tissues were further homogenized using five UFO beads per tissue sample and a Next Advance Bullet Blender. The total RNA was isolated from homogenized tissues using phenol/chloroform extraction and standardized to 70ng/uL before reverse transcription.

Extracted RNA was used to prepare double-stranded copies (cDNA). cDNA was quantified via qPCR measuring the expression levels of various candidate genes found to be differentially expressed or differentially accessible. Precise levels of gene expression were obtained via SYBR Green fluorescence. Primer sequences for qPCR were as follows: talpid3_for2: CCTGGATCAGCAGGTTAAGCA, talpid3_rev2: CTGATGGCAAGGCAGAAAAGG, asb5_for4: CAGTGTGATGGTACCCGTCC, asb5_rev4: CACAAAACGAGCAGCTCAGAAA, actr6_for1: GCAAATCCCGTCTGTTACGC, actr6_rev1: CATAGTCCTCCCGCATCACC, Cdc20_for3: GGCTCCTGTATCAGTTCGCT, Cdc20_rev3: AGGCGAATGGTCTCATCTGC, capn1_for1: ATGTTTAGGGTTGGGACTCCAG, capn1_rev1: GGTGACAACCAACTGATCCCT, sp7_for2: GGCCGCATCTATTCTGGAGG, sp7_rev2: GGCAGTCTTACCGGGTGTAG. The detailed protocol for RNA extraction, RT-PCR, and qPCR will be attached as supplementary information. qPCR data was analyzed using the comparative Ct method. Relative quantification of gene expression compared to -actin was analyzed. The ANOVA and T-tests were also implemented as another means of comparing Ct values.

Trait Imaging and Analysis

To determine whether the geometry of the 4-bar linkage of the cichlid oral jaw changes in response to alternate foraging treatment, we landmarked and measured the coupler and output linkages. We captured images of the right lateral surface of each specimen using a Leica M165 FC microscope with an attached Leica DFC450 camera. Images were processed using Photoshop and analyzed in ImageJ.

Results

Gene expression (qPCR) shows similar patterns to the genome-wide analyses.

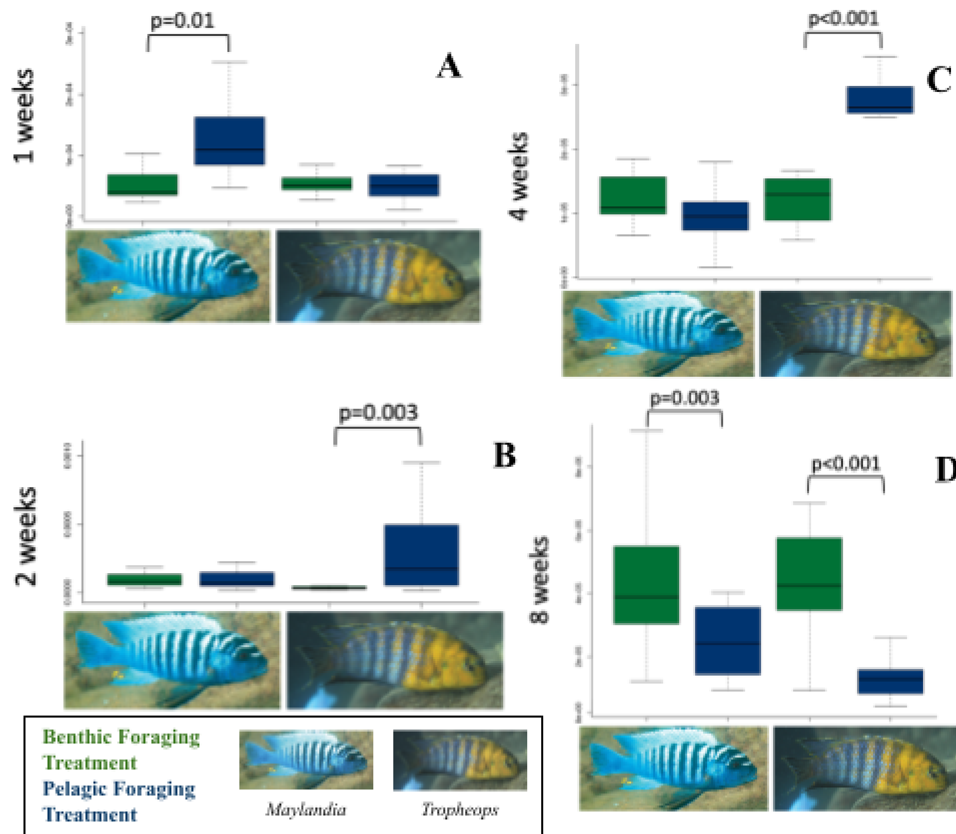

Figure 1. *Talpid3* gene expression over time. Panel A is the one-week time point. Panel B is the two-week time point. Panel C is the four-week time point. Panel D is the eight-week time point.

In our genetic experiments, we predicted that gene expression output by qPCR will show similar patterns to our previous genome wide analyses (RNA- and ATAC-seq), which were performed between two and three weeks. In general, this prediction holds as illustrated by *Talpid3/Ta3,* where the two and four week gene expression time points (Figure 1B&C) were similar to the genome wide analyses. We also found that expression in *Maylandia* was plastic at one week while *Tropheops* was not but at four weeks *Tropheops* was plastic and *Maylandia* was not (Figure 1). Plasticity in *Talpid3*, along with other genes of interest, was higher in the pelagic environment and in *Tropheops*, aligning with the genome wide analyses. Overall, plasticity in an isolated gene was dynamic over time (Figure 1A-D)

<u>*Gene expression is dynamic over time.*</u>

We next generated a summary of expression data for the six environmentally sensitive genes: *Actr6, Asb5, Capn1, Cdc20, Sp7,* and *Talpid3*. Over the course of the experiment, gene expression was dynamic where plasticity in gene expression peaked early in *Maylandia* and manifested later in *Tropheops* (Figure 2). Timing is important in the plastic response of bone.

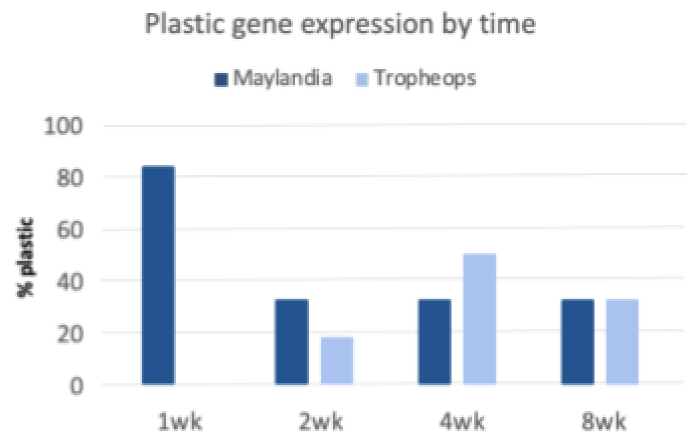

Figure 2. Compilation of plasticity for environmentally sensitive genes. "% plastic" refers to the number of genes, of 6 assessed, that were plastic.

Skeletal structures used for genetic analyses are plastic & plasticity manifests earlier in Maylandia.

We used the same animals for both qPCR and morphological analyses. For the latter, we took measurements of two parts of the four-bar linkage system: the output (retroarticular) and coupler links (interopericle) (Figure 3A-B). These structures were also the same structures used for the aforementioned genetic study. The coupler link, which makes up most of the tissue dissected for the transcriptional studies, was plastic in *Maylandia* by four weeks and the output link was plastic at two weeks, but not at any other time points (Table 1). The coupler link was generally longer in the pelagic environment than in the benthic, which aligns with the optimal mechanics of the linkage system in the pelagic environment. In *Tropheops*, size differences between environments in the coupler link were not significant at any time (Table 1). Size differences between environments in the output link were significant at eight weeks (Table 1) and were trending in that direction by week two with the benthic output link being longer than pelagic. Again, this

Table 1. Morphological summary of the lengths of the coupler and output links in *Maylandia* and *Tropheops*. Data represent the earliest signs of plasticity in the lengths of the coupler and output links.

	Coupler Link	Output Link
Maylandia	4 weeks	2 weeks
Tropheops	N/S	8 weeks

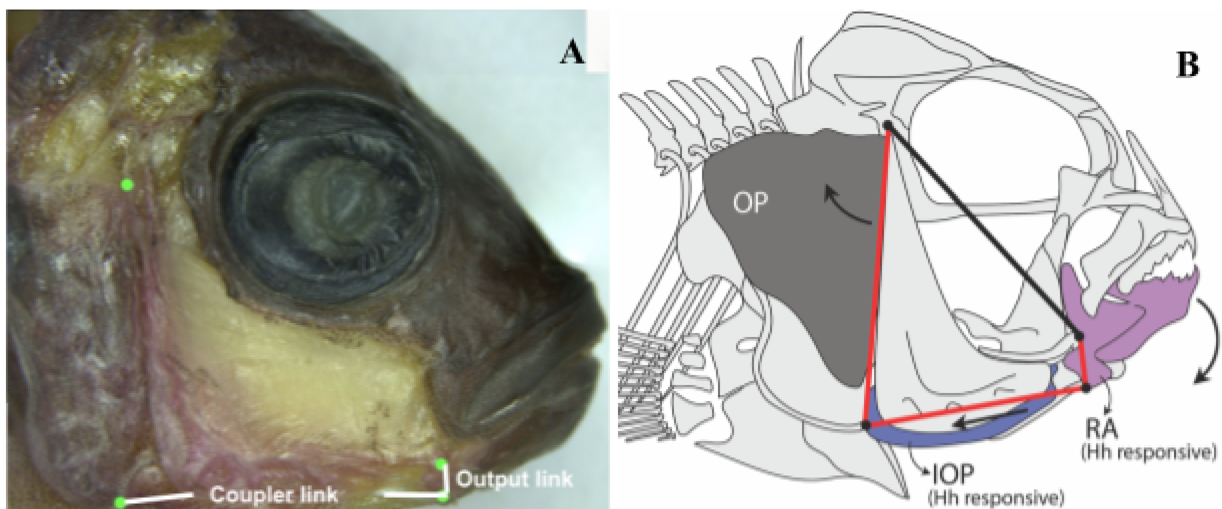

Figure 3. A: Structures in the 4-bar system, "coupler link" and "output link," where morphological differences occur. These were the same used for the genetic study of plasticity. B: "IOP" refers to the interopericle bone and "RA" refers to the retroarticular bone.

aligns with the optimal mechanics of the linkage system in the benthic environment. Importantly, morphological plasticity will track with genetic plasticity in *Maylandia* and *Tropheops*, and thus changes in gene expression are coincident to changes in phenotype.

Discussion

Differences in gene expression that occur over time (Figure 2) during normal bone formation have not only been documented in Cichlids. In mice, bone-related genes like *Osx*, *Runx2*, *Sox9*, and various collagen genes were shown to be differentially expressed over time (Kaback et al., 2007). In hMSCs (human mesenchymal stem cells), these same genes were differentially expressed between three, eight, and 15 days (Valenti et al., 2008). These data combined with previous studies underline the significance of differential gene expression over time and is an important factor when conducting plasticity studies.

Given the dynamic expression of bone genes over time under “normal” conditions, we wanted to understand here the effects of different foraging environments on the feeding apparatus over time. 1, 2, 4, and 8 week time points were necessary in order to understand how dynamic bone-related gene expression was. We found that one generalist species (*Maylandia*) showed a robust plastic response in gene expression in the first and second weeks of the experiment while the other generalist species (*Tropheops*) showed a plastic response in the fourth and eighth weeks (Figure 1A-D and 2). The overall trend that plastic signatures reduce over the eight week study possibly suggests that the genes analyzed here are necessary for an early plasticity response. Our experimental design may be limited to finding “later” acting genes, as our list was determined by genome-wide analyses conducted at 3-4 weeks. Either way, our data show that “plasticity genes” are specific to certain time periods. In future plasticity studies not

only in cichlids but also mice or human stem cells, these data may provide a road map for extended time series experiments guiding where plastic signatures will be most evident.

Another notable finding is that *Maylandia* exhibits a plastic response and shape changes in the feeding apparatus earlier than *Tropheops* (Table 1 and Figure 3A-B). Thus, *Maylandia* responds, anatomically and genetically, to changes in their environment quicker than *Tropheops*. It is possible that *Maylandia* may be more plastic than *Tropheops*. Alternatively, *Maylandia* may be able to respond faster to environmental change, but *Tropheops* will eventually show the same amount of plasticity (Figure 2). Either possibility suggests that *Maylandia* is better able to rapidly adapt to a new environment, which in turn, could affect overall fitness.

Previous work with cichlids has elucidated the relationship between plasticity and specialized versus non-specialized species, with generalist cichlid species overall are more plastic than specialized species (Navon et al., 2020). This is also seen in other species. For example, the generalist species of *Nasonia vitripennis*, showed more behavioral plasticity than in three other specialist sister taxa (Kalyanaraman et al., 2021). In this study we show that this may translate to plasticity at the genetic level, with *Maylandia* possibly being more plastic than *Tropheops.* In the future we recommend examining true cichlid specialists, such as *Labeotropheus fuelleborni,* that are not plasticity at the morphological level, at the transcript level.

Conclusion

All in all, these data outline several important findings. Differences in gene expression over time are indicative of plastic signatures in environmentally sensitive genes. These 'plastic' genes are associated with plastic morphological changes between environments. Further, we

established that *Maylandia* are better able to rapidly respond to changes in the environment than *Tropheops*. This suggests that *Maylandia* may be a more plastic species, and thus by extension may be more able to respond to a rapid environmental shift. In future work, we hope to shed light on the types of mechanisms that occur molecularly to cause plastic responses (i.e. cell cycle/division, cell signaling, cell proliferation/differentiation, epigenetic mechanisms). Our work also has an extrapolated extension to medical science. Developmental plasticity is known to occur in humans and is the reason why continued experimentation with phenotypic plasticity is necessary.

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Bibliography

R. C. Albertson, & M. J Pauers, (2019). Morphological disparity in ecologically diverse versus constrained lineages of Lake Malaŵi rock-dwelling cichlids. *Hydrobiologia*, *832*(1), 153–174.

J. Ben, S. Elworthy, A. S. M. Ng, F. van Eeden, & P.W. Ingham, (2011). Targeted mutation of the talpid3 gene in zebrafish reveals its conserved requirement for ciliogenesis and Hedgehog signaling across the vertebrates. *Development, 138(22), 4969–4978.*

J. Collier, (1998). Information increase in biological systems: how does adaptation fit? In van der Vijver, G., S. N. Salthe & M. Delpos (eds.). *Evolutionary Systems.* Kluwer, Dordrecht: 129–140.

M. G. Davey, I.R. Paton, Y. Yin, M. Schmidt, F.K. Bangs, D. R. Morrice, T.G. Smith, P. Buxton, D. Stamataki, M. Tanaka, A.E. Münsterberg, J. Briscoe, C. Tickle, & D.W Burt, (2006). The chicken *talpid*^{3} gene encodes a novel protein essential for Hedgehog signaling. *Genes & Development*, *20*(10), 1365–1377.

J. M. Gibert, (2017). The flexible stem hypothesis: Evidence from genetic data. *Development Genes and Evolution*, *227*(5), 297–307.

S.C. Goetz, P. J. R. Ocbina, & K. V. Anderson, (2009). Chapter 10—The Primary Cilium as a Hedgehog Signal Transduction Machine. *Methods in Cell Biology*, *94*, 199–222.

L. A. Kaback, D. Y. Soung, A. Naik, N. Smith, E. M. Schwarz, R. J. O'Keefe, & H. Drissi. (2008). Osterix/Sp7 regulates mesenchymal stem cell mediated endochondral ossification. *Journal of Cellular Physiology*, *214*(1), 173–182.

D. Kalyanaraman, J. Gadau., & M. Lammers. (2021). The generalist parasitoid Nasonia vitripennis shows more behavioural plasticity in host preference than its three specialist sister species. *Ethology*, *127*(11), 964–978.

D. Navon, I. Male, E. R. Tetrault, B. Aaronson, R. O. Karlstrom, & R.C. Albertson, (2020). Hedgehog signaling is necessary and sufficient to mediate craniofacial plasticity in teleosts. *Proceedings of the National Academy of Sciences*, *117*(32), 19321–19327.

T. Manousaki, P.M. Hull, H. Kusche, G. Machado-Schiaffino, P. Franchini, C. Harrod, K. R. Elmer, & A. Meyer, (2013). Parsing parallel evolution: Ecological divergence and differential gene expression in the adaptive radiations of thick-lipped Midas cichlid fishes from Nicaragua. *Molecular Ecology*, *22*(3), 650–669.

E.R. Moore, Y. X. Zhu, H.S. Ryu, C.R. Jacobs, (2018). Periosteal progenitors contribute to load-induced bone formation in adult mice and require primary cilia to sense mechanical stimulation. *Stem Cell Research & Therapy*, *9*(1), 1–15.

P. Niewiadomski, J. H. Kong, R. Ahrends, Y. Ma, E. W. Humke, S. Khan S, M. N. Teruel, B. G. Novitch, & R. Rohatgi, (2014). Gli protein activity is controlled by multisite phosphorylation in vertebrate Hedgehog signaling. *Cell Rep*, 6:168–181.

K. J. Parsons, M. Concannon, D. Navon, J. Wang, I. Ea, K. Groveas, C. Campbell, & R. C. Albertson, (2016). Foraging environment determines the genetic architecture and evolutionary potential of trophic morphology in cichlid fishes. *Molecular Ecology*, *25*(24), 6012–6023.

K. J. Parsons, A. Trent Taylor, K. E. Powder, & R. C. Albertson, (2014). Wnt signalling underlies the evolution of new phenotypes and craniofacial variability in Lake Malawi cichlids. *Nature Communications*, *5*(1), 3629.

V. Ribes & J. Briscoe, (2009). Establishing and Interpreting Graded Sonic Hedgehog Signaling during Vertebrate Neural Tube Patterning: The Role of Negative Feedback. *Cold Spring Harbor Perspectives in Biology*, *1*(2), a002014.

D. Schluter, (2000). The ecology of adaptive radiation. *Oxford: Oxford University Press.*

R. F. Schneider & A. Meyer, (2017). How plasticity, genetic assimilation and cryptic genetic variation may contribute to adaptive radiations. *Molecular Ecology*, *26*(1), 330–350.

R. F. Schneider, Y. Li, A. Meyer, & H. M. Gunter (2014). Regulatory gene networks that shape the development of adaptive phenotypic plasticity in a cichlid fish. *Molecular Ecology*, *23*(18), 4511–4526.

O. Seehausen, (2006). African cichlid fish: a model system in adaptive radiation research. *Proceedings of the Royal Society B: Biological Sciences,* 273(1597), 1987–1998.

S. Tebbich, K. Sterelny, & I. Teschke, (2010). The tale of the finch: Adaptive radiation and behavioural flexibility. *Philosophical Transactions: Biological Sciences*, *365*(1543), 1099–1109.

E. R. Tetrault, (2021). Investigating the complex regulation of the craniofacial profile. *Molecular and Cellular Biology Program at the University of Massachusetts, Amherst.*

M. T. Valenti, L. D. Carbonare, L. Donatelli, F. Bertoldo, M. Zanatta, & V. Lo Cascio. (2008). Gene expression analysis in osteoblastic differentiation from peripheral blood mesenchymal stem cells. *Bone*, 43(6), 1084–1092.

Z. Wang, M. Gerstein, & M. Snyder, (2009). RNA-Seq: A revolutionary tool for transcriptomics. *Nature Reviews Genetics*, *10*(1), 57–63.

M. J. West-Eberhard, (2003). Developmental Plasticity and Evolution. *Oxford University Press.*

L. Zogbaum, P.G. Friend, & R.C. Albertson (2021). Plasticity and genetic basis of cichlid gill arch anatomy reveal novel roles for Hedgehog signaling. *Molecular Ecology*, *30*(3), 761–774.

J. Li, C. Wang, C. Wu, T. Cao, G. Xu, Q. Meng, B. Wang (2017). PKA-mediated Gli2 and Gli3 phosphorylation is inhibited by Hedgehog signaling in cilia and reduced in Talpid3 mutant. *Developmental Biology*, *429*(1), 147–157.

Appendix

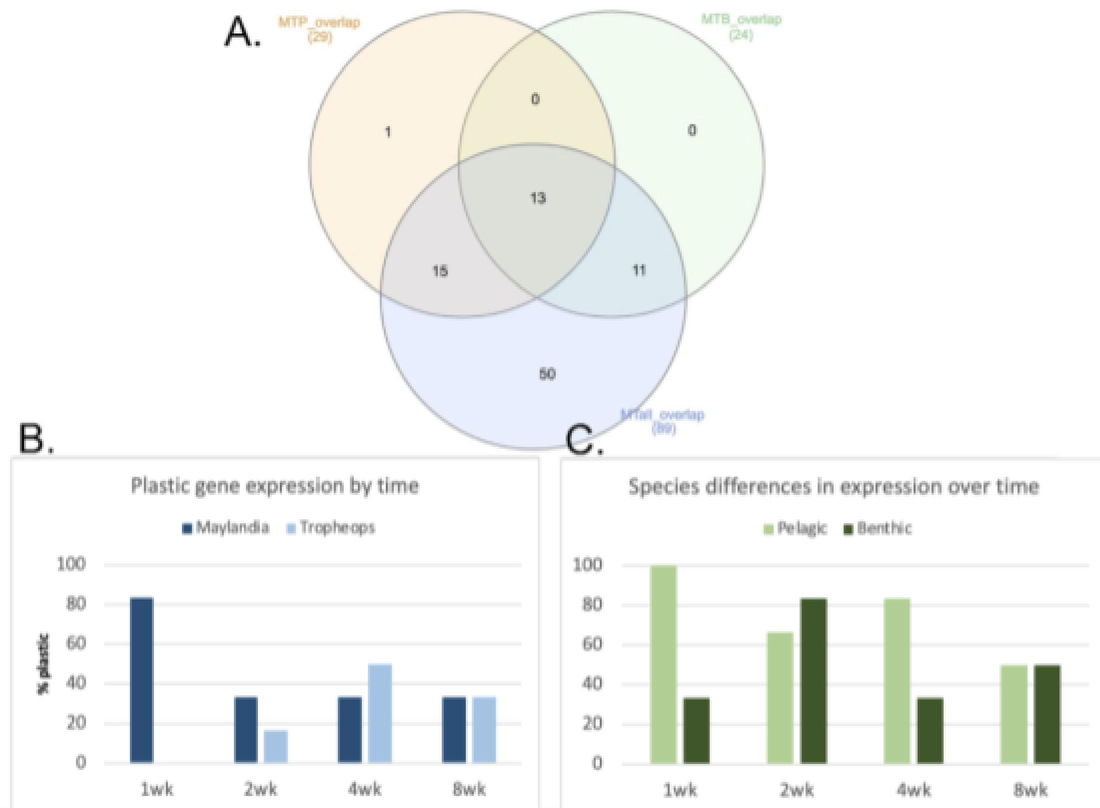

Figure 1a. A: venn-diagram of genes of interest based on genome wide analyses. B: compilation of plasticity for environmentally sensitive genes. "% plastic" refers to the number of genes, of 6 assessed, that were plastic. C: compilation of plasticity for environmentally sensitive genes showing species differences between *Maylandia* and *Tropheops*. "% plastic" refers to the number of genes, of 6 assessed, that were plastic.

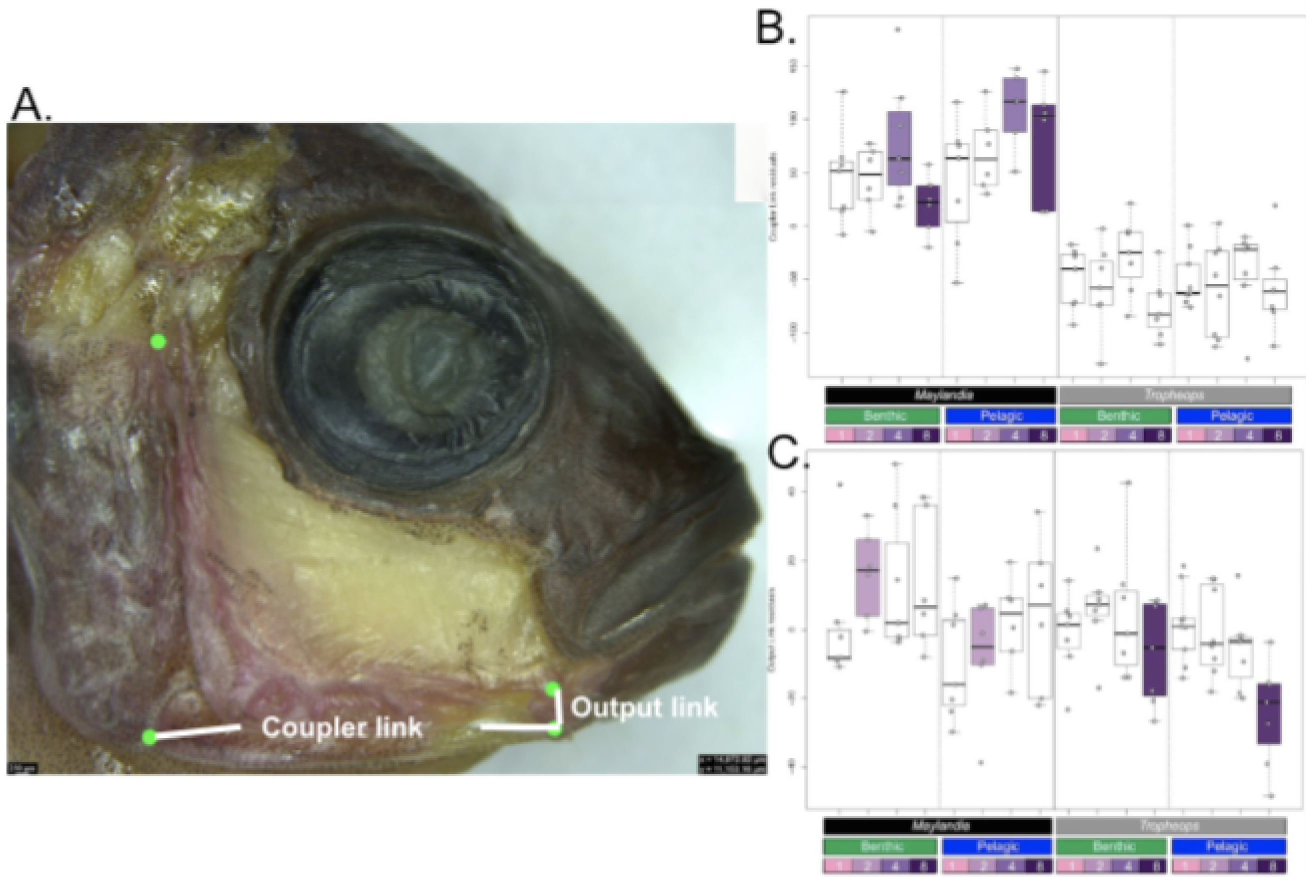

Figure 2a. A: Structures in the 4-bar system, “coupler link” and “output link,” where morphological differences occur. B: Morphological differences in the coupler link by species and over time. C: Morphological differences in the output link by species and over time.

Supplementary Information

Extracting RNA from field gathered tissue samples for RT-qPCR

Tissue collection:

Into TRIzol (Ambion Life Technologies, Catalogue #15596-026)

Keep as cold as possible.

Cut up tissue as much as possible to allow the TRIzol to permeate the cells.

Always do any work with TRIzol under the hood.

Any material that touches the TRIzol must go into the toxic waste containers.

Into RNAlater

One dimension of the tissue must be kept at <0.5cm to allow RNAlater to reach cells.

Can be kept at room temperature for around a week.

If kept cold or frozen can last months to years.

Upon return from the field place samples into TRIzol then into the freezer.

STAGE 1

RNA Extraction:

Tips

- Don't try to do more than 5-6 extractions at once. It'll get difficult.
- Store tissue samples in the controlled freezer overnight – the freezing process will cause more tissue break-up allowing for easier homogenization of the tissue.
- Label all tubes. Either have a system code that refers to something in your notebook or write the information directly onto the tube.
- All materials should come from the RNA cabinet. If run out of tips, take them from the autoclaved area, remove tape and stick on top, then write 'RNA' on the tape.

1. Homogenize samples in 500µl Trizol

   *Step occurs in fume-hood

   The homogenizer is a large glass rod that works like a mortar and pestle using friction to break apart the cells. In the Albertson lab it is found in the top cabinet by the sink.

   Pour (or pipette if sample is small) the sample into the homogenizer. Smash the sample.

   The homogenizer must be rinsed using 70% ethanol to remove additional tissue and finished with 95% ethanol.

   All waste ethanol must go into the toxic bin, including pipette tips.

   Return homogenized samples to the original screw-cap tubes.
2. Add 120 ul chloroform and invert 4-6 times to mix

   *Step occurs in the fume-hood

   Located in the left fume-hood cabinet at the back. Evaporates quickly.
3. Incubate at room temp for 2 min
4. Spin at 13000x for 3 min

   Remember to balance the centrifuge.

   General rule for centrifuging – Place the hinge on the outside. Allows for easier pipetting when you gain a pellet.

   *Remove glycogen from freezer to thaw for step six.
5. Transfer top (aqueous) layer to a new 1.5ml Eppendorf tube.

Will produce two primary layers. The top layer is the one we want. Extract gently using a pipette to transfer small amounts into the new Eppendorf. Pipetting can be done multiple times, once or twice should be enough to get the aqueous layer.

If you get the central or bottom layer (you'll know, it's a different colour), push the liquid back in and spin down again. It's better to get a pure mixture than one with some contamination.

6. Add 1ul glycogen (optional) and a volume of isopropanol equal to that of the aqueous layer (usually ~400ul) into the Eppendorf. Invert 4-6 times to mix.
   Isopropanol goes in at a 1:1 mix.
7. Incubate at room temp for 30 min
   Turn on cooler centrifuge to allow it to reach temperature. May need to swap some power cords around.
8. Spin at 13000x at 4°C for 20 minutes
   Kara's addition: invert a few times and spin an additional 20minutes.
9. Decant sup (may or may not save)
   Use pipette to remove on opposite side of the hinge to save the pellet.
10. Add 500ul cold 80-100% EtOH
    Located in the control freezer in a Falcon tube.
    *If doing the DNase step, remove the reaction buffer and EDTA now (not enzyme).
11. Spin at 13000xg at 4°C for 5 min
12. Decant sup (may or may not save)
    Use pipette to remove on opposite side of the hinge to save the pellet.
    May start to see small white precipitate on hinge side – this is the RNA.
13. Touchspin and remove any remaining supernatant.
    Use standard centrifuge – touch the start button to get to ~6000rpm, then release.
14. Airdry pellet
    Can do this by inverting and jamming under a tube rack, or leave on side in fume-hood.
      Do not want dust to enter the tube.
    Takes around 10-15 minutes – precipitate turns clear.
15. Resuspend total RNA pellet in 8ul RNase-free water
    RNase-free water is located in the RNA cabinet.
    *Can stop at this stage, but not recommended – getting into DNA is far more stable.

STAGE 2

DNase: (Invitrogen 18068-015)

This stage removes any genomic DNA that can negatively affect the qPCR result.

Only remove DNase enzyme from freezer when ready to use, and keep on ice.

Vortex and spin down (in centrifuge) the buffer prior to starting.

In PCR tube, mix (using pipette)

Total RNA from step 15	8uL
10x reaction buffer	1uL
DNase	1uL
	10uL

1. Incubate at room temp for 15 min

2. Add 1ul 25mM EDTA and incubate at 65°C for 10 min
   Use the PCR machine to do this – program: 'Sixty Five 10'

STAGE 3

RT-PCR (Ambion High Capacity cDNA Reverse Transcription Kit 200rxns, 4368814 from Invitrogen)

In same PCR tube used for DNase step, assemble

DNased RNA from STAGE 2	11uL
10x reaction buffer (Yellow cap)	2uL
10x random primers (White cap)	2.5uL
10mM dNTPs (Eppendorf)	1.3uL
RNA water	2.2uL
RT enzyme (On ice)	1uL
Total	20uL

*Good to use a Master mix here.

Multiply by the number of reactions you wish to run. If you have 4 extractions the multiply by 4, you may want to add enough for an extra if something goes wrong.

Again, enzyme comes out last and place on-ice.

The Reverse Transcriptase enzyme makes DNA

PCR machine program – hc RT PCR protocol

1. Incubation:

25°C	10min
37°C	2hr
85°C	1min
4°C	hold (can go overnight, or place in control freezer)

2. To degrade rRNA, add 3.5uL 0.5M NaOH/50mM EDTA and incubate at 65°C for 10 min, then bring to 4°C
   This step makes the rRNA more unstable, EDTA protects the cDNA.
3. To neutralize, add 5uL 1M Tris-HCl pH 7.5
4. Best to test cDNA by using 1uL as template in a PCR reaction.

STAGE 4

Amplification using PCR

In new PCR tubes add

cDNA template from STAGE 3	1uL
Forward Primer	1uL
Reverse Primer	1uL
Red Taq polymerase	10uL
RNA water	7uL
Total	20uL

Again, can make Master mix.

This involves placing 1ul of the cDNA template into new PCR tubes. Then, in a new Eppendorf mix F-R primers, RNA water, and finally Taq (thawed on-ice). Multiple quantities

above by the number of reactions you need to do. Add 19ul to the cDNA template in the PCR tubes.

Use PCR program – XYZ protocol.

1. Incubation:

    Standard PCR protocol

    4°C hold (can go overnight, or place in control freezer)

STAGE 5

Running a gel to check for RNA product

Amount of Agarose gel depends on sample size.

For <18 samples use the small gel container.

1. For 1% gel use 0.4g agarose with 40ml of new TAE solution.
   Use small weighing scales with measuring paper (in top drawer).
   Open both sides and use the spatula to tip agarose onto the paper.
2. Mix agarose and TAE in flask from the fume hood (not EthBr flask).
3. Microwave for 50 seconds, spin and mix for another 15 seconds
   Add more/less time depending how much agarose dissolved.
   Should see the sides bubbling in the microwave.
4. Allow agarose mixture to cool in flask for ~5 minutes.
   Should be able to hold the bottom of the flask in hands for 1-2 seconds.
5. Add 0.6ul of florescent dye to warm agarose solution. Mix gently.
   Florescent dye is light sensitive, only remove from freezer when needed.
6. Pour into small gel case and add the wells template. Wait 20 minutes.
   Pour some used TAE if hard to insert the gel case.
7. Remove wells template. Take ladder (1KB or 10KB) from freezer and pipette 2ul into a well.
8. Use parafilm to place small dots of loading dye to mix with 3ul of your PCR sample.
   Mix 3ul of PCR product with the loading dye on the parafilm.
9. Pipette dye and product into wells.
   Make a note of what is in each well.
10. Slide the electrode top into place and turn on at the blue power pack. Run for ~20 minutes.
    Power pack should read between 95-97V.
    Small bubbles should rise on the black wire side.
    Place a label by the gel so you know what is contained in each well.
11. Remove gel and place on UV light box, cover with orange sheet.
    Take a picture. Place the label from before in view so you record what is in each well.
    Compare the bands to your product – if it worked your RNA should light up!

Disposal Notes

All TRIzol in the fume-hood waste bins – both solid (i.e., pipette tips) and liquid.

Empty tip cases leave on the small table by the door.

Gels (with no EthBr) can go in the bin.