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Exploring Mechanisms Underlying Plasticity and Variation in Lake Malawi Cichlids

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Abstract

Because natural selection operates on phenotypes, understanding the mechanisms that generate and maintain phenotypic variation is key to explaining evolutionary divergence. In this thesis, I investigate multiple sources of variation that shape diversification, focusing on phenotypic plasticity, the genetic basis of plastic responses, genotype–environment interactions, and the role of mutations. Together, these processes not only provide the raw material for natural selection but also influence the trajectory of adaptive diversification. Lake Malawi cichlids, representing the largest known extant adaptive radiation with extensive variation in their craniofacial morphology, serve as a promising model system to test these ideas.

Chapter 1 outlines the conceptual background and establishes the context for the investigations undertaken in this research. **Chapter 2** tests a key hypothesis that plasticity decreases with an increase in specialisation. For this, I studied eight representative species of Malawi cichlids and conducted a geometric morphometric approach to quantify shape variation in the craniofacial region. I also conducted RNA-seq on a subset of these species to examine gene expression and its variation in response to environmental cues across species. The findings demonstrated that plasticity operates at multiple levels, from developmental gene regulation to morphology, in facilitating adaptive divergence. The integration of morphological and transcriptomic datasets revealed that species positioned centrally along the biting–suction ecomorphological axis display relatively high plasticity, whereas specialists at the ends exhibit canalisation. This correspondence supported models where plasticity is lost during ecological specialisation via genetic assimilation in an adaptive radiation.

In **Chapter 3,** I studied the transcription factor *zeb1* as a differentially expressed gene associated with mandible shape variation and validated its functional relevance in Lake Malawi cichlids and zebrafish. Here, I conducted qPCR on *Labeotropheus fuelleborni* (LF) and *Tropheops* sp. 'red cheek' (TRC) embryos to test for divergence in *zeb1* expression. To further elucidate the role of *zeb1* in mandible formation, I genotyped wild-type and *zeb1* mutant zebrafish and conducted geometric morphometrics. Experiments with *zeb1* mutant zebrafish provided evidence of how *zeb1* correlates with jaw-shape variation and trophic specialisation, illustrating how a specific regulatory mutation can influence ecologically relevant morphology.

Chapter 4 explores the genetic basis of adaptive plasticity by studying a candidate gene, *wnt7ba*. I reared wild-type and *wnt7ba* mutant zebrafish under alternate feeding treatments and conducted geometric morphometrics to quantify shape variation. Although the results did not yield significant genotype effects on plasticity, patterns of differences in mutant plasticity point to *wnt7ba* as a plausible contributor to craniofacial development and adaptive plasticity. Given its clear relevance to trophic morphology, an ecologically critical trait, *wnt7ba* remains a strong candidate.

In **Chapter 5**, I introduce a novel, cost-effective protocol for establishing primary bone cell cultures from cichlids. By isolating primary osteogenic cells from species chosen along the biting-sucking ecomorphological axis, this approach enables characterisation of their mechanotransduction pathways and comparison of osteogenic responses among species with differing trophic ecologies and plasticity. This working protocol fills a significant gap in bone biology by facilitating the study of osteocyte-independent mechanisms of bone formation and remodelling, and provides a valuable framework for linking cellular responses to mechanical stress with evolutionary patterns of craniofacial variation in divergent cichlid species.

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Author's Declaration

The research conducted in this thesis took place between October 2021 and September 2025 and is entirely my own. None of the work in this thesis has been submitted for another degree.

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Abbreviations

AS	*Aulonocara stuartgranti*
bmp4	Bone morphogenetic protein 4
cam1	Calmodulin
chrdl	Chordin-like
CB	*Copadichromis borleyi*
DC	*Dimidiochromis compressiceps*
DE	Differentially expressed
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal Bovine Serum
fst	Follistatin
LC	*Labidochromis caeruleus*
LF	*Labeotropheus fuelleborni*
MZ	*Maylandia zebra*
PBS	Phosphate Buffer Saline
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PLL	Poly-L-Lysine
QTL	Quantitative trait loci
RC	*Rhamphochromis chilingali*
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
TRC	*Tropheops 'red cheek'*
wnt	Wingless
zeb1	Zinc finger homeobox 1

Chapter 1: General Introduction

1.1 Adaptive divergence

Adaptive divergence is the emergence of new forms from a common ancestor by adaptation to environmental conditions. In many clades, this divergence occurs as adaptive radiation, a rapid burst of speciation within a lineage (Schluter, 2000). There are several examples, which include Darwin's finches (Grant & Grant, 2007), Anolis lizards (Losos et al., 1998), limnetic-benthic pairs of sticklebacks (Rundle et al., 2003), Arctic charr (Adams et al., 1998), and African cichlids (Kocher, 2004). A widely accepted three-stage framework explains that radiations typically involve habitat divergence, trophic differentiation, and sexual selection (Fryer & Iles, 1972; Rundle, 2002; Streelman & Danley, 2003; Kocher, 2004). Adaptive divergence is traditionally studied through a genetic perspective, focusing on how selection acts on standing genetic variation (Schluter, 1996; Schluter & Conte, 2009). The Modern Evolutionary Synthesis combines Darwin's theory of natural selection with Mendelian genetics to explain the emergence and regulation of adaptive variation within populations, becoming the dominant framework in evolutionary biology (Mayr, 1993). However, some researchers regard this theory as gene-centric (Bonduriansky, 2012), as it considers genetic mutation the sole source of variation for natural selection. It overlooks developmental processes, including how the environment can instruct the formation of phenotypes (Parsons et al., 2020; Lala et al., 2024). Conversely, the Extended Evolutionary Synthesis (EES) highlights the roles of developmental processes, plasticity, and non-genetic inheritance in shaping evolutionary trajectories (Gilbert & Epel, 2009; Moczek et al., 2011; Laland et al., 2014; Gilbert et al., 2015; Moczek, 2015; Laland et al., 2015).

The environment is known to play a significant role in the evolution of adaptive variation (Laland et al., 2014; Parsons et al., 2016). In this context, phenotypic plasticity has emerged as a key phenomenon and is recognised as a progenitor of variation that allows populations to develop adaptive phenotypes in different environments. It is defined as the ability of a developmental system to produce multiple phenotypes in response to different environmental conditions. Therefore, environmental inputs are not merely "noise" surrounding genes; they influence phenotype formation, determining which genetic variants are expressed, selected, and retained. According to the plasticity-first hypothesis, environmentally induced phenotypes arise initially; then, selection refines and stabilises the underlying developmental and genetic architecture, sometimes resulting in canalised traits

(Levis & Pfennig, 2016). The initial ideas of plasticity-led evolution trace back to the Baldwin effect (Baldwin, 1896, 1902), which emphasises how plasticity can enable populations to survive in new environments, with this variation being acted upon by natural selection (Crispo, 2007). In adaptive radiations, a plastic ancestor repeatedly colonises new niches, expressing alternative phenotypes that eventually become canalised in different lineages (West-Eberhard, 2003; Wund et al., 2008; Schneider & Meyer, 2017). This leads to the emergence of new, complex traits and ecological niches (Pfennig et al., 2010; Moczek et al., 2011; Moczek et al., 2015). Therefore, including plasticity in models of adaptive divergence may offer valuable insights into rapid adaptation and the potential for evolutionary novelty (Pfennig et al., 2010).

1.2 From plasticity to specialisation: patterns of variation in an adaptive radiation

To fully understand the evolutionary implications of phenotypic plasticity, it is essential to explore the mechanisms through which environmentally induced traits become genetically canalised in populations. One such mechanism is genetic accommodation, which refers to the process by which adaptive genetic changes modify the expression or regulation of a phenotype in response to the surrounding environment (Pfenning, 2016). This evolutionary mechanism allows initially plastic traits to become more stable over generations through natural selection. A trait may evolve either an increased or decreased environmental sensitivity (i.e., phenotypic plasticity), enabling organisms to optimise their traits in response to environmental perturbations. Plasticity is adaptive, and as it decreases, an extreme form of genetic accommodation, known as genetic assimilation, occurs (Waddington, 1942; Schmalhausen, 1949; Waddington, 1953). This leads to the formation of a novel, canalised trait- one that is fixed with limited influence from environmental conditions. Conversely, variable environmental conditions may promote plasticity (Waddington, 1953; Pigliucci & Murren, 2003; Masel, 2004). This results in a gradient across radiations, where generalists in variable environments tend to retain higher plasticity, while specialists in stable environments exhibit less plasticity or even developmentally fixed traits (Skúlason & Smith, 1995; Wund et al., 2008; Schneider & Meyer, 2017). Waddington's experiments demonstrated that environmental stresses (e.g., ether vapour, heat shock) could induce rare phenotypic changes, such as the bithorax and cross veinless trait in *Drosophila melanogaster* (Waddington, 1953). By selectively breeding flies that expressed this trait, he increased its frequency in the population. Surprisingly, the trait

became genetically canalised after several generations and appeared even without the environmental trigger (i.e. assimilated). Waddington explained this as the outcome of selection on the activation threshold of the trait, leading to genetic assimilation. By integrating genetic assimilation into modern evolutionary theory, particularly through the Extended Evolutionary Synthesis (EES), we can develop a more comprehensive understanding of how organisms adapt, diversify, and evolve.

1.3 Mechanisms of adaptive variation

While phenotypic plasticity is now widely recognised as a central component of the evolutionary process, we still have very little understanding of the genetic underpinnings of plasticity in natural systems undergoing rapid adaptive diversification (Gilbert, 2017). The genotype is a key predictor of phenotypic variation and thus the form-function relationships that are essential for adaptive divergence. Phenotypic plasticity is associated with changes in gene expression, offering deeper insights into its molecular mechanisms (Aubin-Horth & Renn, 2009; Gilbert & Epel, 2009). Gene expression plasticity, mediated through gene regulatory networks and signalling pathways, allows organisms to dynamically modify their morphology, physiology, and behaviour. Although there is literature on the genetic basis for diet-induced morphology in cichlids (Cooper et al., 2011; Parnell et al., 2012; Parsons et al., 2015, 2016; Hu & Albertson, 2017; Navon et al., 2020), there are not many studies that have explicitly examined how these factors contribute to plasticity in an adaptive radiation. Studies should extend beyond external morphological phenotypes to include molecular phenotypes, aiming to gain a better understanding of canalisation and the mechanisms by which variation occurs and responds to selection.

Genetic assimilation can be promoted by quantitative changes in gene regulation involving one or more loci, with effects arising either in phenotypically relevant genes or their upstream regulators (Ledón-Rettig et al., 2014; Pfennig & Ehrenreich, 2014). Techniques such as RNA-sequencing at different developmental stages can measure genome-wide transcription (Wang et al., 2009; Ehrenreich et al., 2010). Beyond transcriptional activities, molecular mechanisms such as post-translational protein regulation or protein-protein interactions may also contribute to this process (Ehrenreich & Pfennig, 2016).

Genotype-environment (GxE) interactions are also a critical driver in determining phenotypic variation (Diouf et al., 2020). Environmental factors such as diet, light, habitat structure, and water chemistry can interact with underlying genetic variation to shape key traits, often leading to divergent phenotypes (Seehausen et al., 2008; Powder & Albertson, 2016). Although the genetic underpinnings of these interactions are less studied, experimental work has shown that the foraging environment can strongly influence how genetic variation translates to morphology (Parsons et al., 2016; Powder & Albertson, 2016; Zogbaum et al., 2021; Tetrault et al., 2023). Studying candidate genes that might be involved in the occurrence of G×E can help identify mechanisms underlying adaptive variation, thus providing a deeper understanding of the genetic architecture that can evolve and promote diversification.

Mutations are another source of variation leading to evolutionary change (Eyre-Walker & Keightley, 2007; Lynch, 2010). They create new alleles or modify existing ones, providing raw material for natural selection. Although most mutations are deleterious, some confer advantageous effects that natural selection can act upon, enabling populations to adapt and evolve (Orr, 2005; Barrett & Schluter, 2008). It also explains the molecular basis of evolutionary divergence, helping trace the pathways from genetic variation to speciation.

Thus, gaining an understanding of a wide spectrum of sources of variation provides crucial insights into adaptation and diversification, highlighting the mechanisms in an evolutionary framework.

1.4 Malawi cichlids as an evolutionary model

Cichlids from the East African Great Lakes are known to evolve rapidly and are considered an exemplary model for studying the role of adaptive phenotypic plasticity in diversification. It has been observed that phenotypic plasticity plays a significant role in changing morphological traits in the African cichlids (Bouton et al., 2002; Rutjes et al., 2009). The adaptive radiation of African cichlids shows a considerable degree of variation in craniofacial morphology (Kocher, 2004; Cooper et al., 2010), providing an excellent system to study the developmental and genetic processes that promote diversity. Adaptive variation in craniofacial structures is an integral component of habitat divergence and resource specialisation in vertebrates, leading to niche partitioning and the formation of new species. The cichlids exhibit species-specific craniofacial traits that promote ecological

specialisation, making them a suitable model for examining the role of development in producing phenotypic variation. African cichlids display a primary axis of craniofacial variation that aligns with two primary foraging mechanisms. Species on one end of this ecomorphological continuum are limnetic feeders that graze upon mobile prey in the water column via suction feeding. These are characterised by a shallow craniofacial profile and a long, slender jaw, which enhances their ability to capture prey efficiently. On the other end of the continuum are benthic feeders that feed on hard prey such as crustaceans and molluscs, or scrape algae from the rocks. These species rely on biting mechanics, which require forceful jaw closure rather than suction. They are characterised by a steep craniofacial profile and a short, broader jaw that provides the strength needed for crushing hard prey (Albertson et al. 2005; Parsons et al., 2014).

These craniofacial differences are not only genetically determined but also influenced by phenotypic plasticity, which allows individuals to adjust their jaw morphology in response to dietary and feeding demands (Bouton et al., 2002; Parsons et al., 2014, 2016; Navon et al., 2020). Because African cichlids are closely related and share a common genetic background, the remarkable variation in their craniofacial shape provides an ideal system for genetic work (Powder & Albertson, 2016). This makes them a powerful laboratory model for investigating the genetic mechanisms underlying adaptive divergence (Albertson & Kocher, 2006). The interplay of genetic factors and plasticity plays a crucial role in the remarkable adaptive radiation of African cichlids. Studying these adaptations can offer deeper insights into the genetic and developmental mechanisms underlying evolutionary diversification (Albertson et al., 2005; Rutjes et al., 2009).

1.5 Role of mechanosensitivity and bone plasticity in adaptive radiation

It is widely known that the process of bone modelling is carried out by osteoblasts (bone-depositing cells) and osteoclasts (bone-resorbing cells), which are regulated by osteocyte cells (Bonewald, 2011). They are most abundant and constitute 95% of total bone cells. Osteocytes are considered master cells that play a critical role in bone physiology. They function as mechanosensors, detecting variation in bone strain (Witten & Huysseune, 2009; Bonewald, 2011). The surprising thing here is that, unlike mammals, many teleosts are devoid of these osteocyte cells, which has questioned the presumption that osteocytes are necessary for bone formation and remodelling. Some studies have shown evidence of bone

remodelling in response to mechanical loading in anosteocytic fishes like *Astatoreochromis alluaudi* (Huysseune et al., 1994), and *Oreochromis aureus* (Atkins et al., 2015), suggesting the presence of osteocyte-independent mechanisms giving deeper insights into bone biology.

During prey capture, fish experience functional stress on their craniofacial bone structures. Several studies have revealed that exposure of bone cells to mechanical stress can increase their metabolic activities (Pead et al., 1988; Dodds et al., 1993) and affect bone formation, remodelling, and repair (Herrel et al., 2009; Riddle & Donahue, 2009). Wolff (1892) presented the 'law of bone transformation', which stated that bones of vertebrates display a high degree of plasticity and respond to mechanical load. Huysseune et al. (1994) conducted a study where they fed two different diets to cichlids (*Astatoreochromis alluaudi*) and observed that the ones fed with a hard diet comprising snails developed stronger and thicker lower pharyngeal jaws as compared to ones fed with a softer diet. This revealed adaptive remodelling of jaws in response to mechanical loading.

Cichlids are also an exemplary model of bone remodelling (Bouton et al., 2002; Parsons et al., 2016; Navon et al., 2020; McWhinnie et al., 2022). They exhibit considerable variation in their craniofacial morphology with evolutionary divergence enabling swift transitions between biting and suction modes of feeding (Kocher, 2004; Cooper et al., 2010). This adaptability offers extensive evolutionary advantages by creating significant biomechanical variation in feeding, leading to crucial shifts in trophic ecology. These species are spread across an ecomorphological axis, and their morphological variation underlies a continuum from pelagic, water-column suction feeders to benthic biters (Kocher, 2004; Cooper et al., 2010). It is predicted that the generalist species positioned in the middle of the continuum might exhibit higher phenotypic plasticity and shift performances across prey types. Towards the end, this plasticity might reduce, and the specialist species might evolve canalised morphologies optimised for either the suction or biting mode of foraging (Skúlason & Smith, 1995). Since the craniofacial structures of these species remodel in response to mechanical loading, they would provide a good model to look at plasticity and genetic assimilation at a cellular level.

Cichlids respond to mechanical stress, where inherent *in vivo* conditions may play a role in overall responses. This makes it difficult to directly assess the role of mechanical stress on bone tissue. Culturing bone cells under controlled *in vitro* conditions offers a way to determine whether isolated mechanical stress has a different impact on the bone cells of

species. In the fish overall, other factors like nutritional profile, bone density, muscle loading, and jaw speed might influence the response, whereas in the cellular study, mechanical stress will be the sole factor responsible for the observed changes.

Examining genetic assimilation and specialisation in cichlid bone cell cultures would offer a strong, explicit test of whether phenotypes caused by mechanical stress are supported by *in vitro* mechanisms. Additionally, by isolating primary osteogenic cells from species that vary in trophic ecology and plasticity, insights into osteogenic responses and mechanotransduction pathways can be gained. Therefore, this approach could serve as a foundation for understanding how bone cells grow and remodel themselves, offering deeper insights into both bone biology and evolutionary shifts from plasticity to specialisation, and providing relevant context where the genetic influences on plasticity and its assimilation can be demonstrated.

1.6 Thesis overview

In **Chapter 02,** I study eight representative species of Malawi cichlids to test the hypothesis that ecological generalists display greater plasticity than specialists, and that canalisation of morphological traits occurs with increased ecological specialisation through genetic assimilation. For this, I use geometric morphometrics to quantify shape variation in the craniofacial region. I also conduct RNA-seq to examine gene expression and its variation in response to environmental cues between generalists and specialists.

In **Chapter 03,** I study the transcription factor *zeb1* as a differentially expressed gene associated with mandible shape variation and validate its functional relevance in Lake Malawi cichlids and zebrafish. Here, I conduct qPCR on *Labeotropheus fuelleborni* (LF) and *Tropheops* sp. 'red cheek' (TRC) embryos to test for divergence in *zeb1* expression. To further elucidate the role of *zeb1* in mandible formation, I genotyped wild-type and *zeb1* mutant zebrafish and conducted geometric morphometrics.

In **Chapter 04**, I study a candidate gene, *wnt7ba* and its role in adaptive plasticity. I rear wild-type and *wnt7ba* mutant zebrafish under alternate feeding and conduct geometric morphometrics to quantify shape variation.

In **Chapter 05**, I introduce a novel, cost-effective protocol for establishing primary bone cell cultures from African cichlids, an ecologically diverse and evolutionarily significant group of teleost fishes. This working protocol fills a significant gap in bone biology by examining the structure and function of bones in species lacking osteocytes. Conducting direct *in vitro* cultures of cichlid bone cells will provide new opportunities to study osteocyte-independent mechanisms of bone formation and remodelling and give valuable insights into the evolutionary and functional aspects of bone adaptation.

Chapter 2: Does plasticity decrease with increased specialisation? Examining the underlying mechanisms of phenotypic plasticity in African cichlids

2.1 Abstract

Evolutionary biology has primarily focused on genetic mutation and inheritance as sources of variation, often overlooking developmental processes and environmentally induced variation. Phenotypic plasticity is an organism's capacity to produce different phenotypes in response to environmental conditions. Through mechanisms such as genetic accommodation and genetic assimilation, initially plastic traits can become canalised as a potential contribution to evolutionary innovation, speciation, and adaptive radiation. While laboratory experiments have demonstrated assimilation, evidence from natural systems remains limited. Lake Malawi cichlids, with over 500 species, represent the largest extant adaptive radiation and can serve as an ideal model for investigating the role of plasticity in diversification. Their craniofacial morphology underpins a range of feeding strategies, from generalists to obligate specialists, reflecting positions along a biting–suction ecomorphological axis. This chapter examines eight representative cichlid species to test the hypothesis that ecological generalists exhibit greater plasticity than specialists, and that canalisation of morphological traits occurs with increased ecological specialisation. By analysing gene expression in craniofacial tissues, this work also aims to identify the regulatory networks underlying plastic responses, assess their role in trophic specialisation, and explore how plasticity contributes to the fixation of specialised traits.

2.2 Introduction

The Modern Evolutionary Synthesis, which emerged in the mid-twentieth century, combined Darwin's theory of natural selection with Mendelian genetics to explain the evolution of adaptive variation. While this has become the dominant framework in evolutionary biology (Mayr, 1993), it is regarded as gene-centric by several researchers (Bonduriansky, 2012; Muller, 2017) since it views genetic mutation as the only source of variation for selection. Thus, the modern synthesis overlooks developmental processes, including how the environment can instruct the formation of phenotypes (Parsons et al.,

2020; Lala et al., 2024). This creates a need to recognise the importance of how environmental factors and development can be more centrally incorporated into an evolutionary framework (Gilbert & Epel, 2009; Moczek et al., 2011; Gilbert et al., 2015; Moczek, 2015; Laland et al., 2015).

Phenotypic plasticity, defined as the ability of the developmental system to produce multiple phenotypes dependent upon environmental conditions, plays a key conceptual focus for extending evolutionary theory (West-Eberhard, 2003; Laland et al., 2015; Susoy et al., 2015). Plasticity can quickly provide a survival advantage in variable environments by altering gene expression or developmental pathways that produce adaptive phenotypes. Over generations, if plasticity provides a benefit, selection may favour genetic changes that stabilise it or modify plasticity to be more efficient (Pigliucci et al., 2006; Lande, 2009). Plasticity can play an important role in the origin of new and complex traits (Moczek, 2008), leading to speciation (West-Eberhard, 2005; Pfennig & McGee, 2010) and adaptive radiation (Pigliucci & Murren, 2003; Wund et al., 2008; Pfennig et al., 2010; Moczek et al., 2011). However, some core theories for plasticity's role in evolution have not accrued a wealth of evidence. For example, adaptive divergence is traditionally studied through a genetic lens, focusing on how selection acts on standing genetic variation (Schluter, 1996; Schluter & Conte, 2009; Seehausen et al., 2014). However, phenotypic plasticity can allow populations to explore diverse ecological niches, potentially guiding evolutionary trajectories by affecting what genetic variation is exposed through the phenotype to become fixed (Levis & Pfennig, 2019). Therefore, incorporating plasticity into models of adaptive divergence may help explain rapid adaptation and the potential for evolutionary novelty (Pfenning et al., 2010).

While exploring the mechanisms through which environmentally induced traits ultimately drive genetic change in populations is essential, evidence in natural systems is sparse. The process of genetic accommodation refers to how adaptive genetic changes modify the expression or regulation of a phenotype induced by the surrounding environment and could offer insights (West-Eberhard, 2003; West-Eberhard, 2005). This evolutionary mechanism allows initially plastic traits to stabilise and become refined through natural selection. Importantly, genetic accommodation does not necessarily entail complete canalisation rather, it encompasses a range of evolutionary outcomes, including both the refinement of plastic responses and, under relatively stable environmental conditions or when plasticity is costly or constrained, a reduction in environmental sensitivity. In such

cases, selection may favour genotypes that produce a consistently adaptive phenotype with reduced plasticity, thereby increasing developmental stability while maintaining or improving fitness. A trait may evolve either an increased or decreased environmental sensitivity, enabling organisms to optimise trait variation in response to environmental perturbations (Ghalambor et al., 2007). When plasticity is lost, a specific form of genetic accommodation, known as genetic assimilation, occurs (Waddington, 1942; Waddington, 1953; Schmalhausen, 1949). This leads to the canalisation of traits that are less, or no longer influenced, by environmental conditions (Pigliucci & Murren, 2003; Pigliucci et al., 2006; Nijhout et al., 2021). Waddington (1953) demonstrated that environmental stresses (e.g., ether vapour, heat shock) could induce rare phenotypic changes, such as the bithorax and cross veinless trait in *Drosophila melanogaster*. By selectively breeding flies that expressed this trait, he increased its frequency in the population. Surprisingly, the trait became genetically fixed after several generations and appeared even without the environmental trigger (i.e. assimilated). Waddington explained this as the outcome of selection on the activation threshold of the trait, leading to genetic assimilation. It was concluded that a trait initially induced by the environment could become genetically encoded in a population if there is heritable variation in developmental pathways and strong selection. Thus, genetic assimilation challenges the mutationalist view of evolution by demonstrating that environmentally induced variation can drive genetic change. Developmental processes contribute to directionality in evolution because exploratory mechanisms like plasticity allow organisms to refine novel phenotypes (Plotkin & Odling-Smee, 1981). By integrating genetic assimilation and accommodation into modern evolutionary theory, we can develop a more comprehensive understanding of how organisms adapt and evolve (Lala et al., 2024).

This interplay between plasticity, genetic assimilation, and environmental stability is predicted to be closely linked to adaptive radiation (West-Eberhard, 2003). Adaptive radiation is the diversification of species from a common ancestor into a range of ecological niches (Schluter, 2000; Schneider & Meyer, 2017). In novel or unstable environments, increased plasticity can enhance the amount of available phenotypic variation and promote adaptation by allowing a population to survive an initial challenge and affecting which genetic variation is expressed (Laland et al., 2014). As the environment stabilises, genetic assimilation is predicted to lead to specialisation (Pigliucci, 2001; West-Eberhard, 2003; Schneider & Meyer, 2017; Skulason et al., 2019). However, ecological generalists may live in a variable environment and retain higher magnitudes of plasticity to cope. While laboratory studies have provided evidence for genetic assimilation (Waddington, 1953;

Sikkink et al., 2014; Walworth et al., 2016; Lorant et al., 2017), examples from natural systems remain limited. Notably, island tiger snakes (*Notechis scutatus*) show that populations colonising islands with larger prey developed increased head sizes through phenotypic plasticity, which over time became canalised (Aubret & Shine, 2009). Similarly, studies on spadefoot toads have demonstrated that environmentally induced carnivore morphs can undergo frequency-dependent adaptation and genetic assimilation (Levis & Pfenning, 2019). While these examples demonstrate genetic assimilation in nature, the role of plasticity in adaptive radiations remains elusive. To address this gap, this chapter focuses on African cichlids from Lake Malawi, the largest extant adaptive radiation, to investigate the role of genetic assimilation and plastic responses in their diversification.

Cichlids from the East African Great Lakes are known to evolve rapidly and are considered an exemplary model for adaptive phenotypic plasticity in diversification (Parsons et al. 2016; Santos et al., 2023). Phenotypic plasticity plays a significant role in cichlid morphological traits (Bouton et al., 2002; Rutjes et al., 2009; Navon et al., 2020; Gilbert et al., 2021), including the craniofacial apparatus, which is a key aspect of their evolution (Kocher, 2004; Cooper et al., 2010). Cichlids are known to exhibit a remarkable range of feeding specialisations, from highly versatile generalists to obligate specialists (Ribbink et al., 1983). For example, *Maylandia zebra* can employ both suction and biting modes of feeding on a wide range of prey, while other species are more rigid in their feeding strategies, being obligate suction feeders or biters. More recently, Cooper et al. (2010) demonstrated that this functional variation is consistently present across multiple adaptive radiations in African rift lakes, with generalists occupying intermediate positions along a morphological biting-suction axis, with more specialised species falling at the extreme ends of the axis. The biting specialists typically possess shorter, more robust jaws that increase mechanical advantage and bite force, whereas suction specialists exhibit gracile, longer jaws that expand buccal cavity volume and have smaller mechanical advantage (Wainwright & Richard, 1995; Westneat, 2004; Albertson et al., 2005; Hu & Albertson, 2014). I leverage this distribution of feeding modes, from generalists to specialists, as it makes cichlids particularly well-suited for studying genetic assimilation in an adaptive context.

The rich diversity of Malawi cichlids is predicted to stem from a three-stage evolutionary process marked by ecological divergence, trophic specialisation, and sexual selection. Starting with generalised ancestral cichlids colonising Lake Malawi, hybridisation led to a hypervariable population (i.e. a 'hybrid swarm) that rapidly diverged into two

macrohabitat clades- the rock-dwelling *mbuna* and the sand-dwelling lineage (Moran et al., 1994; Danley & Kocher, 2001; Sturmbauer et al., 2011). This enabled further specialisation as within clades, competition for food resources drove differentiation in trophic morphology, as species adapted to exploit distinct ecological niches. (Danley & Kocher, 2001; Seehausen, 2004). While described as a genetic process, this staged pattern of divergence is also consistent with a directional process of genetic assimilation, the main hypothesis of this chapter.

In this study, I investigate how plasticity decreases with an increase in specialisation. To test this, I selected eight species representing positions along a biting-suction ecomorphological axis, intending to capture a spectrum from generalists to specialists (Cooper et al., 2010). Species occupying intermediate positions on this axis exhibit generalist foraging, characterised by using both suction and biting modes of feeding (Ribbink et al., 1983). In contrast, species placed at the extremes of the axis show obligate specialisation, either biting or suction feeding exclusively. Here, I tested the hypothesis that genetic assimilation contributes to the Malawi cichlid radiation. Specifically, I predicted that species from the middle of the morphological distribution of the radiation would be more plastic than species from the extremes. If supported, this would demonstrate that feeding morphology can become canalised with increasing ecological specialisation in an adaptive radiation through genetic assimilation.

While research has elucidated the molecular pathways through which varied feeding regime influences the expression levels of various genes, less is known about how these mechanisms vary across radiations that include both generalists and specialists experiencing different ecological challenges and degrees of canalisation. Previous studies have focused on candidate genes influencing craniofacial morphology, like *ptch1* (Navon et al., 2020), *crocc2* (Gilbert et al., 2021), and *bmp4* (Parsons & Albertson, 2009). This chapter explores the genetic and developmental mechanisms underlying morphological plasticity by examining how gene expression patterns and regulatory networks mediate plastic responses in craniofacial structures. This will help in gaining a comprehensive understanding of the genes that act within the craniofacial region during a plastic response and reveal the regulatory mechanisms that underlie the evolved differences across different species of cichlids with varying phenotypes and degrees of specialisation. Thus, the aim is to better understand the evolutionary dynamics underlying adaptive morphological diversification and the potential for plasticity to contribute to the fixation of specialised traits through genetic assimilation.

2.3 Methods

2.3.1 Fish husbandry

Cichlids were purchased from the aquarium trade and housed and bred in 40-gallon glass tanks at 28°C within the University of Glasgow Aquaria facility. Each tank had a continuous water exchange and aeration, with regular checks on water parameters to ensure consistent water quality. Each brood from the females was raised separately in egg incubators with an air stone to ensure the embryos were well aerated. 2-3 broods were collected per species, depending on the brood size. At around 20 days post-fertilisation, when the yolk was completely absorbed, the broods were transferred to small glass tanks (~25L) and fed flake food. Care was taken to ensure all broods were raised separately and in tanks fitted with a fine mesh over the outflow pipe to prevent fish from escaping. After 5-6 weeks, the fish were moved into larger (40-gallon) tanks to start the plasticity experiments.

2.3.2 Plasticity experiment (two modes of feeding- sucking and biting)

Plasticity experiments were conducted on eight species of cichlids spanning the biting-suction axis in Malawi. They were chosen based on their position along the major eco-morphological axes of divergence for African cichlids (Cooper et al., 2010). The aim was to select species from extreme ends and the middle of the axis, comprising the biters with short jaws and the suction feeders with long jaws. The species included were *Rhamphochromis chilingali, Dimidiochromis compressiceps, Maylandia zebra*, *Copadichromis borleyi, Aulonocara stuartgranti,* and *Labidochromis caeruleus*. For *Labeotropheus fuelleborni* and *Tropheops 'red cheek'*, morphometric data were available from a previous plasticity study that was conducted at Glasgow using the same protocol (McWhinnie et al., 2022) **(Figure 2-1)**.

Figure 2-1: Left to Right: RC- *Rhamphochromis chilingali,* DC- *Dimidiochromis compressiceps,* AS- *Aulonocara stuartgranti,* CB- *Copadichromis borleyi,* LC- *Labidochromis caeruleus,* MZ- *Maylandia zebra,* TRC- *Tropheops 'red cheek',* LF- *Labeotropheus fuelleborni*

The cichlid broods were equally divided and reared in foraging environments that mimicked either benthic or limnetic modes of feeding (Parsons et al., 2014, 2016). Density was standardised across treatments, with each 40-gallon tank housing 17-22 individuals, and each treatment was replicated. For *Labidochromis caeruleus,* a replicate was not included due to an insufficient number of available broods.

The food given to the fish mimicked two types of foraging. For the benthic treatment, equal proportions of high-quality algae flake (Aquadip) and algae wafers (Hikari Cichlid Excel mini, United Kingdom) were mixed and spread over lava rocks. These rocks were left overnight to air-dry and then sunk to the tank bottom. This encouraged the fish to scrape food from the rock surface using a combination of biting, twisting, and scraping methods. For the limnetic treatment, the same mixture in powdered form was sprinkled into the tanks, prompting the fish to suction food from the water **(Figure 2-2)**. The nutritional composition of the limnetic and benthic diets was standardised to ensure similar nutrient content across treatments. Studies have shown that a Vitamin C deficient diet alters the morphology and jaw shape in fish (Wimberger, 1993). The powdered food was supplied three times daily, and rocks were provided twice daily for 8-9 months until the fish reached adulthood.

Random sampling (ten individuals were selected randomly per treatment per species, and standard length was measured) was conducted every 3-4 months, to ensure that growth was similar between treatments and the quantity of food was adjusted as needed **(Table 2-1).** Once the treatments concluded, the fish were euthanised using benzocaine solution (10g benzocaine powder, Sigma Life Science, diluted in 950ml ethanol and 50ml water) diluted 1:50 in fish water, following UK Home Office guidelines, labelled, and fixed in 10% neutral buffered formalin.

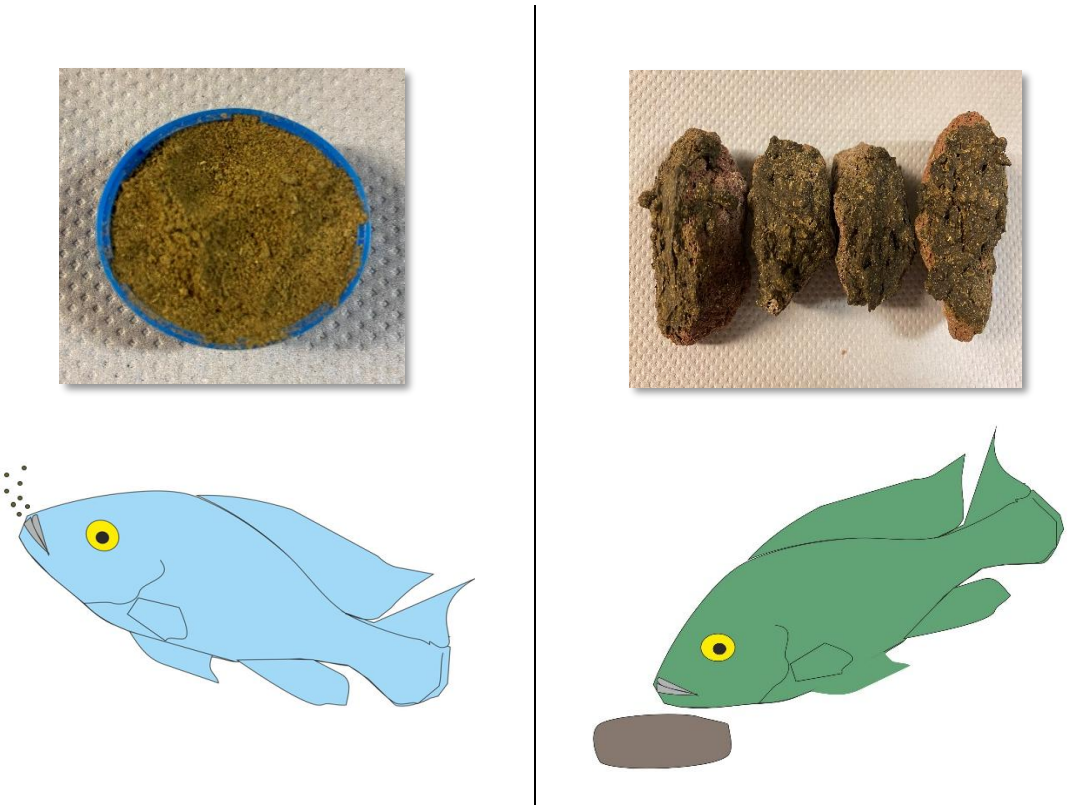


Figure 2-2: Plasticity experiment where the left panel shows limnetic diet treatment in the form of powder food to induce suction feeding. The right panel shows benthic diet treatment in the form of rocks to induce scraping mode of feeding.

Table 2-1: Standard length (mm) of fish individuals measured at the fourth and eighth months

Species	Benthic	Limnetic
	4th month	
Copadichromis borleyi	32.20 ±4.89	33.22 ±1.91
Maylandia zebra	24.12 ±4.42	25.64 ±4.72
Aulonocara stuartgranti	25.28 ±5.33	31.08 ±3.87
Rhamphochromis chilingali	39.10 ±6.14	38.84 ±5.28
Labidochromis caeruleus	24.58 ±2.58	25.25 ±1.58
Dimidiochromis compressiceps	21.80 ±1.98	27.45 ±4.62
	8th month	
Copadichromis borleyi	61.90 ±7.64	58.05 ±5.76
Maylandia zebra	59.66 ±7.78	56.40 ±4.30
Aulonocara stuartgranti	56.12 ±6.92	53.47 ±2.75
Rhamphochromis chilingali	66.05 ±10.05	64.69 ±6.89
Labidochromis caeruleus	58.38 ±4.92	59.10 ±2.64
Dimidiochromis compressiceps	54.82 ±7.74	55.30 ±6.48

2.3.3 Tissue extraction for RNA-sequencing

To investigate the transcriptome-wide plasticity across the species, tissue from the muscle and bone surrounding the left quadrate/articular joint was collected for RNA-seq. This joint is the primary rotational axis of the lower jaw, making it a critical anatomical structure for foraging **(Figure 2-3a)**. Six species comprising sand and rock dwellers (Sand dwellers- *Rhamphochromis chilingali, Dimidiochromis compressiceps, Copadichromis borleyi,* and *Aulonocara stuartgranti) (*Rock dwellers- *Maylandia zebra*, and *Labidochromis caeruleus)* were selected. 12 individuals per treatment and species were randomly selected for tissue collection, and the samples were stored in RNAlater at -80 °C. Tissue extracted from seven individuals per treatment and species was sent for RNA-sequencing to Novogene Co., Ltd. (Cambridge, UK). For *Labidochromis caeruleus*, only six individuals per treatment were sent due to the lack of a replicate group. After tissue collection, the fish were stained with 0.005–0.01% (w/v) alizarin red S in 75% ethanol (Springer & David, 2000) for morphometric analysis and then preserved in 70% ethanol.

2.3.4 Morphometric data collection

The craniofacial region of the fixed specimens was dissected to reveal musculature and to identify functionally relevant anatomical landmarks for geometric morphometrics (Cooper et al., 2010). A set of 15 landmarks was selected based on established protocols in cichlid functional morphology (Cooper et al., 2010; Parsons et al., 2016) to capture variation in structures underlying feeding biomechanics. Landmarks were chosen to represent key functional components of the feeding apparatus, including the neurocranium (e.g., dorsal end of the occipital crest), oral jaws (e.g., posterior tip of the premaxilla; tips of teeth on the premaxilla and mandible; retroarticular), the orbital region (antero-ventral and posterio-ventral margins of the eye socket), and elements of the suspensorium and jaw articulation system (maxillary-palatine joint, articular-quadrate joint, maxillary articulation). This captured functionally important aspects of jaw protrusion, bite force, and craniofacial kinesis (Westneat, 2003, 2004; Grubich et al., 2008). Only landmarks that were consistently identifiable across all specimens were retained.

Each specimen was positioned laterally on a wax plate with the mouth closed and photographed on its right side using a Canon EOS 1100D camera (Canon, UK). A ruler was used while photographing for scale calibration, and the fish were given individual identification. A total of 15 landmarks were digitised on the images using the TPS software suite (TPSdig and TPSUtil; Rohlf, 2015) **(Figure 2-3b)**. The resulting TPS file was imported

into R version 4.3.0 (R Core Team, 2022), and all downstream analyses were performed using the geomorph package version 4.0.6 (Adams & Otárola-Castillo, 2013).

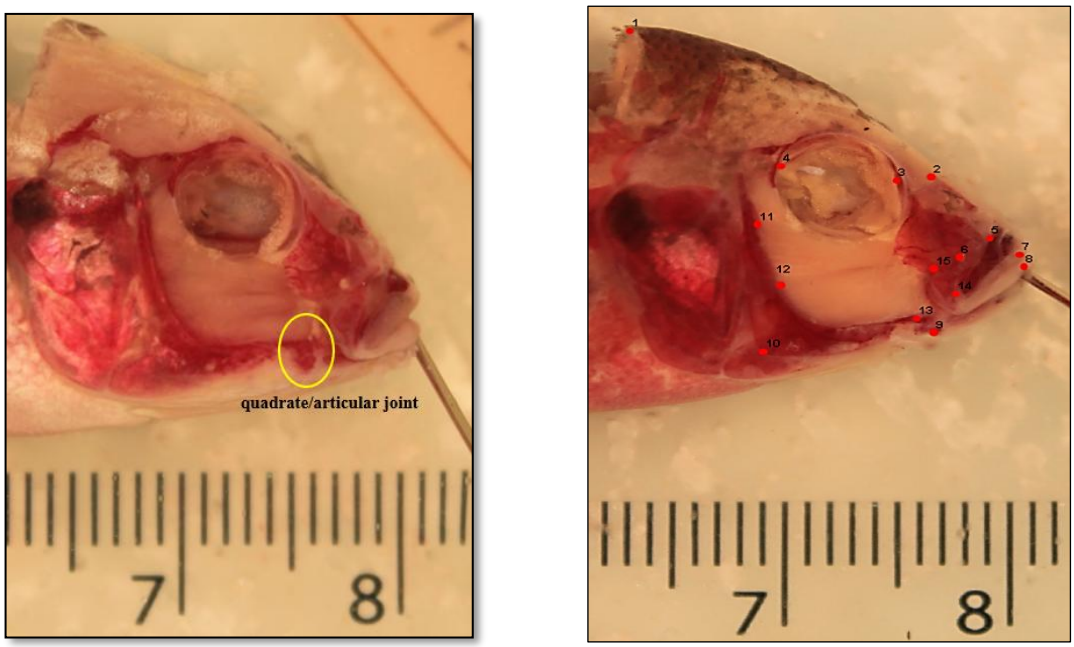


Figure 2-3: (a) Tissue from the muscle and bone surrounding the left quadrate/articular joint was collected for RNA-seq. This joint is the primary rotational axis of the lower jaw, making it a critical anatomical structure for foraging
(b) The landmarks selected for morphometrics based on functional and ecological relevance (Cooper et al., 2010; Parsons et al., 2016). Landmarks represent the following anatomical locations: 1) Dorsal end of the occipital crest; 2) Posterior tip of the premaxilla; 3) Anterio-ventral point of eye socket; 4) Posterio-ventral point of eye socket; 5) Maxillary-palatine joint; 6) Muscle insertion on the maxilla; 7) Tip of the tooth on the pre-maxilla; 8) Tip of the tooth on the mandible; 9) Retroarticular of the mandible; 10) Posterio-ventral corner of preopercular bone; 12) Origin point of muscle insertion on the pre-opercular; 12) Posterio-ventral corner of muscle origin; 13) Articular-quadrate joint; 14) Maxillary-articulation joint; 15) Muscle insertion on the articular process of the mandible.

2.3.5 Analysis of morphological data to test how plasticity varies with specialisation

To remove variations due to size and orientation, a Generalised Procrustes Analysis (GPA) was performed using the *gpagen* function (Zelditch et al., 2012). GPA standardises landmark configurations by translating all specimens to a common centre, scaling them to unit centroid size, and rotating them to minimise the summed squared distances between corresponding landmarks. This procedure isolates shape variation by eliminating the effects of overall size, thereby allowing comparison of craniofacial morphology across individuals.

Landmarks from both the adaptive radiation and plasticity experiments were analysed jointly to ensure all specimens were placed in a common shape space. Data for the adaptive radiation were adapted from Cooper et al. (2010), with two additional species (*Rhamphochromis chilingali,* and *Aulonocara stuartgranti)* included in the analysis. Geometric morphometric data were collected from three adult RC and five adult AS individuals. A GPA was performed, and then the mean landmark configuration across all aligned specimens was calculated using the *mshape* function, providing the consensus craniofacial shape (Adams & Otárola-Castillo, 2013). To ensure compatibility with the dataset of plasticity specimens, one landmark was removed from all adaptive radiation individuals (McWhinnie et al., 2023). For the plasticity experiment subset, a multivariate Procrustes ANOVA was performed using the *procD.lm* function with type I sums of squares to test the effects of diet, species, and their interaction, and log-transformed centroid size on craniofacial shape. This method performed a permutation-based linear model with 999 iterations (Collyer & Adams, 2018). This approach fits a linear model to Procrustes-aligned landmark coordinates by using residual randomisation in a permutation procedure (RRPP). It estimates model coefficients from ordinary least squares, then permutes residuals to generate an empirical null distribution of the test statistic. Significance is evaluated by comparing the observed sums of squares to this permutation-based null distribution, providing inference for multivariate shape data.

To address the prediction that plasticity decreases with increasing specialisation, I quantified the amount of plasticity for each species and later compared these values with their positions along the eco-morphological axis. A larger Procrustes distance relates to relatively higher levels of plasticity. For this, a trajectory analysis was conducted using the *trajectory.analysis* function (Collyer & Adams, 2013). The function quantifies phenotypic shape change trajectories from the specimens and assesses variation in attributes of the trajectories via permutation. A shape change trajectory is defined by a sequence of shapes in tangent space. These trajectories can be quantified for various attributes (their size, orientation, and shape), and comparisons of these attributes enable the statistical comparison of shape change trajectories. For each species, the trajectory length (path through Procrustes shape space) was used to quantify the magnitude of morphological change.

While I leveraged data from Cooper et al. (2010), it was important to assess where the fish (and landmarks) from this experiment were positioned in this morphospace to assess ecological relevance. Therefore, I performed a Principal Component Analysis to determine

how my study species were distributed. To achieve this, I used the Procrustes-aligned coordinates of the adaptive radiation and experimental groups using the *gm.prcomp* function.

To assess the relationship between the magnitude of plasticity possessed by each species relative to their position in the adaptive radiation, I modelled the relationship between PC1 and Procrustes distance using a quadratic regression. Because plasticity may not change in a strictly linear way along the ecomorphological axis, a comparison of the quadratic model, linear and generalised additive models (GAM) was made using AIC scores.

To visualise shape differences due to plastic responses between the two foraging treatments across species, deformation grids for species *Rhamphochromis chilingali, Dimidiochromis compressiceps, Copadichromis borleyi, Aulonocara stuartgranti, Labidochromis caeruleus, Maylandia zebra, Tropheops 'red cheek',* and *Labeotropheus fuelleborni* were created using thin-plate spline deformation analyses using the *plotRefToTarget* function. These deformation grids were magnified by 2.5 to enable easier interpretation.

2.3.6 RNA-seq for jaw tissue

My experiment exposed the anatomy of cichlids to mechanical stress, which provided the opportunity to assess how gene expression in relevant structures was impacted by plasticity. The total RNA extracted from jaw joint tissue (~20 mg of tissue per sample) was done using the Qiagen RNeasy Mini Kit (Qiagen, Cat No. 74104), following the manufacturer's standard protocol. RNA purity and concentration were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). Only samples with A260/280 and A260/230 absorbance ratios greater than 1.8 were retained for downstream processing to ensure high purity and minimal contamination. RNA library preparation and whole transcriptome sequencing were performed by Novogene Co., Ltd. (Cambridge, UK). Samples were sent out in two batches. A replicate was included in batch 2 to enable investigation of technical batch effects. Libraries were constructed using poly(A) selection, and paired-end sequencing was performed on the Illumina NovaSeq X Plus platform (Illumina Inc., San Diego, CA, USA), generating 150 bp paired-end reads (PE150). Each sample was sequenced to a target depth of ~20 million reads.

Quality of raw reads from RNA-seq was assessed using FastQC (Andrews, 2010) and MultiQC (Ewels et al., 2016) software. The obtained Fastq files were trimmed to remove

Illumina adapter sequences using Cutadapt version 1.2.1 (Martin, 2011). Cleaned reads were aligned to the *Maylandia zebra* genome version (Genbank GCA_000238955.5) (Conte & Kocher, 2015) using the Hisat2 aligner version 2.2.0 (Kim et al., 2019). The resulting SAM files, which contained the alignment information for each sample, were then sorted and converted to BAM format, compressed binary versions of SAM files, which were sorted and indexed using Samtools index (Danecek et al., 2021). A matrix of read counts was obtained from the alignments using HTSeq-count2 (Anders et al., 2015). For batch correction, ComBat-Seq (a function in the sva R package) was used by modelling count data with a negative binomial regression. This retained the integer nature of count data, making the batch-adjusted data compatible with common differential expression software (Zhang et al., 2020). It removes the batch-specific effects while retaining variation due to biological covariates (diet and species). This was followed by the removal of the replicate from the second batch to avoid duplication of the data. In EdgeRv3.30.3 (Robinson et al., 2010; Chen et al., 2014), batch corrected read counts were imported, filtered to remove lowly expressed genes (*filterByExpr* function with min.count=10), normalised by TMM (*calcNormFactors)* and modelled with a design matrix including the main effects of species, diet, and their interaction. In limma, normalised counts were transformed with *voom* (Law et al., 2014). Differential expression (DE) in each contrast was tested for each gene by applying empirical Bayes moderation with *eBayes* (Smyth, 2004). Genes were ranked for evidence of differential expression (DE) using *topTable*, and significance was determined by controlling the false discovery rate (FDR) with the Benjamini–Hochberg method (Benjamini & Hochberg, 1995). Genes with adjusted $p \leq 0.05$ were considered differentially expressed.
To visualise variation in DE genes across the species, volcano plots were made using ggplot2 v3.3.5 (Wickham, 2016) and ggrepel v0.9.1 (Slowikowski et al., 2021), showing log fold change on the x-axis versus $\log_{10}$ P-value on the y-axis.

2.4 Results

2.4.1 Eco-morphological axis of divergence

The primary axis of morphological variation among African cichlids is associated with changes in the preorbital region, which are linked to biomechanical shifts in jaw function, distinguishing benthic from pelagic-feeding fishes. Here, PC1 (24.67% variation) represented the primary axis of craniofacial divergence and corresponded to the benthic–pelagic trophic gradient- species with more negative PC1 scores exhibited traits typical of

pelagic feeders (e.g., elongate preorbital regions and more protrusible jaws), whereas species with more positive PC1 scores displayed features associated with benthic feeding (e.g., shorter, more robust jaws adapted for forceful biting). PC2 (14.71% variation) captured secondary aspects of craniofacial shape variation, including differences in skull depth and jaw proportions that are not strictly aligned with the benthopelagic axis. Accordingly, species were distributed along PC1 according to their trophic ecology, forming a continuum from pelagic to benthic morphologies, while variation along PC2 reflected additional, more subtle morphological differentiation within these groups **(Figure 2-4)**.

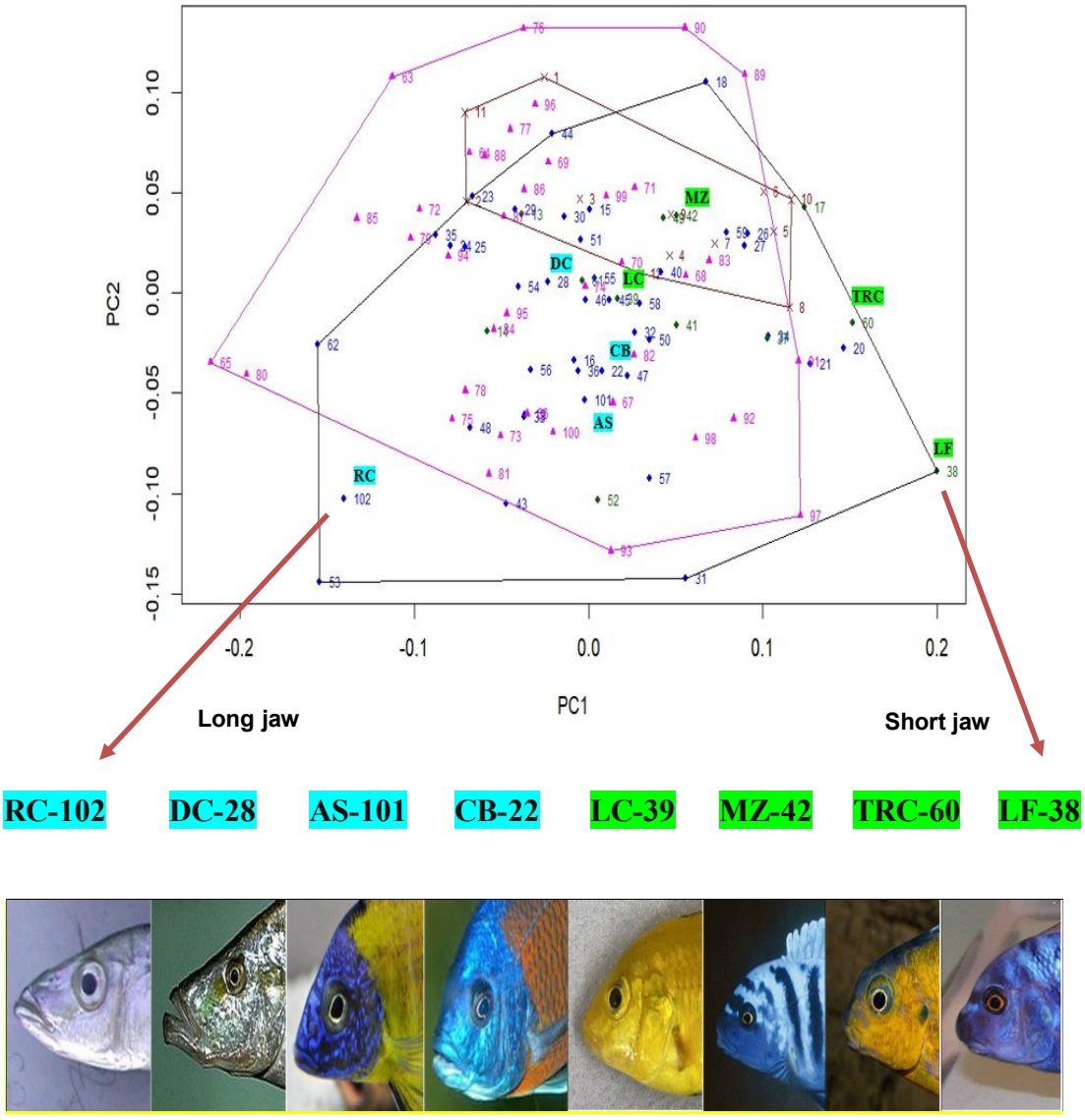


Figure 2-4: PC (Principal component) score plot of all Lakes data with ecomorphological groupings. LV (Lake Victoria) -red, LT (Lake Tanganyika) -magenta, LM (Lake Malawi)- black. LM rock dwellers- green, LM sand dwellers- blue (Figure modified from Cooper et al., 2010). Species selected for the study are labelled with their names. Blue-highlighted species are the sand-dwellers, while the green-highlighted ones are the rock-dwellers.

Left to Right: RC- *Rhamphochromis chilingali,* DC- *Dimidiochromis compressiceps,* AS- *Aulonocara stuartgranti,* CB- *Copadichromis borleyi,* LC- *Labidochromis caeruleus,* MZ- *Maylandia zebra,* TRC- *Tropheops 'red cheek',* LF- *Labeotropheus fuelleborni*

2.4.2 Geometric morphometrics to study plasticity

The average survival rate recorded across the plasticity experiments for the species was 91%. My Procrustes ANOVA showed that species had a significant effect on craniofacial shape ($F = 217.05$, $p < 0.001$), accounting for the greatest degree of shape variation. Diet showed a marginally significant influence ($F = 2.60$, $p = 0.051$), suggesting that shape is impacted by different foraging treatments. It is the effect of diet (as a single factor) on the shape. The diet × species interaction was strongly significant ($F = 2.99$, $p < 0.001$), demonstrating that the shape responded to diet in ways that varied across species **(Table 2-2)**.

Because Procrustes ANOVA operates on multivariate shape data using permutation procedures, traditional parameter estimates and associated standard errors can't be defined. In Procrustes ANOVA, the Z-score is a standardised statistic derived from permutation testing that describes how far the observed F-statistic deviates from the null distribution. A larger Z indicates the effect is increasingly improbable under the null hypothesis. The effect size is represented by R^2, which quantifies the proportion of total shape variance explained by each factor.

Table 2-2: Summary of output from the Procrustes ANOVA model for the effect of diet, species, size and interaction on craniofacial shape. Asterisks highlight statistically significant P values.

Factors	DF	Sum Sq	Mean Sq	R Sq	F	Z	p
Diet	1	0.0173	0.01726	0.00122	2.6028	1.7259	0.051.
Species	7	10.0754	1.43934	0.71079	217.0510	10.7747	0.001***
Size	1	0.0152	0.01522	0.00107	2.2954	1.6439	0.050*
Diet: Species	7	0.1392	0.01988	0.00982	2.9979	4.9080	0.001***
Diet: Size	1	0.0399	0.03989	0.00281	6.0160	3.6690	0.001***
Species: Size	7	0.1738	0.02483	0.01226	3.7443	6.0261	0.001***
Diet: Species: Size	7	0.0736	0.01051	0.00519	1.5853	2.3819	0.012 *
Residuals	549	3.6406	0.00663	0.25683			

The magnitude of plasticity varied across species as revealed by the trajectory analysis **(Table 2-3)**. Differences in diet-induced craniofacial shape trajectory reflect how species vary in plasticity along the biting-sucking continuum. For the rock-dwellers, LC exhibited the largest Procrustes distance (0.266) while LF showed the smallest distance (0.054), representing a five-fold difference in the magnitude of plasticity from the middle to the edge of the radiation. Among the sand dwellers, DC exhibited the largest distance (0.165), and RC showed the smallest distance (0.079), representing a two-fold difference in the magnitude of plasticity. These findings of differences in the magnitude of plasticity between species in the centre of the radiation to the extremes were highlighted by the quadratic regression.

The AIC scores for the linear, GAM and quadratic regression models were -15.38, -15.63 and -15.95, respectively. Since the quadratic model had the lowest AIC score, it was selected. Quadratic regression revealed a non-linear relationship between shape (PC1) and Procrustes distance. The model indicated that plasticity was highest among species at intermediate PC1 values, with both extreme negative and positive PC1 values showing reduced plasticity **(Figure 2-5)**.

Table 2-3: This table shows the Procrustes distances, which relate to the level of plasticity in rock and sand dwelling Malawi cichlids (LF- *Labeotropheus fuelleborni,* TRC- *Tropheops 'red cheek',* MZ- *Maylandia zebra,* LC- *Labidochromis caeruleus*, CB- *Copadichromis borleyi*, AS- *Aulonocara stuartgranti,* DC- *Dimidiochromis compressiceps,* RC- *Rhamphochromis chilingali*)

Species	Procrustes distance	PC1 score
Rock-dwellers		
LF	0.054	0.200
TRC	0.069	0.152
MZ	0.057	0.0503
LC	0.266	0.0165
Sand-dwellers		
CB	0.123	0.00774
AS	0.119	-0.00164
DC	0.165	-0.0236
RC	0.079	-0.140

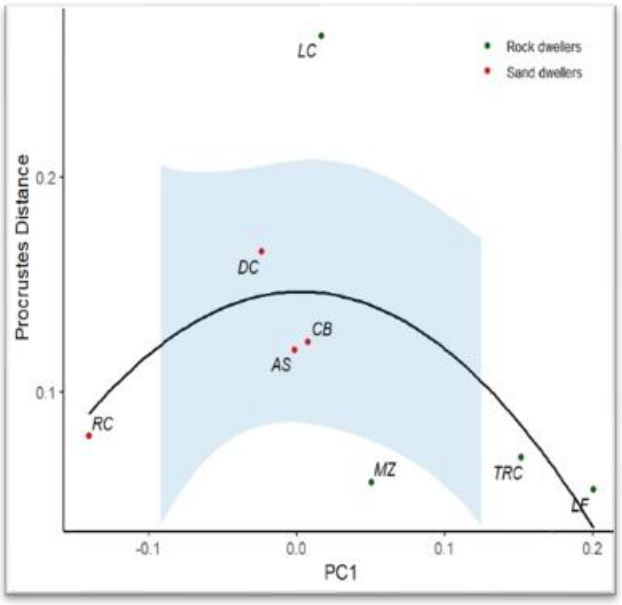


Figure 2-5: To determine the position of cichlid species on the ecomorphological axis with respect to the magnitude of plasticity, a relationship between mean shape (PC1) and Procrustes distance was done. The fitted black curve represents a quadratic regression model, with a shaded blue ribbon indicating the 95% confidence interval. Species are colour-coded, where red points indicate sand-dwelling species while dark green points represent rock-dwelling species.

Pairwise comparisons of morphological trajectory paths revealed significant differences in magnitudes of plasticity (Procrustes distances) between species **(Figure 2-6)**. Notably, DC exhibited significantly different trajectory length compared to LF (d = 0.111, p = 0.002), MZ (d = 0.107, p = 0.010), RC (d = 0.086, p = 0.010), and TRC (d = 0.096, p = 0.003). LF also differed significantly from CB (d = 0.069, p = 0.038) while LC differed from LF (d = 0.212, p = 0.041) **(Appendix, Supplementary table 1)**.

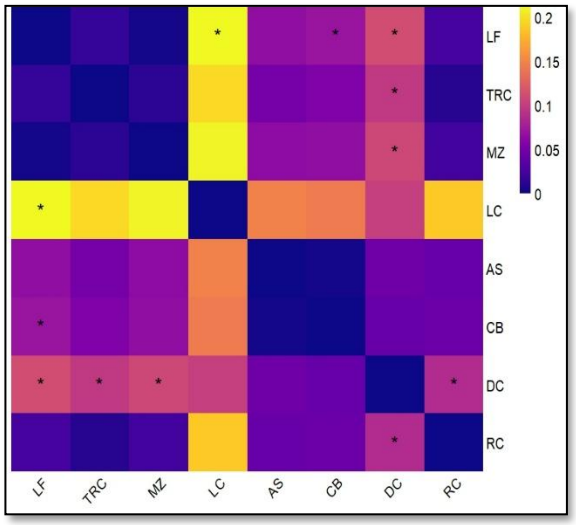


Figure 2-6: Heatmap of Pairwise differences in plasticity among species in response to diet treatments. The colour gradient (from dark blue to bright yellow) indicates the magnitude of morphological trajectory distance difference between species pairs, with yellow indicating the largest differences and dark blue representing smaller differences. Asterisks (*) mark statistically significant pairwise differences ($p < 0.05$).

Corresponding to quantitative results, deformation grids of sand-dwelling species revealed more shape differences between the limnetic and benthic treatments as compared to the rock-dwellers **(Figure 2-7).**

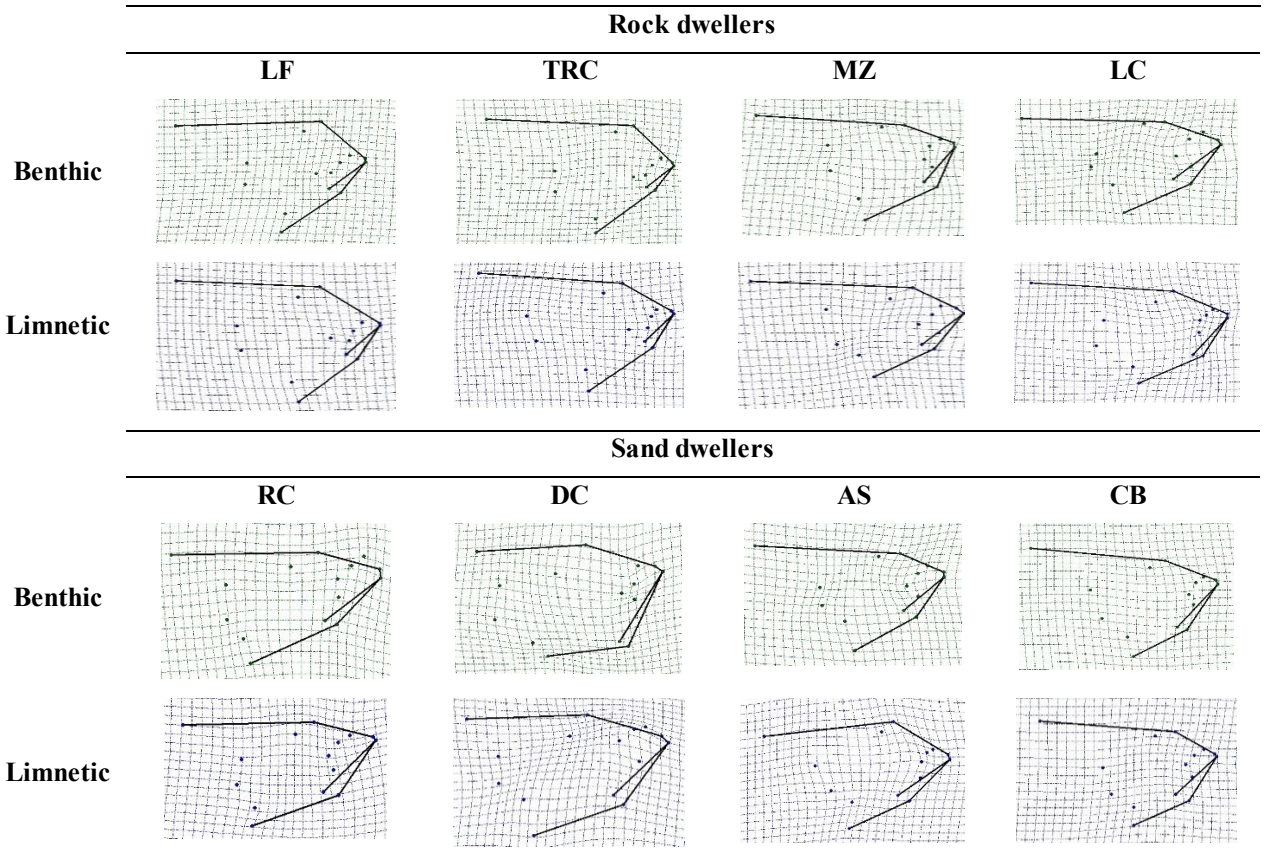


Figure 2-7: Deformation grids (with 2.5 magnification) depicting craniofacial plasticity across rock and sand dwelling Malawi cichlids. Green colour represents benthic treatment and blue colour represents limnetic treatment. LF- *Labeotropheus fuelleborni,* TRC- *Tropheops 'red cheek',* MZ- *Maylandia zebra,* LC- *Labidochromis caeruleus*, CB- *Copadichromis borleyi*, AS- *Aulonocara stuartgranti,* DC- *Dimidiochromis compressiceps,* RC- *Rhamphochromis chilingali*

2.4.3 Differential gene expression across species relative to diet

Following the generation of RNAseq data quality filtering, adapter trimming, and mapping, 22,523 expressed genes were retained for downstream analysis. The number of diet-induced DE genes (accounting for an FDR <0.05) varied widely among species. Within the sand dwellers, *Dimidiochromis compressiceps* (DC) showed 3,069 DE genes, *Aulonocara stuartgranti* (AS) showed 158, *Copadichromis borleyi* (CB) 26 and *Rhamphochromis chilingali* (RC) 5. Among the rock dwellers, there were only 2 DE genes in *Labidochromis caeruleus* (LC) and 1 in *Maylandia zebra* (MZ) **(Figure 2-8)**. Thus, these findings revealed a striking degree of interspecific variation in transcriptional plasticity, with

the sand dwellers exhibiting substantially more differentially expressed genes as part of their plastic response to foraging treatments compared to the rock dwellers.

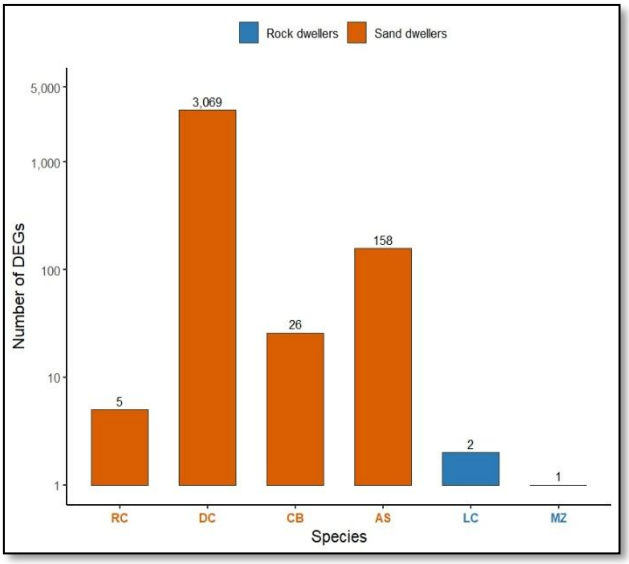


Figure 2-8: Number of differentially expressed genes (DEGs) across the rock and sand-dwelling Malawi cichlids. RC- *Rhamphochromis chilingali,* DC- *Dimidiochromis compressiceps,* CB- *Copadichromis borleyi*, AS- *Aulonocara stuartgranti,* LC- *Labidochromis caeruleus*, MZ- *Maylandia zebra*

To understand the effect of the environment on the genes, their upregulation under each treatment was seen. Within DC, 1995 DE genes were upregulated under the limnetic treatment relative to the benthic treatment. In AS, there were 77 and 81 upregulated genes in the limnetic and benthic conditions, respectively. CB exhibited 7 limnetic and 19 in benthic upregulated genes, whereas RC exhibited 5 upregulated genes in the benthic condition but none in the limnetic. For the rock dwellers, LC had 1 upregulated gene in each treatment, and MZ had 1 under the benthic and none under the limnetic treatment. It showed that the upregulation was species-specific and that only one environment was not directing it.

To visually study which genes showed up under the limnetic and benthic treatment across the species, volcano plots were made **(Figure 2-9)**. Among the sand-dwellers, RC had the lowest number of named DE genes, followed by CB, AS and DC, having the highest number of DE genes. On the other hand, the rock-dwellers LC and MZ had 1 and zero named DE genes, respectively.

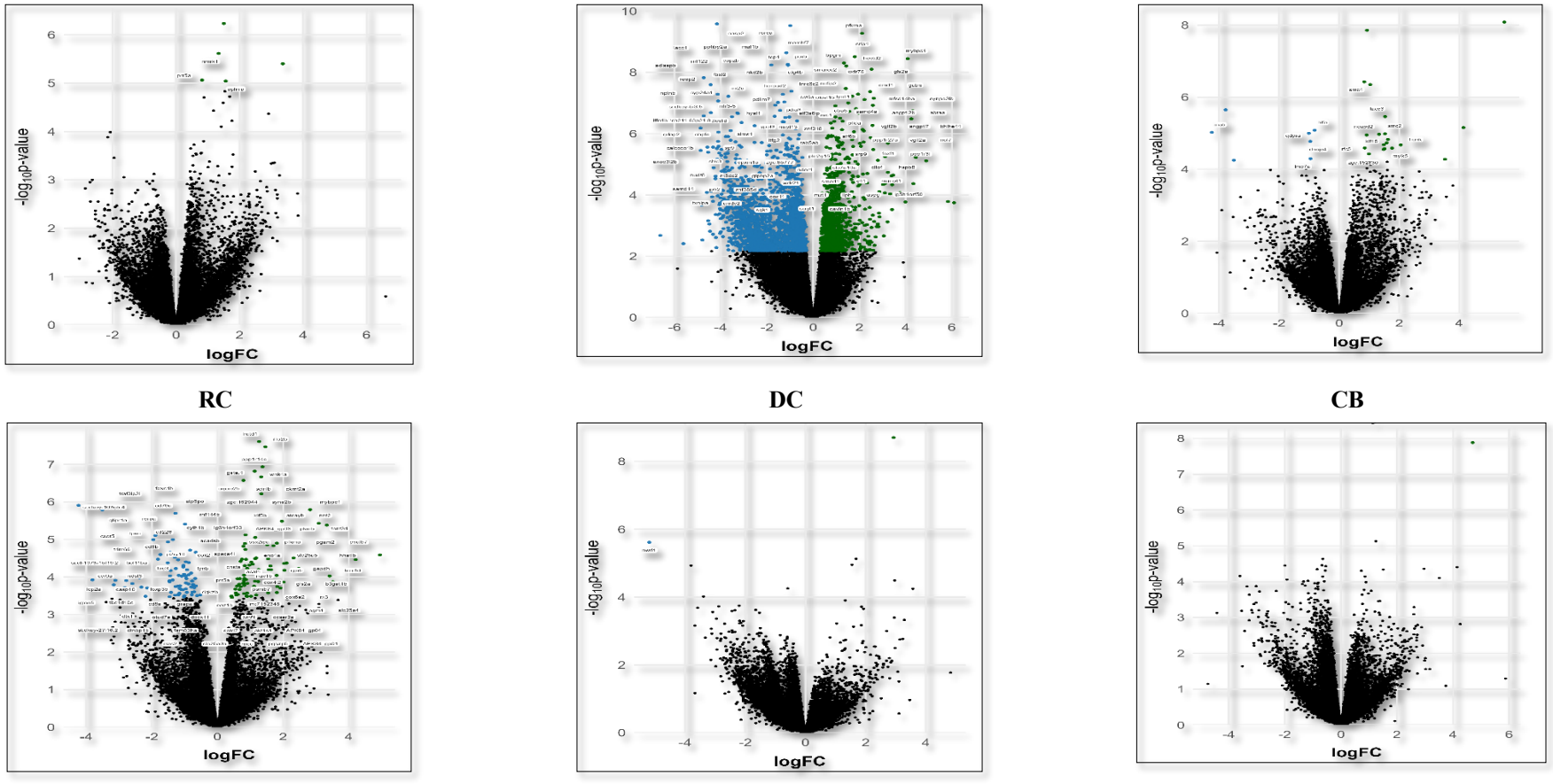


Figure 2-9: Volcano plots of DEGs between limnetic and benthic treatments for each of six species of Malawi cichlids. Each plot displays the log fold change (x-axis) versus the $-\log_{10}$ p-value (y-axis). Genes significantly differentially expressed at FDR < 0.05 are highlighted: green points correspond to genes upregulated in benthic treatments, blue points correspond to genes upregulated in limnetic treatments, and black points represent non-significant genes. The limnetic treatment was set as the reference, so positive log fold changes indicate genes with higher expression in benthic treatments, and negative log fold changes indicate genes with higher expression in limnetic treatments. This identifies genes whose expression differs significantly between treatments. Both up and downregulation are therefore defined relative to the comparison being made. Top 100 significant genes are shown with gene labels for clarity. RC- *Rhamphochromis chilingali,* DC- *Dimidiochromis compressiceps,* CB- *Copadichromis borleyi*, AS- *Aulonocara stuartgranti,* LC- *Labidochromis caeruleus*, MZ- *Maylandia zebra.*

In LC, the *nwd1* gene was identified under the limnetic treatment. For CB, 14 named genes were identified (*smc4, tacc3, hlfa, smc2, ncapd2, qdpra, dnajc4, hunk, mylk5, rfc5, kif15, zgc:152830, lmo7a, inab*). For RC, three named genes (*nmrk1*, *prr5a*, *aplnra*) were revealed. The potential functions of each gene are mentioned in **the Appendix, Supplementary Table 2.**

2.5 Discussion

In this chapter, I have tested the hypothesis that plasticity would decrease as specialisation increased. My findings support the hypothesis that plasticity in craniofacial morphology is greater in generalist species occupying intermediate positions along the biting–suction ecomorphological axis, and declines towards both ends of the continuum, where the specialists are found (Schluter, 2000; Pfennig et al., 2010). This pattern is reinforced by responses in gene expression. Among the sand-dwellers, the more plastic species showed a higher number of DE genes, which decreased for the specialists. The rock-dwellers, on the other hand, did not show a big difference in the number of DE genes. While these patterns are suggestive of a relationship between plasticity and ecological specialisation, they do not provide direct evidence that the observed responses are adaptive. Malawi cichlids represent one of the most extensively studied examples of adaptive radiation, characterised by rapid diversification in morphology, ecology, and behaviour (Kocher, 2004; Seehausen, 2006). This radiation has produced a wide range of trophic specialisations, making it a powerful system for investigating the mechanisms underlying phenotypic diversification. Within this context, the patterns observed here contribute to our understanding of how plasticity may be associated with ecological divergence, even if its direct adaptive significance remains to be established.

Below, I discuss the implications of these findings in relation to cichlid radiations and the broader implications this has for our understanding of adaptive radiations.

Across an adaptive radiation, we have generalists and specialists depending on their ecological and behavioural preferences. Here, I predict that generalists maintain higher phenotypic plasticity to cope with variable environments, whilst specialists display canalised morphologies adapted to stable, narrow ecological niches via genetic assimilation (Pigliucci & Murren, 2003; Schneider & Meyer, 2017). This suggests that the extent of shape change relates to ecological specialisation (Schluter, 2000; Muschick et al., 2011). The findings from this chapter reveal that among rock dwellers, *Labidochromis caeruleus* (LC) occupies

a middle position, acting as a generalist, and exhibits a high Procrustes distance, suggesting that its broad dietary preferences (Albertson, 2008) are supported by higher craniofacial plasticity. In contrast, *Maylandia zebra* (MZ) and *Tropheops 'red cheek'* (TRC), foraging generalists (Navon et al., 2020) that inhabit more specialised rock-dwelling niches, exhibited a lower plastic response. MZ is known to be a more generalised forager, feeding from both the rocky substrate and water column (Albertson et al., 2003; Rupp & Hulsey, 2014). *Labeotropheus fuelleborni* (LF), an obligate benthic forager (Ribbink et al., 1983), positioned at the extreme biting-specialist end, displayed the least plasticity, indicating a canalisation of traits. MZ showed less plasticity than TRC. This contradicts previous studies where TRC is shown to have a feeding morphology between LF and MZ (Ribbink et al., 1983; Albertson & Kocher, 2001; Navon et al., 2020). This could be explained by differences in their geographic distributions within Lake Malawi. LF and MZ occupy habitats that share similar ecological features, such as rocky littoral zones. Such a niche provides stable environments that may select for morphological canalisation, where traits are finely tuned to optimise performance in a specific habitat, reducing the need for high plasticity (West-Eberhard, 2003). MZ are known to inhabit small rocky zones based on colouration and markings (Ribbink, 1983), which points towards their confined environment. Interestingly, despite MZ being more centrally positioned on the ecomorphological axis than TRC, suggesting a more generalist morphology, its presence as a subpopulation within stable rocky habitats may reduce the selection pressure to maintain high plasticity, resulting in a pattern more similar to LF, a biting specialist. Among sand dwellers, *Dimidiochromis compressiceps* (DC) is positioned closer to the centre of the axis and shows the highest levels of plasticity within this group of species. Conversely, *Rhamphochromis chilingali* (RC), a specialised suction-feeding piscivore at the end of the axis, exhibits the lowest plasticity. Interestingly, this trend suggests that as the specialisation increases, the plasticity decreases, and this is repeated in both the clades- rock and sand dwellers.

The transcriptomic analyses also support the hypothesis that plasticity is greater in generalist species occupying intermediate positions along the biting–suction ecomorphological axis, and declines towards the specialists. The results revealed variation in diet-induced gene expression plasticity, which aligned with the patterns observed in craniofacial plasticity. Bone plasticity has been related to changes in gene expression (Navon et al., 2020). Under the sand dweller group, *Dimidiochromis compressiceps* (DC) exhibited 600-fold more genes compared to the specialist *Rhamphochromis chilingali* (RC). Among the rock dwellers, a difference in the number of DE genes was not found. The difference in diet-induced differentially expressed genes between sand-dwelling and rock-dwelling

cichlids can be explained by their distinct ecological adaptations and anatomical characteristics (Husley et al., 2019). Wolff (1892) presented the 'law of bone transformation,' which stated that vertebrates' bones display a high degree of plasticity and respond to mechanical load. During foraging, fish experience mechanical stress on their craniofacial bones, which induces adaptive remodelling (Albertson & Yelick, 2007). The rock-dwellers inhabit the rocky littoral substrates where strong, biting and plucking modes select for high rates of mineral deposition (Hu & Albertson, 2014; Navon et al., 2020). These highly dense, ossified bones require less remodelling, which might require reduced transcriptional response. On the contrary, the sand-dwellers employ a suction feeding mode that induces lower mechanical load on the craniofacial bones (Ribbink et al., 1983). Their thinner, gracile, less ossified bones are easily remodelled, which might demand a higher transcriptomic plasticity. Here, there is no 'more or less effect', and this relates to the impossibility of having control. The mechanical stresses are simply different, and the responses are relative, this does not provide a situation where asymmetry can be defined.

The large transcriptional response in sand dwellers- DC, AS, CB parallels their intermediate ecomorphological position and high plasticity in jaw morphology, supporting the link between molecular and phenotypic plasticity (Schluter, 2000; Pigliucci & Murren, 2003; Schneider & Meyer, 2017). This concordance suggests that transcriptional variation may underlie the capacity for morphological plasticity, with higher DE gene counts potentially enabling broader phenotypic responses to environmental variation. Conversely, the low DE gene counts in highly specialised species align with the expectation that morphological traits adapted to narrow ecological niches may be developmentally canalised, limiting both gene expression changes and phenotypic plasticity (West-Eberhard, 2003; Muschick et al., 2011; Schneider & Meyer, 2017). Together, these findings support the view that plasticity at the molecular level can be a crucial mechanism facilitating plastic responses in generalists, while specialists exhibit less plasticity in both transcriptomic and phenotypic responses. The results are in line with Tetrault et al. (2023), where 38 DE genes were observed between foraging environments in MZ, and none were observed for TRC. This suggests that the rock dwellers might respond more simply compared to the sand dwellers when it comes to gene expression.

Amongst the genes identified in the sand dwellers, several are linked to cell proliferation and growth (*prr5a, smc4, smc2, tacc3, ncapd2, rfc5, kif15).* Prior studies have shown that cell proliferation contributes to bone formation and growth (Capecchi et al., 2018; Shekhar et al., 2019; Le Pabic et al., 2022). These observations imply that proliferation is an important mediator of bone plasticity in this species. I also detected genes like *mylk5*

and lmo7a, known for muscle contraction and Wnt signalling, alongside additional genes involved in neurotransmission, metabolic processes, and stress responses, together indicating that diet-induced plasticity is complex and engages a broad range of genes. These genes represent compelling candidates for the fundamental molecular framework that drives plastic responses, providing the raw material to further understand plasticity and genetic assimilation (Waddington, 1953; Levis & Pfennig, 2019). While I highlight a few genes as starting points, many additional genes showed up whose functions are not entirely known, underscoring the need for future studies to elucidate their roles in diet-induced plasticity.

2.6 Conclusion

This chapter indicates that phenotypic plasticity decreases with an increase in specialisation, at both the morphological and molecular levels. It has been studied outside the lab in the largest extant adaptive radiation. The findings demonstrate that plasticity operates at multiple levels, from developmental gene regulation to morphology. The integration of morphological and transcriptomic datasets reveals that species positioned centrally along the biting–suction ecomorphological axis display high plasticity, whereas specialists at the ends exhibit canalisation. This correspondence supports models where plasticity is reduced with ecological specialisation (West-Eberhard, 2003; Schneider & Meyer, 2017; Levis & Pfennig, 2019).

Chapter 3: Shaping the bite: Role of *zeb1* in jaw bone formation

3.1 Abstract

Morphological diversification in vertebrate craniofacial structures underlies key ecological adaptations. This chapter identifies the transcription factor *zeb1* as a differentially expressed gene associated with mandible shape variation and validates its functional relevance in East African cichlids and zebrafish. Here, we bridge molecular regulation with ecological diversification. By investigating *zeb1* expression and function across two complementary model systems, our study addresses fundamental questions about the genetic architecture of morphological evolution. Quantitative PCR revealed a significant difference in *zeb1* expression between *Labeotropheus fuelleborni* (LF) and *Tropheops* sp. 'red cheek' (TRC) (p-value= 0.036). The two species differ in jaw width, with TRC having a narrower mandible, which exhibits a plucking mode of feeding, and LF having a broader mandible for scraping low-growing algae from rock surfaces. These species inhabit a similar ecological niche but use different tactics to exploit food resources. In contrast, BMP antagonists *follistatin* and *chordin-like* showed no significant expression differences. Further, we observed that zebrafish heterozygous *zeb1* mutants showed genotype-dependent shape variation, supporting its role in craniofacial development. These findings highlight *zeb1* as a potential modulator of jaw ossification and functional morphology. This integrative evo-devo study connects gene expression variation to adaptive craniofacial diversification, providing insight into the regulatory mechanisms underlying adaptive morphological evolution.

3.2 Introduction

Morphological variation has long been recognised as a fundamental driver of evolutionary diversification (Heard & Hauser, 1995; Schluter, 2000). Such variation often serves as the basis for adaptive radiations and represents a significant component of phenotypic biodiversity (Parsons et al., 2015). Adaptive radiation is a process where several species evolve from a common ancestor to exploit different ecological niches (Schluter, 2000; Irschick et al., 2013; Skúlason et al., 2019). Interestingly, a significant portion of the morphological and functional divergence among vertebrates has been observed in the

craniofacial region (Gans & Northcutt, 1983). This facilitates trophic specialisation and habitat divergence in vertebrates, which in turn contributes to niche partitioning and speciation (Hu & Albertson, 2014). Such modifications influence the food type used and processing efficiency (Parsons & Albertson, 2009). Understanding the genetic and developmental mechanisms underlying these adaptive differences is central to evo-devo research, providing critical insights into how complex trait variation emerges and diversifies. Craniofacial divergence has been extensively studied in various animals, including birds (Abzhanov et al., 2006; Brugmann et al., 2010), squamates (Watanabe et al., 2019), dogs (Drake & Klingenberg, 2010; Schoenebeck et al., 2012), foxes (Parsons et al., 2020), phyllostomid bats (Dumont et al., 2012), and cichlids (Roberts et al., 2011; Muschick et al., 2011; Parsons et al., 2016; Gilbert et al., 2023, McWhinnie et al., 2023).

In teleost fishes, especially within the adaptive radiations of East African cichlids, craniofacial variation among species plays a vital role in trophic specialisation, reflecting niche-specific feeding strategies influenced by both genetic and developmental mechanisms (Albertson et al., 2005; Cooper et al., 2010; Parsons et al., 2011, Parsons et al., 2014; Powder & Albertson, 2016). Cichlids are recognised as a valuable model for studying the genetic basis of morphological evolution due to their remarkable degree of phenotypic variation and rapid speciation (Kocher, 2004; Salzburger, 2018). They diverged along a bentho-pelagic eco-morphological axis that aligns with two primary foraging mechanisms. Species on one end of this ecomorphological continuum are limnetic feeders that graze upon mobile prey in the water column via suction feeding. These are characterised by a shallow craniofacial profile and a long mandible. Species on the other end are benthic feeders that scrape prey from the rocks by biting and are characterised by a steep craniofacial profile and a short mandible (Albertson et al., 2005; Parsons et al., 2014). Within this main axis of Malawi cichlid divergence lie highly specialised foraging tactics between species sharing similar ecologies (Ribbink et al. 1983; Albertson, 2008; Parsons et al. 2015).

A key to this type of trophic divergence lies in functional modifications of the mandible, particularly its shape and biomechanical structure, which directly influence feeding mechanics and ecological specialisation (Westneat, 1995; Wainwright et al., 2004; Hulsey et al., 2006; Albertson & Kocher, 2006; Gidmark et al., 2013). Changes in jaw morphology can alter bite force, kinematics, and mechanical advantage, thereby enabling organisms to exploit distinct dietary niches and contributing to evolutionary diversification. For example, *Tropheops 'Red Cheek'* (TRC) and *Labeotropheus fuelleborni* (LF) are both algae biters and immediate neighbours in morphospace (Cooper et al., 2010). However, TRC

has a narrower mandible and employs a plucking mode of feeding to focus on long strands of algae, whereas LF has a broader mandible and scrapes low-growing algae from rock surfaces (Parsons et al., 2016; Albertson & Pauers, 2019). Thus, we can come to the understanding that among the morphological traits under selection, the mandible and its related developmental pathways offer a unique opportunity to understand how variation in gene regulation drives the evolution of ecologically relevant form and function.

Numerous studies have identified key genes involved in generating adaptive morphological differences in craniofacial structures, including *bmp4*, *calmodulin1 (cam1)*, *patched1 (ptch1)*, and components of the *wnt* signalling pathway (Semba et al., 2000; Wu et al., 2004; Abzhanov et al., 2004; Albertson et al., 2005; Parsons & Albertson, 2009; Roberts et al., 2011; Hu & Albertson, 2014; Parsons et al., 2014; Powder et al., 2015). These genes have been implicated in modulating bone growth and integration during craniofacial development. Despite the mandible's critical role in feeding biomechanics and ecological specialisation, it has received scant attention as a focal trait in studies of the genetic architecture underlying adaptive variation. A QTL study on cichlids identified a new candidate gene, *zeb1* (zinc finger E-box-binding homeobox 1), in the genetic architecture of the mandible (McWhinnie, 2020). Here, the genomic region surrounding *zeb1* showed strong evidence of selection with a high F_{st} value of 0.95. Discriminant function analysis (DFA) at the nearest markers flanking *zeb1* showed that individuals corresponding to LF displayed a wider mandible shape along the negative end of the LD1 axis, whereas those corresponding to TRC exhibited a narrower mandible exhibiting evidence of shape divergence between the two species. However, direct evidence linking *zeb1* expression to mandibular variation remains limited. Studies have shown that *zeb1* can induce the expression of BMP inhibitors, like *follistatin (fst)* and *chordin-like (chrdl)* and influence bone formation (Postigo et al., 2003; Liu et al., 2008; Mock et al., 2015). Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily, are multifunctional growth factors and known to induce bone formation (Attisano & Wrana, 1996; Streelman et al., 2003). The activity of the BMP pathway is modulated by BMP inhibitors, which enables osteogenic differentiation (Abe et al., 2004; Kawabata et al., 2007; Plouhinec et al., 2013). These proteins are secreted to the extracellular space where they bind to BMPs and antagonise their function (Balemans & Van Hul, 2002). *fst* and *chordin* are known to antagonise BMP function (Fainsod et al., 1997; Iemura et al., 1998) and influence bone growth and formation (Rosen, 2006). *follistatin,* a known BMP antagonist, was found to be expressed in developing enamel-knot–like signalling centres in bicuspid and tricuspid teeth of cichlids, suggesting its role in shaping craniofacial structures, such as dentition (Bloomquist, 2025). It has also been seen to inhibit

BMP signalling within the enamel knots in mice (Wang et al., 2004). This suggests that *zeb1*, *fst* and *chordin* may act as developmental regulators whose activity could drive subtle morphological differences in jaw shape, particularly in systems where trophic morphology is under strong selection pressure.

Furthermore, to test the hypothesis that *zeb1* modulates mandibular shape during development and has an ecological relevance, I complemented this study with a genetic approach in zebrafish (*Danio rerio*), a model organism with established tools for genetic manipulation and morphometric analysis (Kimmel et al., 1995; Parichy, 2015). Zebrafish offer an ideal comparative system due to their conserved craniofacial developmental pathways and the availability of morphometric techniques (Cooper et al., 2013). This integrative approach, combining expression analysis in cichlids with mutant phenotyping in zebrafish, enables a better understanding of how *zeb1* influences mandible shape variation.

3.3 Methods

3.3.1 Gene expression in two morphologically diverged cichlids

To test for divergence in *zeb1* expression, qPCR was conducted. Three genes were selected for qRT-PCR: *zeb1*, *follistatin* (*fst*) and *chordin-like* (*chrdl*). Studies have demonstrated that *zeb1* induces the expression of these BMP inhibitors (Mock et al., 2015). Therefore, it was of interest to examine the expression levels of these genes, given their established association with *zeb1*.

For this study, LF and TRC embryos were collected, and a key point in development was chosen when the mandible begins to differentiate between species and genes that interact with *zeb1* were likely to be expressed (stage 16, approximately 4-5 days post-fertilisation) (Fujimura & Okada, 2007; Parsons et al., 2014; Hu & Albertson, 2017; Bloomquist et al., 2017) **(Figure 3-1)**. Three individual embryos were pooled per sample, with the head and mandible collected and a total of 15 such pooled samples were generated for each species to provide sufficient RNA. These tissues were stored in RNAlater at room temperature overnight before freezing at -80˚C.

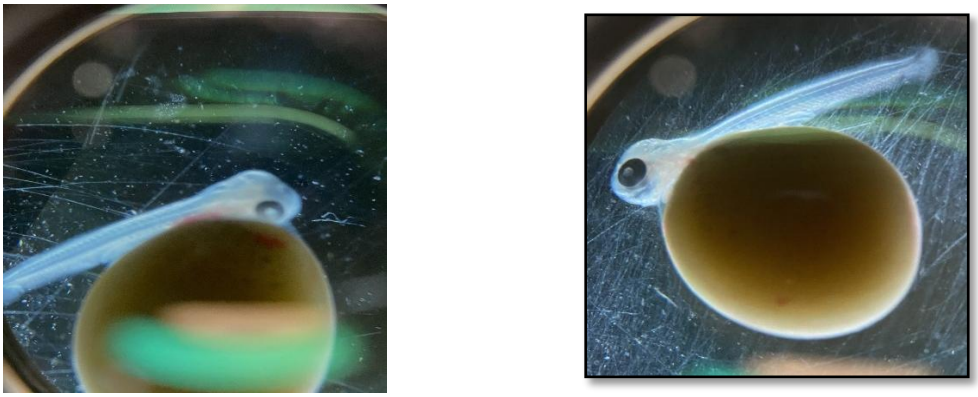


Figure 3-1: Embryos at stage 16 corresponding to 4-5 dpf; based on the staging of Nile Tilapia (*Oreochromis niloticus*) by Fujimura & Okada (2007).

3.3.1.1 RNA extraction, cDNA synthesis and primer design

RNA was extracted from approximately 30mg of tissue using an RNeasy Mini Kit (Qiagen). RNA purity and quantity were determined using a Nanodrop spectrophotometer (ThermoFisher Scientific, Massachusetts, USA), and it was ensured that the 260/280 ratio was ~2.0. Next, a QuantiTect Reverse Transcription Kit (Qiagen) was used for the synthesis of cDNA from RNA (template RNA: 0.8-1 µg). cDNA of each sample was diluted 1:10 times in nuclease-free water to be used for qRT-PCR steps.

For primer design, candidate genes were identified using the *Maylandia zebra* annotated genome on Ensembl Genome Browser (https://www.ensembl.org), and the cDNA sequence was downloaded. Next, primer3 (Applied Biosystems, CA, USA) was used to design primers keeping a short amplicon size (50- 200bp) suitable for qPCR quantification (Fleige & Pfaffl, 2006). The primer concentration and annealing temperatures were optimised, and OligoAnalyzer 3.1 (Integrated DNA Technology) was used to check the occurrence of dimerisation and secondary structures. The primers were validated by running a PCR using target cDNA, and amplification efficiency was assessed through gel electrophoresis. The primer details are mentioned in **Table 3-1**.

Table 3-1: Primer sequences for the candidate genes *zeb1*, *chordin-like* (*chrdl*), *follistatin* (*fst*) and housekeeping gene (*β-actin)*

Gene	Sequence	Tm (˚C)	GC (%)	Product size (bp)
zeb1	F: TTCGCAGTCAACAGGAGGAT	59.02	50.00	199
	R: TTTGCTACTGATGTGCGAGC	58.92	50.00	
fst	F: TGCGACAATGTTGACTGTGG	59.06	50.00	59
	R: GGCTTGCTTCTTCGGTTCAA	59.05	50.00	
chrdl	F: ACCTCTTCCCATCCAAGCAA	58.92	50.00	86
	R: TTGGCAGGTTTTCAGAGCAC	58.97	50.00	
β-actin	F: GTATGTGCAAGGCCGGATT		51.30	106
	R: TTCTGACCCATACCCACCAT		51.30	

3.3.1.2 qRT-PCR and analysis

Reactions were performed on 15 samples per species in triplicate using an Agilent AriaMx Real-Time PCR cycler with Fast SYBR Green Master Mix (Applied Biosystems). The conditions included an initial hot start for 95˚C 3 min, followed by 40 cycles of 95˚C 15 sec, 57˚C 1 min and 72˚C 1 min. The primer concentration was 10µM, and all samples were run on a single 96-well plate with a standard curve for each gene with *β-actin* as a reference housekeeping gene (Navon et al., 2020). For the standard curve, a 1:10 serial dilution was used with a pooled sample of LF and TRC. The R^2 value and efficiency (%) from the melt curve were used to determine the potency of the candidate genes.

The Cq value of the reference gene was used as a normalisation factor (Cq reference) (Livak & Schmittgen, 2001), and the ΔCq values of the control (LF) and experimental (TRC) were calculated (ΔCq control =Cq target- Cq reference). To compare gene expression between the two cichlid species, LF was treated as the control group and TRC as the experimental group and ΔΔCq values (ΔCq control- ΔCq experimental) were calculated. Relative expression quantities (RQ) were calculated for the normalised values using $e^{-\Delta\Delta Cq}$, and the fold difference values were derived by transforming RQ values to logarithmic base 2 values to enable further statistical analysis (Livak & Schmittgen, 2001).

Linear models were initially fitted to assess differences in gene expression between groups, and model assumptions were evaluated through residual diagnostic plots. These indicated some deviations from normality and the presence of outliers, which may influence model performance. Therefore, a non-parametric Mann-Whitney U test was used in R (http://www.r-project.org).

to assess group differences. To account for multiple testing across the three candidate genes, p-values were adjusted using the false discovery rate (FDR) method.

3.3.2 Genotyping in zebrafish mutants to explore the role of zeb1 in mandible formation

To further elucidate the role of *zeb1* in mandible formation, we used zebrafish (*Danio rerio*) with *zeb1* mutant alleles (sa15743) obtained from the European Zebrafish Resource Centre (EZRC). Larvae were reared until they reached sexual maturity, during which time they were fed a diet consisting of *Artemia nauplii* and ZM000 powdered feed (ZM Fish Food) for the first month. Then, the zebrafish were moved to a diet comprising ZM100 and ZM200 powders to support optimal growth and reproductive health. Adult fish were bred in-house to obtain sufficient individuals for the experimental setup. The experiment was conducted in replicates for three months, and a total of 61 individuals were collected for morphometric measurements and genotyping. At the time of sampling, the standard length of fish in both tanks was measured to be 31.10 ± 2.11mm and 31.14 ± 2.06mm.

3.3.2.1 Genotyping

The fish were euthanised using Schedule 1 killing methods, following UK Home Office guidelines, with an overdose of benzocaine solution (10g benzocaine powder, Sigma Life Science, diluted in 950ml ethanol and 50ml water) and diluted 1:50 in fish water. Death was confirmed by cervical dislocation. Caudal and pectoral fins were collected for genotyping and stored in 100% ethanol at -80˚C. The rest of the fish were fixed in 10% NBF (Neutral Buffered Formalin) and labelled for morphometric analysis.

Genomic DNA was extracted from fin tissue using the standard phenol-chloroform method (Sambrook & Russell, 2006). DNA purity and concentrations were assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA), ensuring 260/280 absorbance ratios within the range of 1.8-2.0. Amplification of the *zeb1* gene was done using a PCR with the following primers to capture the site of mutation: forward 5'-ATCCATTGCCACATTCAGCG-3' and reverse 5'-ATCAAACAGGAGCCAGTCGA-3'. PCR conditions included an initial denaturation step at 94˚C for 3 min, followed by 40 cycles at 94˚C for 45 sec, 52˚C for 30 sec, 72˚C for 30 sec, with a final extension step for 10 min at 72˚C. Amplified products were visualised on a 1.6% agarose gel to confirm the presence

of target bands and then submitted to DNA Sequencing and Services (University of Dundee, UK) for Sanger sequencing.

3.3.2.2 Geometric morphometrics

Adult zebrafish were cleared and stained (Potthoff, 1984) and imaged using a dissecting microscope (Leica M165, Leica, Wetzlar, Germany) mounted with a digital camera (Leica DFC450 C, Leica, Wetzlar, Germany). The ventral surface was photographed for morphological analyses, focusing on the mandible and head width. tpsDig2 was used for landmarking (10 fixed and 30 semi-landmarks), and tpsUtil (Rohlf, 2015) was used to form tps files for further analysis **(Figure 3-4b)**. Semi-landmarks helped to capture the shape properly. They were superimposed by the thin-plate spline sliding method along curves bounded by landmarks (Bookstein, 1997). Morphometric analyses were conducted in R version 4.3.1 (R Core Team, 2022) using the *geomorph* package (Adams & Otárola-Castillo, 2013; Adams et al., 2020). Landmark coordinates were imported from TPS files and subjected to Generalised Procrustes Analysis (GPA), which standardises shape by translating, rotating, and scaling specimens to a common centroid size (Zelditch et al., 2012). The semi-landmarks of each specimen were allowed to slide relative to one arbitrary specimen. Subsequently, a Procrustes superimposition was performed on these coordinates to derive a mean shape. All semi-landmarks were then permitted to slide in relation to the average Procrustes shape (Gunz & Mitteroecker, 2013).

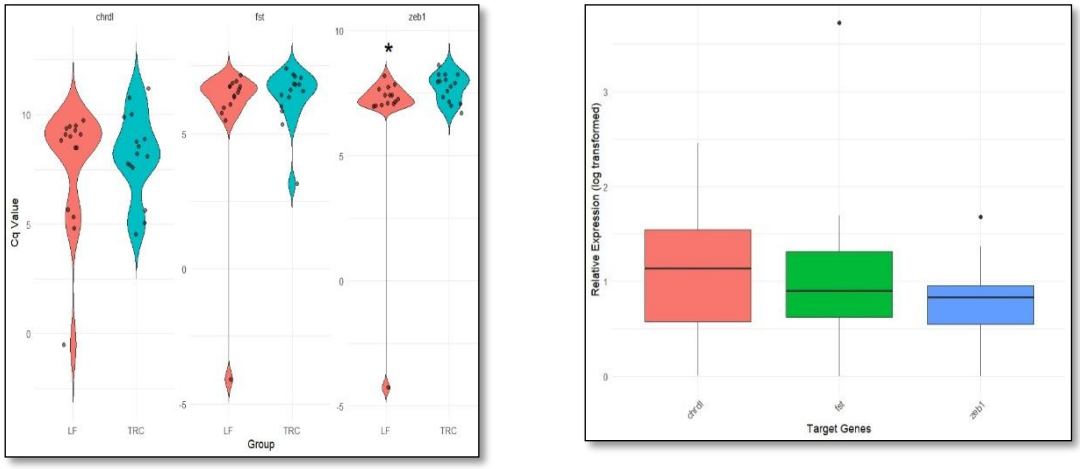
The resulting shape coordinates and centroid sizes were incorporated into a geomorph data frame alongside genotype data, with genotype treated as a categorical factor. A Procrustes ANOVA was performed using a Procrustes distance-based linear model (*procD.lm*) with 999 permutations to test for the interaction between shape, genotype, and size. To assess whether the relationship between size and shape differed by genotype, a principal component analysis (PCA) was conducted on the Procrustes-aligned landmark data, and the first principal component (PC1), representing the primary axis of shape variation, was extracted.

3.4 Results

3.4.1 Gene expression in two morphologically diverged cichlids

All candidate genes exhibited quantifiable expression levels, with Cq values below 34, indicating reliable detection suitable for downstream analyses, including ΔCt, ΔΔCt, fold change calculations, and statistical comparisons. The Cq value of the reference gene *β-actin* was used as a normalisation factor (NF) to assess relative expression levels of the target genes. Statistical analysis using the Mann–Whitney U test revealed a significant difference in *zeb1* expression between *Labeotropheus fuelleborni* (LF) and *Tropheops* sp. 'red cheek' (TRC), with a p-value of 0.036. In contrast, *follistatin* (*fst*) and *chordin-like* (*chrdl*) did not show statistically significant differences between species **(Figure 3-2a)**. However, after correcting for multiple testing using the false discovery rate (FDR) method, none of the genes remained statistically significant (*zeb1*- adjusted p = 0.108; *fst*- adjusted p = 0.664; *chrdl*- adjusted p = 0.772).

The Relative quantification (RQ) values showed that *zeb1* expression was reduced in TRC compared to LF, with an RQ value of 0.81, corresponding to a 25% downregulation **(Figure 3-2b)**. Conversely, *fst* and *chrdl* were slightly upregulated in TRC, reflecting increases of 12% and 14.36%, respectively.



(a) **(b)**

Figure 3-2 (a) Expression levels of candidate target genes based on raw Cq values in *Labeotropheus fuelleborni* (LF) and *Tropheops* sp. 'red cheek' (TRC) **(b)** Comparison of relative expression levels of the three target genes (*chordin-like* (*chrdl*), *follistatin* (*fst*) and *zeb1*). The Relative Quantification (RQ) is a measure of gene expression that compares the gene expression levels between the experimental and the control groups. RQ > 1 means upregulation and RQ < 1 means downregulation.

3.4.2 Genotyping in zebrafish mutants for the role of zeb1 in mandible formation

Genotyping data revealed that, out of 61 individuals, 17 were heterozygous carriers of the *zeb1* mutation. Procrustes ANOVA revealed no significant effects of genotype ($F_{1,57}$ = 0.77, p = 0.566) on the shape. However, the interaction between genotype and centroid size indicated marginal effects ($F_{1,57}$ = 1.99, p = 0.071), suggesting a potential genotype-dependent effect of size on shape variation, otherwise known as allometric effects **(Table 3-2)**.

Table 3-2: The results of a Procrustes ANOVA for effects of genotype and size on shape variation

	df	SS	Rsq	F	Z	p-value
Genotype	1	0.002224	0.01274	0.7721	-0.19670	0.566
Centroid size	1	0.002377	0.01362	0.8252	-0.06464	0.528
Genotype: Centroid Size	**1**	**0.005744**	**0.03291**	**1.9939**	**1.47893**	**0.071 .**
Residuals	57	0.164190	0.94073			

An interaction plot of centroid size versus shape score (PC1), colored by genotype, is a visual representation of the model trends between wild-type and mutant zebrafish. While wild-type individuals exhibited a slight decrease in shape score with increasing centroid size, mutant individuals showed a subtle increase **(Figure 3-3)**. Although the genotype x centroid size interaction almost reached statistical significance (p = 0.071), the opposing directions of the regression slopes suggest a genotype-dependent pattern of allometric shape variation.

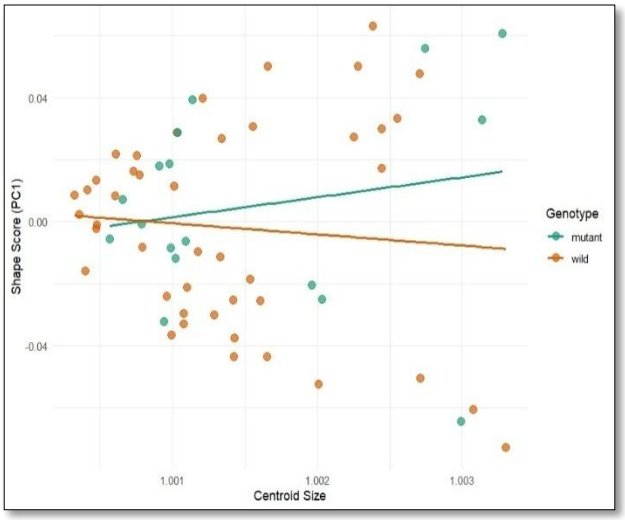
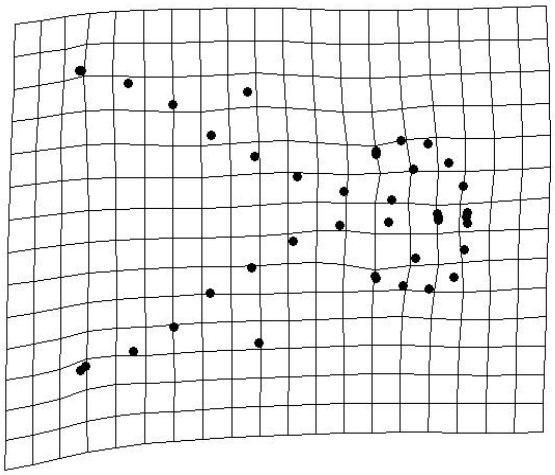


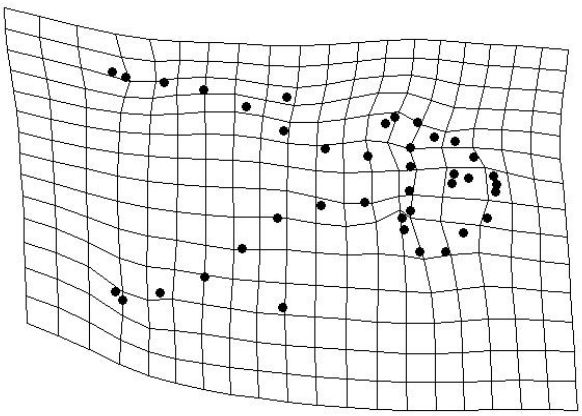
Figure 3-3: Interaction plot showing the relationship between centroid size and shape (PC1) for wild-type and *zeb1* mutant zebrafish.

Deformation grids were generated to visualise shape variation between genotypes using Geomorph's *PlotReftoTarget* function. The overall mean shape, calculated from all specimens, was analysed and compared to the mean shape of each genotype using thin-plate spline deformation analysis. The visualisations revealed genotype-specific shifts in landmark configurations, highlighting regions of morphological divergence. Shape differences were magnified by 8x to accentuate differences **(Figure 3-4a)**. Here, more warping was observed in the anterior region (including the mandible) of the *zeb1* mutant zebrafish, which looked narrower, pointing towards higher variation in that region.

Deformation grid for wild-type



Deformation grid for *zeb1* mutant



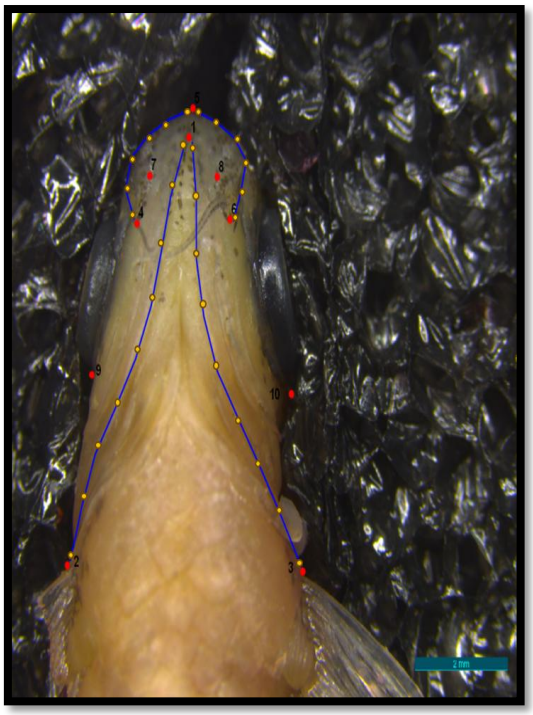


Figure 3-4 (a) Deformation grids (with 8x magnification) depicting associated shape variation for wild-type and *zeb1* mutant zebrafish **(b)** 10 regular (red colour) and 30 semi-landmarks (yellow colour) on the ventral surface of zebrafish.

3.5 Discussion

This study revealed a distinct pattern of *zeb1* gene expression associated with divergent trophic morphologies of cichlid species during the late larval stage, just before the onset of the juvenile phase and initiation of active feeding. While the observed differences should be interpreted with caution following correction for multiple testing, they suggest that *zeb1* may play a role in shaping species-specific mandible formation and could contribute to morphological divergence linked to trophic diversification and ecological specialisation.

Although not previously studied in cichlids, *zeb1* is known to be associated with neural crest-derived tissues (Vandewalle et al., 2009), including the craniofacial region. It also plays a key role in epithelial-to-mesenchymal transition (EMT), a process essential for the formation of various morphological structures (Shin et al., 2012; Zhang et al., 2015; Francou & Anderson, 2020). Expression of *zeb1* has been shown to increase during odontoblast differentiation in mice. It was expressed in the mandible and mesenchyme-derived tissues, including odontoblast and dental pulp (Xiao et al., 2021). Furthermore, *zeb1* null mutant mice have exhibited various oral and maxillofacial developmental abnormalities (Takagi et al., 1998; Jin et al., 2008), supporting a broader role in craniofacial development.

The significant downregulation of *zeb1* in TRC compared to LF aligns with previously characterised morphological and functional differences in craniofacial structures between these species (Parsons et al., 2014; Parsons et al., 2016; Albertson & Pauers, 2019). LF has a broader mandible used for scraping feeding modes, while TRC possess a narrower mandible adapted for plucking. Such variations have been shown to directly relate to bite mechanics and ecological specialisation (Hulsey et al., 2006; Albertson & Kocher, 2006). This suggests *zeb1* could be a regulator involved in mediating such divergence by influencing craniofacial ossification patterns during a critical period of larval development.

Our results suggest that elevated *zeb1* expression in LF may be associated with differences in bone remodelling dynamics, potentially influencing osteoblast activity or osteoclast-mediated resorption, and thereby contributing to a more robust jaw morphology suited for scraping. However, given the lack of statistical significance after multiple testing correction, this interpretation remains tentative. In other vertebrate systems, *zeb1* has been implicated in promoting osteoclast differentiation (Zhu et al., 2023). Bone marrow mesenchymal stem cells (BMSCs) are multipotent progenitors capable of differentiating into

osteogenic, chondrogenic, and adipogenic lineages (Bartscherer et al., 2006). Upregulation of *zeb1* has been shown to suppress osteogenic differentiation in human BMSCs (hBMSCs), whereas *zeb1* knockdown enhanced their osteogenic potential (Xu et al., 2021). In this case, I speculate that the elevated *zeb1* expression in LF could cause bone formation or retention via increased osteoblast activity or reduced osteoclast resorption, since bone remodelling is a coupled cycle of osteoclast resorption and osteoblast formation. Here, the LF, which is less plastic than TRC, could not be resorbing bone, reflecting a reduction in its remodelling capacity or the ability of the bone to be plastic, although further work is required to elucidate its functional role and confirm this interpretation.

Interestingly, although *zeb1* has been implicated in regulating the expression of BMP antagonists like *fst* and *chrdl* (Mock et al., 2015), their expression levels did not vary significantly between LF and TRC. This observation may reflect complex feedback or compensatory regulatory networks that buffer developmental pathways, as seen in other vertebrate systems (Wang et al., 2004; O'Connell et al., 2012).

The findings from our zebrafish experiment provide additional support for this hypothesis. While homozygous *zeb1* mutants were not recovered, suggesting a potentially lethal phenotype or developmental arrest, the analysis of heterozygous individuals revealed subtle, genotype-dependent shape changes. Although the overall shape variation did not differ significantly between genotypes, the marginal genotype × size interaction and opposing trends in shape allometry suggest an emerging pattern of *zeb1*-dependent morphological development. These results align with prior studies demonstrating that small regulatory changes in transcription factors can yield ecologically meaningful shape variation (Powder et al., 2015; Parsons et al., 2016). This also points towards evolutionary tuning of transcriptional regulation, rather than disruption of gene function that can underlie adaptive morphological diversification (Hoekstra & Coyne, 2007; Li et al., 2023; Jones et al., 2024).

Notably, the integration of expression profiling in ecologically divergent cichlid species with functional validation in a genetically tractable model organism adds robustness to our conclusions. While the statistical support for differential expression is limited, the combined evidence suggests that *zeb1* represents a promising candidate gene for further investigation into the genetic basis of craniofacial diversification. This integrative evo-devo approach has previously proven valuable in elucidating the genetic basis of craniofacial traits (Abzhanov et al., 2006; Powder & Albertson, 2016), and our study builds on this framework by highlighting *zeb1* as a novel, ecologically relevant modulator of jaw shape variation.

3.6 Conclusion

To conclude, this study provides new insights into the molecular mechanisms underlying adaptive morphological divergence in teleost fishes. By identifying *zeb1* as a potential differentially expressed gene associated with mandible shape variation and validating its functional relevance in zebrafish, we bridge molecular regulation with ecological diversification. By investigating *zeb1* expression and function across two complementary model systems, our study addresses fundamental questions about the genetic architecture of morphological evolution. Specifically, we test whether species differences in gene expression reflect adaptive divergence in a developmental regulator and whether such divergence can be functionally linked to phenotypic effects. This work contributes to a growing body of research that seeks to bridge the gap between genotype and phenotype in evolutionary developmental biology, with implications for understanding how regulatory networks evolve to generate the diversity of vertebrate form. We observed that species-specific shapes can be detected at the earliest stage of development examined, which suggests that early developmental patterning events play an important role in determining adult morphology in cichlids. Additionally, these are species-specific developmental paths (Powder et al., 2015).

This study has looked at a relevant small-scale adaptive divergence between TRC and LF, but this work could be expanded to include other species from the other end of the Malawi feeding axis, like *Rhamphochromis chilingali* (a specialist suction feeder) or *Maylandia zebra* (a suction-feeding generalist), to comparatively explore *zeb1* further. By expanding and looking at additional species, this would help us further understand how the mandible and craniofacial morphology have diversified (Irschick et al., 2013).

Mutations are a source of novel genetic variation leading to evolutionary change (Lynch, 2010; Eyre-Walker & Keightley, 2007). Although most mutations are deleterious, some confer advantageous effects that natural selection can act upon, enabling populations to adapt and evolve (Orr, 2005; Barrett & Schluter, 2008). Experiments with *zeb1* mutant zebrafish provide evidence of how *zeb1* correlates with jaw-shape variation and trophic specialisation, illustrating how a specific regulatory mutation can influence ecologically relevant morphology.

Chapter 04: Examining the underlying role of a candidate gene, *wnt7ba*, in adaptive phenotypic plasticity

4.1 Abstract

The Wnt/β-catenin signalling pathway is a key regulator of craniofacial development, and variation in pathway components has been linked to trophic morphology in rapidly diversifying cichlids. Through QTL mapping of craniofacial traits, *wnt7ba* emerged as a candidate gene in craniofacial plasticity. Allelic divergence within this gene was observed by studying a range of different cichlid genomes. This suggested that adaptive divergence of *wnt7ba* may contribute to environmentally induced craniofacial variation. Here, I test the role of *wnt7ba* in diet-induced plasticity using zebrafish as a tractable model and rearing wild-type and *wnt7ba* mutants under benthic and limnetic feeding treatments. A permutation-based Procrustes ANOVA was conducted, and a diet by size interaction was found, indicating diet-dependent allometry. Genotype and its interaction were not, however, significant despite a larger magnitude of plasticity indicated by mutants. This points to a limitation of sample size, although there could be no effect of the mutation. Craniofacial variation in deformation grids did qualitatively support a role for *wnt7ba* as a contributor to craniofacial development and ecologically relevant plasticity.

4.2 Introduction

Phenotypic plasticity refers to an organism's ability to produce different phenotypes in response to environmental cues. As a trait, plasticity is vital for the emergence of new and complex characteristics (Moczek, 2008), which can lead to speciation (West-Eberhard, 2005; Pfenning & McGee, 2010) and adaptation (Pigliucci & Murren, 2003; Wund et al., 2008; Sultan, 2010; Pfennig et al., 2010; Moczek et al., 2011). Plasticity itself is an evolvable and adaptive trait, allowing populations to respond to novel environments (Scheiner, 1993; Robinson & Parsons, 2002; Dewitt & Scheiner, 2004; Pigliucci, 2005; Nicotra et al., 2010; Svanbäck & Schluter, 2012). This process involves interactions between developmental and genetic architectures. The genetic architecture, also called the genotype-phenotype map, explains how genetic variation manifests as phenotypic traits (Pigliucci, 2008). The interplay between environment and genes across development shapes phenotypic variation, which is acted upon by natural selection (Le Rouzic & Carlborg, 2008; Parsons et al., 2010). As a

result, it opens pathways to novel variation that could contribute to adaptive divergence. Early views suggested that genes specifically dedicated to plasticity might not exist (Via, 1993). More recent research has identified loci that influence plastic responses (Gibson & Dworkin, 2004; Snell-Rood et al., 2010); however, how these loci contribute to adaptive divergence remains poorly understood (Moczek et al., 2011). Most studies on this topic have been conducted on models like *Saccharomyces cerevisiae* (Kovuri et al., 2023), *Caenorhabditis elegans* (Gutteling et al., 2007; Sommer & Ogawa, 2011), *Drosophila melanogaster* (Zhou et al., 2012), *Arabidopsis thaliana* (Bloomer et al., 2014), *Brassica napus* (Fletcher et al., 2015), and *Triticum aestivum* (Zhai et al., 2014). While these models have been invaluable for identifying candidate mechanisms, they provide limited insight into the genetic control of plasticity in natural populations experiencing adaptive diversification (Ledón-Rettig et al., 2014). Therefore, our understanding of how the molecular basis of environmentally induced plastic responses relates to broader evolutionary patterns remains limited.

Morphological variation has long been recognised as a fundamental driver of evolutionary diversification (Heard & Hauser, 1995; Schluter, 2000). Notably, a significant portion of morphological and functional divergence among vertebrates has been observed in the craniofacial region (Roberts et al., 2011). In fishes, plasticity in feeding structures and morphology has facilitated exploitation of various ecological niches, supporting trophic specialisation and resource polymorphisms (Skúlason and Smith, 1995; Parsons et al., 2010; Skúlason et al., 2019). Such modifications influence the type of food utilised and the efficiency of processing (Parsons & Albertson, 2009). Therefore, understanding the molecular and developmental mechanisms underlying these adaptive differences is essential to evo-devo research, providing vital insights into how complex trait variation arises and diversifies. However, our understanding of the genetic basis of environmentally induced phenotypic variation and how it may evolve in conjunction with adaptive divergence remains limited (West-Eberhard, 2003; Zhai et al., 2014).

Morphometric and molecular approaches have been employed to test the genetic mechanisms underlying plastic traits in plants, insects, fish, birds and mammals (Parsons & Albertson, 2013). Genetic mapping was developed and widely used as a powerful tool (Ungerer et al., 2003; Li et al., 2006; Li et al., 2010; Ward et al., 2012). Quantitative trait locus (QTL) studies have provided valuable insights into the genomic regions underlying adaptive phenotypic variation, identifying candidate loci that shape craniofacial morphology across various fish species (Parsons et al., 2014; Küttner et al., 2014; Parsons et al., 2016).

These findings highlight how the genetic basis of plasticity can evolve and contribute to adaptive radiation.

In vertebrates, the Wnt/β-catenin signalling pathway is a vital regulator of facial morphogenesis and development (Brugmann et al., 2010; Parsons et al., 2014; Powder et al., 2015). Early research recognised Wnt/β-catenin as a widely conserved developmental pathway with roles in axis formation, proliferation, and cell fate determination (Cadigan & Nusse, 1997; Schmidt & Patel, 2005). Notably, for craniofacial development, the pathway is involved in neural crest cell formation and migration, which are precursors to the facial skeleton and connective tissue (Schmidt & Patel, 2005). Studies on vertebrates have demonstrated that Wnt/β-catenin signalling influences facial development and craniofacial variation (Brugmann et al., 2007; Brugmann et al., 2010). Liu et al. (2010) discuss species-specific facial features and how disruptions in Wnt/β-catenin activity can alter the proliferation and differentiation of craniofacial progenitors. Overall, these studies highlight the essential role of Wnt/β-catenin signalling in molecular processes that underpin facial development.

Lake Malawi cichlids are recognised as one of the most dramatic adaptive radiations and a model for investigating the genetic basis of morphological evolution, owing to their remarkable phenotypic diversity and trophic specialisation (Kocher, 2004; Cooper et al., 2010; Parsons et al., 2011, Parsons et al., 2014; Salzburger, 2018). Genomic studies in Lake Malawi cichlids have revealed divergence in Wnt/β-catenin signalling pathway genes, pointing to its broad role in shaping trophic diversity (Loh et al., 2008). Experimental work has implicated the Wnt/β-catenin signalling pathway as a key regulator of morphological variation in cichlids (Parsons et al., 2014; Powder et al., 2015) and other adaptive traits such as fin rays (Navon et al., 2017) and hypertrophied lips (Machii et al., 2025), hinting that changes in the activity of the Wnt/β-catenin pathway might influence ecologically relevant morphological traits, making it a promising candidate for further research.

This chapter focuses on the hypothesis that adaptive divergence of *wnt7ba* may contribute to environmentally induced craniofacial plasticity. Here, I compare wild-type and mutant zebrafish under alternative feeding regimes. By integrating insights from cichlid QTL mapping to mutant zebrafish, this study provides a powerful framework for linking the function of candidate genes to the genetic basis of adaptive plasticity.

4.3 Methods

4.3.1 Selection of candidate gene wnt7ba

Previously, candidate loci underlying craniofacial plasticity were identified through a quantitative trait loci (QTL) approach (Parsons et al., 2016), in which F3 hybrids of two Malawi cichlid species- *Labeotropheus fuelleborni* (LF) and *Tropheops "red cheek"* (TRC) were reared and kept under different feeding regimes to induce phenotypic plasticity in craniofacial traits. Within the traits examined in this research, I chose to focus on the lateral shape of the craniofacial region, which showed a plastic response and provided a locus of interest on linkage group 17. I examined the genomic region surrounding this QTL peak (~29.1 Mb), where genetic differentiation was highest (F_{st} = 0.95). Marker sequences flanking this QTL were aligned to the *Maylandia zebra* reference genome (Ensembl, M_zebra_UMD2a) using BLAST to convert linkage map positions to physical coordinates. The resulting interval ranged from approximately 23.7 Mb to 30.7 Mb on chromosome 17. Genes within this interval were recorded from Ensembl annotations, and *wnt7ba* was identified at 29.67–29.68 Mb, approximately 0.5 Mb from the QTL peak, making it a strong candidate for involvement in diet-induced craniofacial plasticity.

Next, the *wnt7ba* genomic region obtained from the *Maylandia zebra* genome was compared to several cichlid genomes. A single-nucleotide polymorphism was identified at coordinate LG17:29,679,737 that was present in *Labeotropheus fuelleborni* but absent in *Tropheops*, indicating allelic divergence at this site. In summary, the gene was identified and selected based on its (1) position relative to the QTL peak region for plastic craniofacial trait, (2) allelic differentiation between the divergent species, and (3) known functional roles in morphological development and bone biology, making *wnt7ba* a strong candidate for understanding ecologically relevant craniofacial plasticity.

4.3.2 Plasticity experiment

To test the function of *wnt7ba,* I identified mutant alleles (sa26281) available in zebrafish that were obtained as embryos from the European Zebrafish Resource Centre (https://www.ezrc.kit.edu/). Larvae were fed a diet consisting of Artemia nauplii and ZM000 powdered feed (ZM Fish Food) for the first month. Then, the zebrafish were transitioned to a diet consisting of ZM100 and ZM200 powders (ZM systems, Twyford, UK) to support optimal growth and reproductive health. Once individuals reached sexual maturity, the adults

were bred to produce sufficient offspring to start the experiments. The fertilised eggs were kept in an incubator and treated with 1-2 drops of methylene blue until hatching to reduce the risk of fungal infection. The fry were reared for three months and then subjected to benthic (n = 51) and limnetic (n = 49) plasticity treatments. Before dividing, individuals were selected randomly, and the average SL was measured, which was 19.14 ± 2.53 mm.

For limnetic feeding, powdered ZM100 and ZM200 were given to induce a suction mode of foraging. Conversely, for benthic feeding, the powder was spread on ceramic tiles and left to dry overnight. The tiles were then placed at the bottom of the tank to induce a scraping mode of foraging. The experiment was conducted in replicates for two months, and later tissues were collected for morphometric measurements and genotyping. At the time of sampling, the standard length of fish in B1, B2, L1, and L2 tanks was measured to be 26.41 ± 1.88 mm, 27.08 ± 2.13 mm, 28.32 ± 1.99 mm, and 27.27 ± 1.52 mm, respectively. The fish were euthanised following Schedule 1 killing methods, in accordance with UK Home Office guidelines, using an overdose of benzocaine solution (10g benzocaine powder, Sigma Life Science, diluted in 950ml ethanol and 50ml water), diluted 1:50 in fish water. Caudal and pectoral fins were collected for genotyping and stored in 100% ethanol at -80˚c. The fish were fixed in 10% NBF (Neutral Buffered Formalin) and labelled for morphometric analysis.

4.3.3 Genotyping

Genomic DNA was extracted from fin tissue using the standard phenol-chloroform method (Sambrook & Russell, 2006). DNA purity and concentrations were measured with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA), ensuring 260/280 absorbance ratios between 1.8 and 2.0. Amplification of the *wnt7ba* region was performed using PCR with the following primers to target amplification of the mutation site: forward 5'-CTGAAGGAGCGTTACACCAC-3' and reverse 5'-ACAGTGTCCTCTTCGCAGTA-3'. PCR conditions included an initial denaturation at 94˚C for 3 minutes, followed by 40 cycles at 94˚C for 45 seconds, 52˚C for 30 seconds, 72˚C for 30 seconds, and a final extension of 10 minutes at 72˚C. The amplified products were visualised on a 1.6% agarose gel to confirm the presence of target bands, then submitted to DNA Sequencing and Services (University of Dundee, UK) for Sanger sequencing.

4.3.4 Geometric morphometrics

The specimens were cleared and stained (Potthoff, 1984) and imaged using a dissecting microscope (Leica M165, Leica, Wetzlar, Germany) mounted with a digital camera (Leica DFC450 C, Leica, Wetzlar, Germany). Each specimen was positioned laterally with the mouth closed to focus on the craniofacial structures and photographed for morphological analyses. For landmarking tpsDig2 was used (27 fixed and 92 semi-landmarks), and tpsUtil (Rohlf, 2015) was used to generate tps files for further analysis **(Figure 4-1)**. Statistical analyses of morphometric data were conducted in R version 4.3.1 (R Core Team, 2022) using the geomorph package (Adams & Otárola-Castillo, 2013; Adams et al., 2020). Semi-landmarks were employed to accurately capture the shape, superimposed by a thin-plate spline sliding method along curves bounded by landmarks (Bookstein, 1997). Landmark coordinates were imported from TPS files and subjected to Generalised Procrustes Analysis (GPA), which standardises shape by translating, rotating, and scaling specimens to a common centroid size (Zelditch et al., 2012). The semi-landmarks of each specimen were allowed to slide relative to one arbitrary specimen. Subsequently, a Procrustes superimposition was performed on these coordinates to derive a mean shape. All semi-landmarks were then permitted to slide in relation to the average Procrustes shape (Gunz & Mitteroecker, 2013). The resulting shape coordinates and centroid sizes were incorporated into a geomorph data frame alongside genotype data, with genotype treated as a categorical factor. A Procrustes ANOVA was performed using a Procrustes linear model (coords~ Genotype* Diet* Centroid size, 999 permutations) to assess the shape variation with respect to diet, genotype, size and their interaction.

Next, to quantify and compare plasticity between wild types and mutants with respect to diet treatments, a trajectory analysis was conducted using the *trajectory.analysis* function (Collyer & Adams, 2013). This function quantifies phenotypic shape change trajectories from the specimens and assesses variation in attributes of the trajectories via permutation. A shape change trajectory is defined by a sequence of shapes in tangent space. These trajectories can be quantified for various attributes (their size, orientation, and shape), and comparisons of these attributes enable the statistical comparison of shape change trajectories.

Deformation grids were generated to visualise shape variation between genotypes under alternate feeding conditions using Geomorph's *PlotReftoTarget* function. The overall mean shape, calculated from all specimens, was analysed and compared to the mean shape

for each diet × genotype group using thin-plate spline deformation analysis. Shape differences were magnified by 2x to accentuate differences.

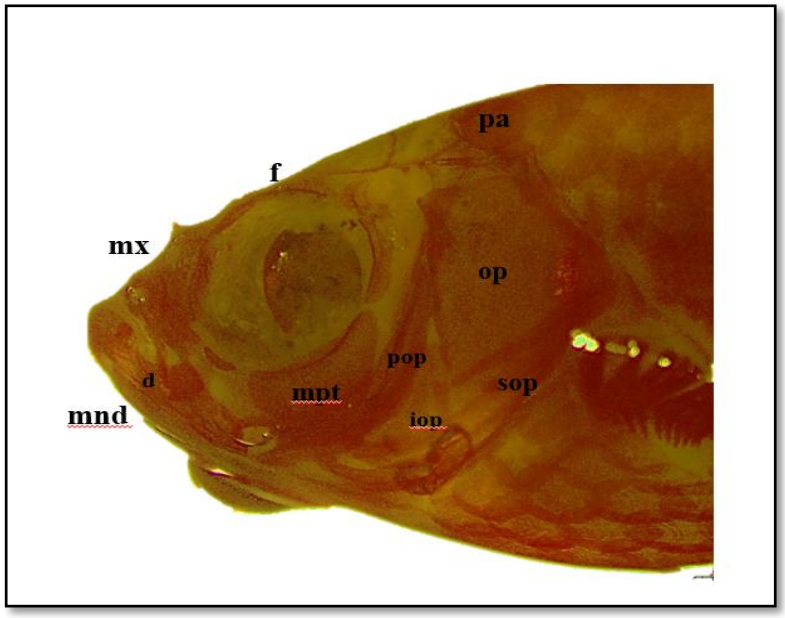
4.4 Results

Genotyping revealed that, out of 100 individuals, there were 12 and 9 carriers of the *wnt7ba* mutation in benthic and limnetic treatments, respectively. Procrustes ANOVA revealed a significant effect ($F_{1,92}$ = 3.28, p = 0.001) on shape, with no significant effects of genotype x diet ($F_{1,92}$ = 0.95, p = 0.454). The interaction between diet and size indicated significant effects ($F_{1,92}$ = 2.15, p = 0.024), suggesting that allometric effects differed across diets **(Table 4-1)**.

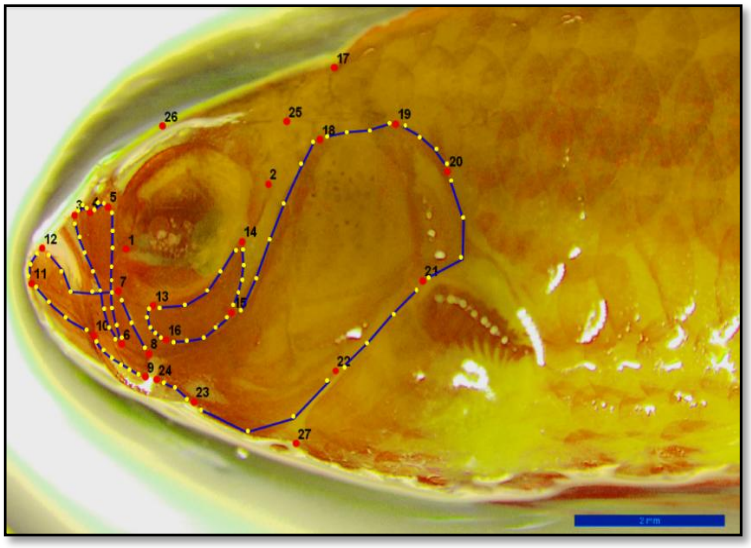
Table 4-1: The results of a Procrustes ANOVA for effects of diet, genotype and size on shape variation. Asterisks highlight statistically significant P values**.**

	df	**SS**	**Rsq**	**F**	**Z**	**p-value**
Diet	1	0.03480	0.02908	3.2837	2.8197	**0.001*** **
Genotype	1	0.00714	0.00596	0.6734	-0.7196	0.775
Centroid size	1	0.11854	0.09903	11.1837	6.2998	0.001***
Genotype: Diet	1	0.01009	0.00843	0.9518	0.1150	**0.454**
Genotype: Centroid Size	1	0.01568	0.01310	1.4795	1.2227	0.106
Diet: Centroid Size	1	0.02279	0.01904	2.1499	1.9528	0.024*
Genotype: Diet: Centroid Size	1	0.01278	0.01068	1.2060	0.6913	0.246
Residuals	92	0.97511	0.81468			

Although not significant, the magnitude of plasticity was larger in mutants than in wild type (0.061 and 0.036, respectively). Here, more warping of the deformation grids was observed in the preorbital region and the opercular area of *wnt7ba* mutants across limnetic and benthic treatments, pointing towards higher plasticity **(Figure 4-2)**.



(A)



(B)

Figure 4-1: Anatomy of the zebrafish head from a left lateral view. **(A)** The anatomical regions (mnd = mandible, mx = maxillae, f = frontal, pa = parietal, mpt = metapterygoid, op = opercle, pop = preopercle, iop = interopercle, sop = subopercle, d = dentary) **(B)** landmarks **(**27 regular- red colour, 92 semi-landmarks- yellow colour).

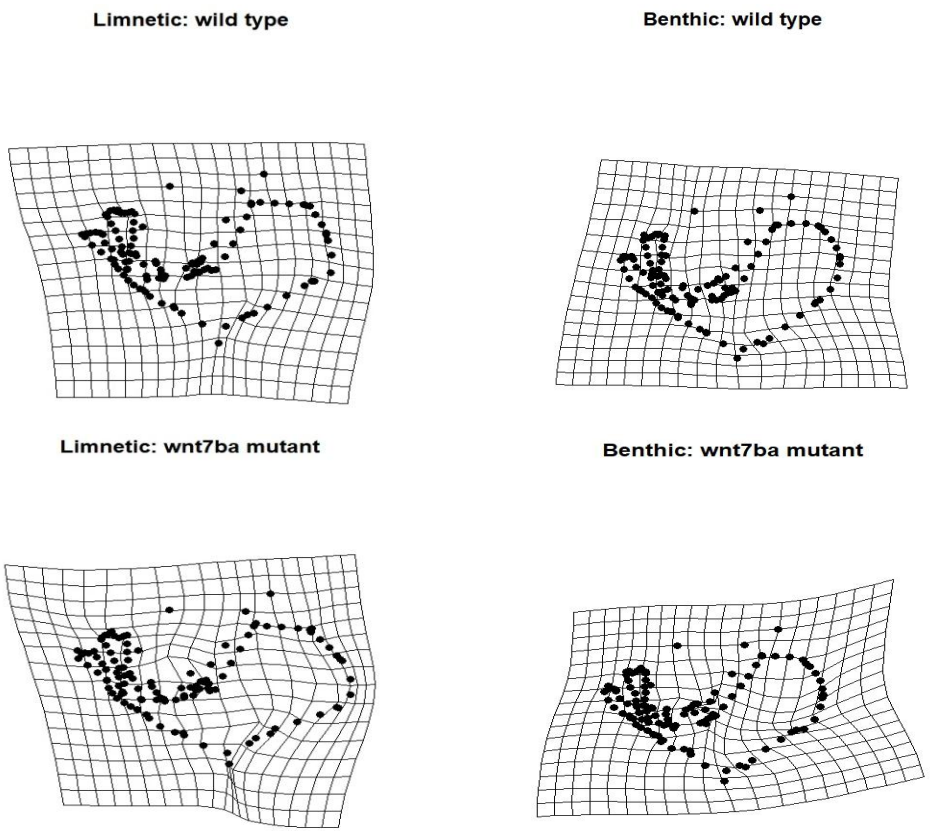


Figure 4-2: Deformation grids (with 2x magnification) depicting associated shape variation for wild-type and *wnt7ba* mutant zebrafish under limnetic and benthic treatments

4.5 Discussion

The findings provided limited support for the hypothesis stating that adaptive divergence of *wnt7ba* may contribute to environmentally induced craniofacial plasticity. Although no significant interaction between the genotype and diet was found, the trajectory analysis and deformation grids indicated some variation in the magnitude of plasticity between wild-type and mutant zebrafish. However, these patterns were not statistically significant and require further investigation to provide conclusive evidence for a role of *wnt7ba* in mediating craniofacial plasticity.

There are a few plausible reasons that point towards a role of *wnt7ba* in morphological plasticity. Firstly, vertebrates carry several Wnt ligands and receptors (frizzled/ LRP5/6) that can signal through overlapping cascades; these ligands and receptors can substitute for each other and are context-dependent (Van Amerongen & Nusse, 2009; MacDonald et al., 2009; Wiese et al., 2018), buffering the effect of a single gene. There may also be transcriptional adaptation where related genes are upregulated, leading to a dampening of the phenotypes (El-Brolosy et al., 2019). This makes it plausible that a mutation in one of the Wnt components, such as *wnt7ba*, could lead to non-significant or subtle shape variations even though the pathway is a key mediator of craniofacial

development (Reid et al., 2011; Alexander et al., 2014). Another way to look at subtle or non-significant shape effects could be through developmental robustness (Nijhout, 2002; Green et al., 2017; Arcuschin et al., 2023), where developmental systems can buffer genetic and environmental perturbations, forming stable phenotypes. Secondly, since the mutation found in the zebrafish used is synonymous, and therefore should not alter the *wnt7ba* amino acid sequence itself. However, synonymous substitutions can alter codon optimality, mRNA stability and translation efficiency, thereby changing the abundance of the *wnt7ba* protein, which might have subtle effects on the Wnt/β-catenin signalling dynamics during a plastic response (Oelschlaeger, 2024). Lastly, reaction norms for craniofacial traits may be polygenic, with *wnt7ba* contributing modestly alongside other loci that may involve genes in other pathways or Wnt components (Albertson et al., 2005; Parsons et al., 2014; Küttner et al., 2014; Parsons et al., 2016). In mice, several small-effect loci for mandible and skull shape have been identified, demonstrating a highly polygenic architecture (Pallares et al., 2016).

Limitations and next steps

A key limitation for the detection of differences in the degree of plasticity was the sample size of mutant carriers. Therefore, a lack of a genotype by diet effect on shape should not be taken as absolute. Indeed, while diet-induced variation in shape differences was of a greater magnitude in mutants, in line with increased differences in craniofacial variation in my deformation grids, this suggests the presence of *wnt7ba* gene regulation. This study can therefore be regarded as a pilot experiment that motivates a comprehensive study, which should include increased mutant sample sizes with balanced groups to enhance statistical power and precision.

4.6 Conclusion

This chapter combined insights from a basis in cichlid QTL mapping to *wnt7ba* mutant zebrafish and tested the hypothesis that *wnt7ba* plays a role in diet-induced craniofacial plasticity. Although the results did not yield significant genotype effects on plasticity, the evidence from cichlid QTLs, the central role of Wnt/β-catenin signalling, and the subtle pattern in mutant trajectories all point to *wnt7ba* as a plausible contributor to craniofacial development and adaptive plasticity. Given its clear relevance to trophic morphology, an ecologically critical trait, *wnt7ba* remains a strong candidate. Building on this foundation, a clear next step would be to increase the sample size and look deeper into the subtle effects of *wnt7ba* in contributing to ecologically relevant craniofacial plasticity.

Chapter 5: Development of primary cell cultures from haplochromine cichlid bone-derived tissues

5.1 Abstract

Bone is a dynamic tissue with ecological and evolutionary importance, as it can grow and remodel itself in response to mechanical stimuli. In mammals, osteocytes are widely recognised as the central regulators of bone formation and mechanotransduction. However, many advanced teleosts lack these cells yet still exhibit evidence of bone formation and remodelling. This challenges the prevailing view that osteocytes are indispensable for these processes. Notably, these anosteocytic teleosts exhibit clear responses to mechanical loading, suggesting alternative mechanisms at play. African cichlids, known for their remarkable ecological diversification and have varied craniofacial morphologies that represent an ideal model system for investigating bone biology in the absence of osteocytes. This protocol outlines the development of primary cell cultures from cichlid jaw bones, establishing a foundation for future research aimed at elucidating the cellular and molecular mechanisms underlying bone formation and remodelling in anosteocytic systems. Such insights have the potential to reshape our understanding of bone function and its evolution across vertebrates.

5.2 Introduction

Bone is a metabolically active tissue that is key to many examples of adaptive evolution due to its ability to change into many different forms (Robling et al., 2006; Parsons & Albertson, 2009). This ability to adapt to a range of environments is essential for understanding bone structure, growth and repair. Several studies have revealed that exposure of bone cells to mechanical stress can increase their metabolic activities (Dodds et al., 1993; Pead et al., 1988) and affect bone formation, remodelling, and repair (Herrel et al., 2009; Riddle & Donahue, 2009). Wolff (1892) presented the ‘law of bone transformation,’ which stated that vertebrates' bones display a high degree of plasticity and respond to mechanical load.

Osteocytes are considered master cells that play a critical role in bone physiology through sensing mechanical stress. They are known to orchestrate adaptation and work with the effector cells (bone-resorbing and bone-forming cells) to regulate bone size, mass, and shape in response to mechanical stimuli (Bonewald, 2011; Xiong et al., 2011). In adult

mammalian bones, the osteocytes are most abundant and constitute 95% of total bone cells (Hellmich & Ulm, 2002; Bonewald, 2011). They originate from mature osteoblast cells. Osteoblasts, or bone-forming cells, are differentiated and specialised derivatives of mesenchymal stem cells. Their primary function is to produce bone through a process called osteogenesis. The osteoblasts continuously produce bone matrix, which eventually mineralises, causing embedding of these cells, leading to terminal differentiation to bone, maintaining osteocytes (Manolagas, 2000). During the embedding stage, these cells extend cellular projections, which eventually form the dendritic processes of the mature osteocytes. These dendritic processes elongate and contract till contact is made with older embedded cells (Dallas & Veno, 2012) **(Figure 5-1)**. These osteocytes work as mechanosensors and respond to variations in mechanical loading (Witten & Huysseune, 2009; Bonewald, 2011).

Mechanically active osteocytes influence bone remodelling through direct cell-to-cell interactions and secretion of signalling factors (like Wnts wingless-int-1), prostaglandin, bone morphogenetic proteins (BMPs), and nitric oxide) that regulate the initiation, differentiation and function of osteoblast and osteoclast cells (You et al., 2008; Santos et al., 2009; Bonewald, 2011; Goldring, 2015). The function and number of these osteoblasts and osteoclasts are regulated by several factors like cytokines, hormones and signalling molecules produced by the osteocytes when subject to mechanical stimuli (Vezerides et al., 2006; Onal et al., 2012).

According to several studies, osteocytes exhibit higher responsiveness to mechanical loading than osteoblasts. They induce a higher influx of calcium (Lu et al., 2012), more release of prostaglandin (Klein-Nulend et al., 1995; Kamel et al., 2010), and nitric oxide (Klein-Nulend et al., 1995) along with faster β-catenin-(Wnt) mediated transcription (Kamel et al., 2010). The bone tissue is remodelled throughout life, where the mature bone tissue is removed by osteoclasts (bone-resorbing cells), and new bone tissue is formed by the osteoblasts (bone-forming cells). Another vital function of the osteocytes is mineral homeostasis, where the bone matrix is broken down, and calcium and phosphorus are released into the bloodstream. This process is led by the osteocytes and is known as osteocytic osteolysis (Witten & Huysseune, 2009; Shahar & Dean, 2013; Doherty et al., 2015).

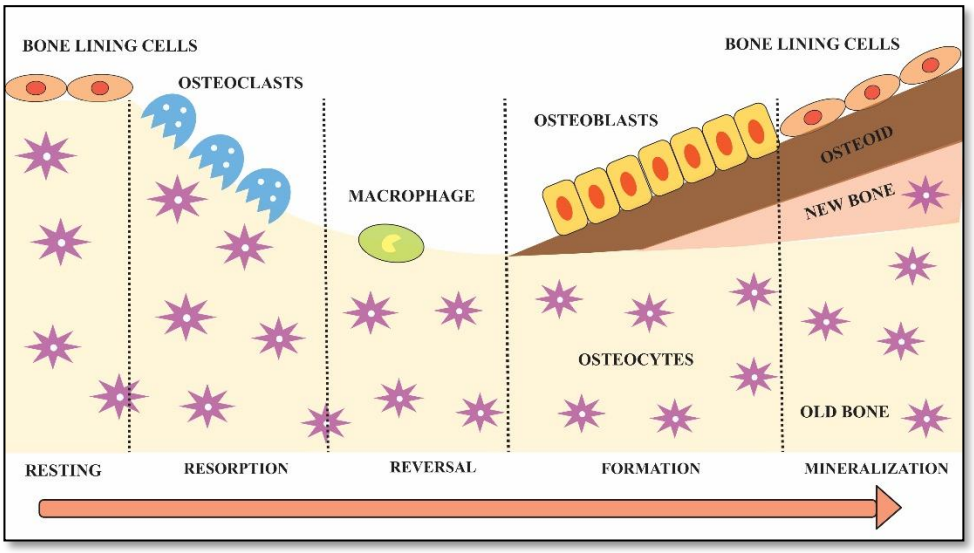


Figure 5-1: Steps involved in the process of bone remodelling- resorption, reversal, formation, mineralisation and resting. The osteoblasts and osteoclasts work together to form a balance between bone formation and bone dissipation (modified from Lisowska et al., 2018).

Researchers made a striking observation while studying teleost bone physiology and investigating the indispensable role of osteocytes in strain detection, bone formation, and mineral homeostasis. They found that, unlike mammals and basal teleosts, osteocytes are absent in advanced teleosts, which form a major group of bony fish and account for about half of all vertebrate species (Moss, 1963; Ekanayake & Hall, 1987; Witten et al., 2004; Shahar & Dean, 2013). This raises questions around the central axiom that osteocytes are most critical for bone formation and homeostasis, as the bones of anosteocytic teleost fishes are well known for their responses to mechanical stress (Robinson & Parsons 2002; Skulason et al. 2019). These fish demonstrate mechanoadaptation, where mechanical loads from rapid swimming (Ofer et al., 2019) or feeding (Gunter et al., 2013) are known to stimulate both bone resorption and deposition. It has been observed that even without osteocytes, bone-surface osteoblasts can detect mechanical loading and induce a mechanical response (Ofer et al., 2019).

The morphological responses of fish to varying mechanical loads have been well characterised. For example, the rostral bone of billfishes (marlin, swordfish) exhibits intense remodelling (Atkins et al., 2014; Currey et al., 2017). Also, during prey capture and processing, fish experience mechanical stress on their craniofacial bones, which induces adaptive remodelling (Albertson & Yelick, 2007). Fish anosteocytic bones have shown evidence of bone remodelling in response to mechanical loading in several species, like *Geophagus sps*. (Wimberger, 1991), *Neochromis greenwoodi* (Bouton et al., 2002),

Astatoreochromis alluaudi (Huysseune et al., 1994) and *Oreochromis aureus* (Atkins et al., 2015), suggesting the presence of osteocyte-independent mechanisms that would give deeper insights into bone biology. Thus, we can see how anosteocytic bone is quite similar to osteocytic bone with respect to its biomechanics, function and mechanisms of bone resorption and remodelling. This suggests that achieving these functions does not necessarily require the presence of osteocytes. However, the anosteocytic bone varies from the osteocytic bone in terms of mineral regulation. In teleosts, the absence of osteocytes prevents mineral homeostasis, which is compensated for by the phosphorus and calcium available in ambient waters and dietary sources (Cohen et al., 2012; Doherty et al., 2015). This suggests that the fundamental role of osteocytes is in mineral homeostasis (via osteocytic osteolysis) rather than in detecting mechanical loading and bone reshaping.

Thus, it can be concluded that anosteocytic bone is a great model to advance our understanding of bone biology and its evolution within vertebrates (Witten et al., 2004; Currey et al., 2017). This is due to its absence of a cell type traditionally thought to have a significant role in bone growth and remodelling. To delve deeper into the structure and function of anosteocytic bone, more studies on teleosts should be conducted, as they are the largest group representing anoesteocytic bone cells. The calcified structures of teleosts have been studied to advance our understanding of the physiological, cellular, and evolutionary mechanisms in vertebrates (Dornburg et al., 2021). In line with this idea, this paper aims to develop a comprehensive protocol for establishing bone cells derived from mechanically active fish species that would help conduct diverse bone studies and add to the existing knowledge in the field.

Resources from the Cellosaurus database (https://www.cellosaurus.org/) have revealed that the majority of the fish cell lines are represented by marine species (52%) compared to freshwater species (Laizé et al., 2022). African cichlids (Perciformes: Teleostei) from Lake Malawi (~1.5 Myrs) are an interesting freshwater species known to have high evolutionary relevance, diversifying into several hundred ecologically specialised species in less than a million years (Brawand et al., 2014; Schneider & Meyer, 2017). These cichlids are also an exemplary model of bone remodelling (Bouton et al., 2002; Parsons et al., 2014; Parsons et al., 2016; Navon et al., 2020; McWhinnie et al., 2022). They exhibit considerable variation in their craniofacial morphology with evolutionary divergence enabling swift transitions between biting and suction modes of feeding (Kocher, 2004; Cooper et al., 2010). This adaptability offers extensive evolutionary advantages by creating significant biomechanical variations in feeding, leading to crucial shifts in trophic ecology. They

display a primary axis of craniofacial differences that aligns with the two primary foraging mechanisms (biting and suction feeding).

Species on one end of this ecomorphological continuum are limnetic feeders that graze upon mobile prey in the water column via suction feeding. These are characterised by a shallow craniofacial profile and a relatively long mandible. Species on the other end are benthic feeders that scrape prey from the rocks by biting and are characterised by a steep craniofacial profile and a short mandible (Parsons et al., 2014). For example, *Labeotropheous fuelleborni*, a biter, feeds by biting algae off rocks and has a short, wide, U-shaped lower jaw. This is conducive to a greater bite force/ mechanical loading. On the other hand, *Maylandia zebra*, a suction feeder, feeds by suctioning the water column and has a long, thin, V-shaped lower jaw, which requires faster speed (Albertson & Kocher, 2006; Navon et al., 2020). Thus, looking at the craniofacial bones of this teleost fish species could be a cornerstone in revealing how bone cells grow and remodel themselves and provide deeper insights into both bone and evo-devo studies.

At present, there is a lack of published methodology explaining the preparation of primary bone cell cultures from the evolutionary relevant cichlids. This article presents a simple and cost-effective protocol for establishing primary cultures from cichlid calcified bone tissues.

5.3 Methods

5.3.1 Six-well plate preparation for primary cell culture

Due to the difficulty in establishing primary cell cultures from these fish, 6-well plates (Corning, USA, catalog number: 3516) are treated with oxygen plasma (for 30 sec).

1) The plates are UV sterilised for 10-15 min (remove the lid).
2) To improve cell adhesion further the plate is coated with PLL solution (Poly-L-lysine, 0.01%, Sigma-Aldrich, catalog number: P4707).
3) 2ml solution is poured into each well and kept for 1 hr with gentle shaking to ensure that the coating is done evenly on the culture surface.
4) The solution is then discarded and rinsed twice thoroughly with sterile plain PBS.

5.3.2 Collection of bone material

Jaw bone samples, including the mandible, maxillae, and premaxillae, were collected from juvenile fish using sterile instruments. While collecting, care must be taken to ensure that the tissue is free of mucus and surface contaminants.

1) Juvenile cichlids (*Labeotropheus fuelleborni* and *Maylandia zebra*) are reared at 28˚C in 40-gallon tanks. Fish are fed thrice a day with crushed algae flakes (Aquadip) for up to 2 months, followed by floating algae pellets (Hikari Cichlid Excel mini, United Kingdom).
2) Adult (8-10 months) disease-free individuals are selected and euthanised with benzocaine (10g benzocaine powder, Sigma Life Science, diluted in 950ml ethanol and 50ml water) diluted 1:50 in fish water.
3) 70% ethanol is sprayed all over the fish body (including the mouth and buccal cavity) to remove surface contaminants like bacteria and fungi.
4) Calcified tissues (lower and upper jaws) are collected using sterile instruments (scalpels, scissors, and forceps), cleaned, and then transferred to 1.5 ml Eppendorf tubes containing 1ml filter-sterilised PBS (Dulbecco's Phosphate Buffered Saline 1x, Gibco; pH 7.0-7.3, catalog number: 14190-094) supplemented with 5% antibiotic (Penicillin-Streptomycin Sigma-Aldrich, catalog number: P0781) and antimycotic (Amphotericin Gibco, catalog number: 15290026).
5) Collected bone tissues are washed 3 x 5 min with PBS supplemented with antibiotic antimycotic solution and then minced into small fragments using sterile scalpel blades (Swann-Morton No. 23 carbon steel, catalog number: 0110) **(Figure 5-2)**.

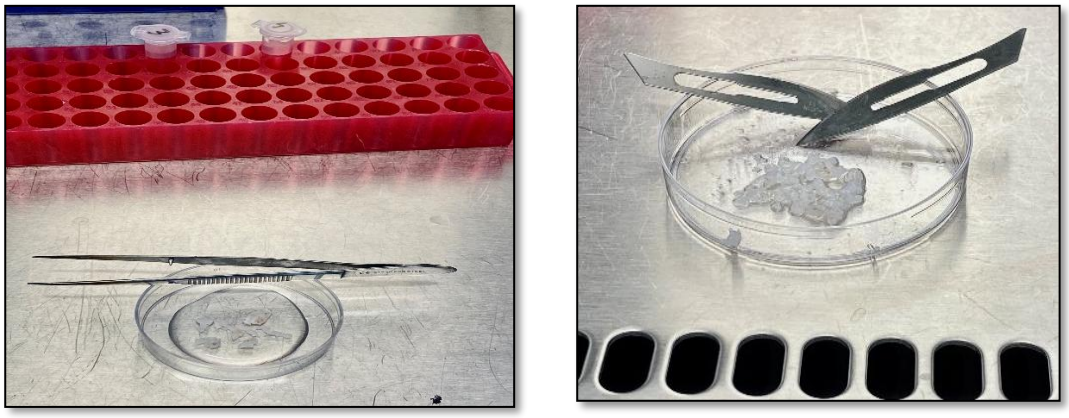
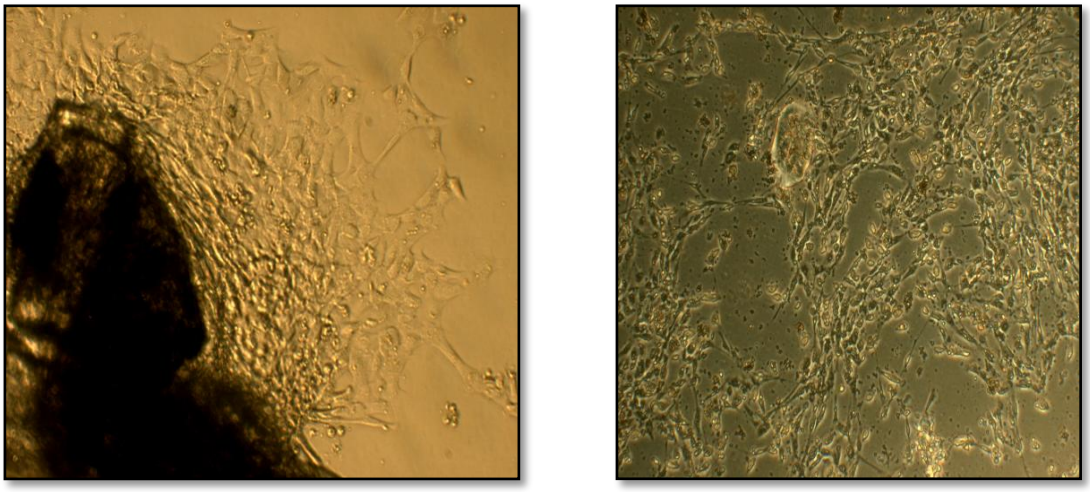


Figure 5-2: Bone tissues minced into small fragments using sterilised instruments.

5.3.3 Initiation of bone cell cultures

Following the detailed steps above, the minced tissue is collagenase-treated to digest the collagen fibres in the bone. Then, the tissue is transferred to a culture plate, and cell culture media containing fetal bovine serum and antibiotic-antimycotic solution is added. The cells are allowed to attach to the substratum with routine media exchange. After a few weeks, when the cells are confluent, healthy, and contamination-free, they are subcultured using trypsin-EDTA to detach them from the plastic surface and re-seed them for expansion or setting up experiments.

1) The fragments are treated with collagenase type II (Gibco, 1000-1200 IU/ml, catalog number: 17101015) for 2 hours at 28˚C with gentle agitation. This step is crucial, as it facilitates the efficient digestion of the collagen matrix, enabling cells to detach easily from the explants.
2) The digestion solution is discarded, and the fragments are washed twice with sterile PBS.
3) These fragments are moved to the PLL-coated six-well plates.
4) 2ml of DMEM culture medium (Dulbecco's modified Eagle's medium, Gibco, catalog number: 21969-035) supplemented with 20% FBS (Fetal bovine serum, Gibco, catalog number: A3382001) and 1% antibiotic-antimycotic solution is poured into the wells, and the fragments are transferred into them.
5) The explants are incubated at 28˚C (CO_2 incubator for DMEM) to allow the cells to leave the bone fragments and attach to the substratum for 2-4 weeks **(Figure 5-3)**. A high fragment density is maintained to help the cells radiate from the explants.
6) 50% exchange of culture media is performed twice weekly to maintain sufficient nutrients and essential growth-promoting factors.



(A) (B)

Figure 5-3: (A) Once the bone fragments are transferred to the culture plates, they are incubated at 28˚C. After 14-28 days, cells radiate from the bone fragments **(B)** Once the cells become confluent, they are passaged and transferred to culture flasks.

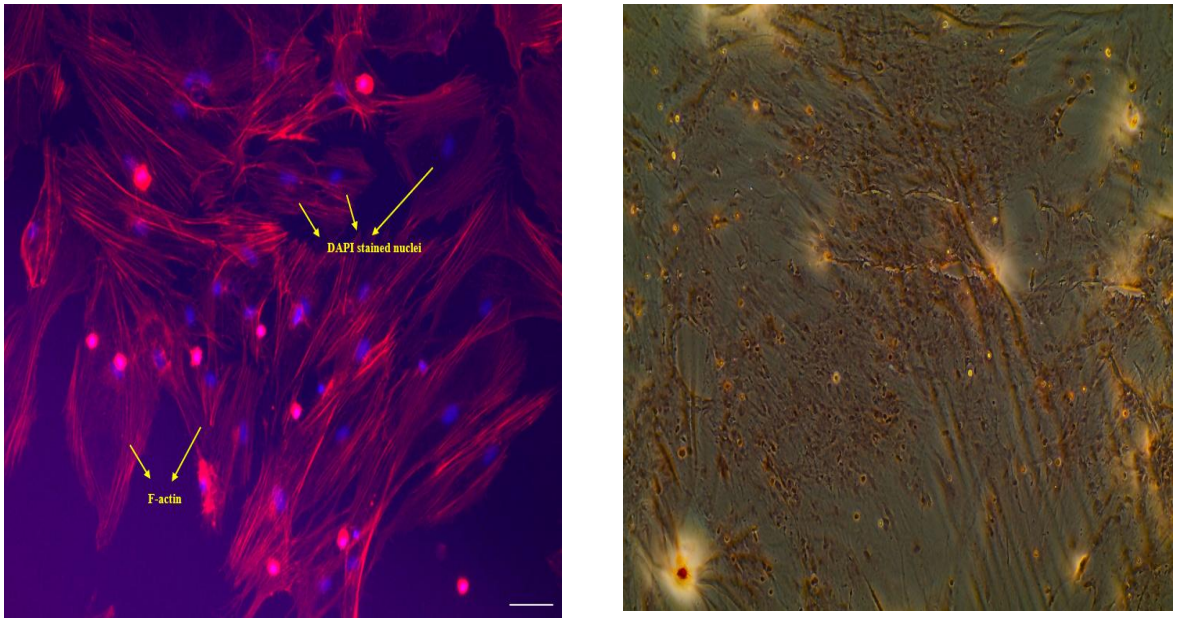
5.3.4 Verification of mineralisation

Testing of bone mineralisation in bone cells can be performed using chemical induction (osteogenic) media. The osteogenic media is prepared by supplementing DMEM with 100Nm dexamethasone (Sigma-Aldrich, catalog number: D2915), 10mM β-glycerophosphate disodium salt hydrate (Sigma-Aldrich, catalog number: G9422), and 200µM L-ascorbic acid-2-phosphate sesquimagnesium salt hydrate (Sigma-Aldrich, catalog number: A8960), which together induce the expression of bone-specific markers and facilitate the deposition of calcium phosphate minerals in the extracellular matrix. The cells are cultured in osteogenic media for 2- 3 weeks, and the media is exchanged 50% twice a week.

5.3.5 Fixation and staining

1) The culture media is removed, and the cells are washed twice with PBS. Cells are then fixed with 10% formaldehyde at 37˚C for 30 mins. Following fixation, the formaldehyde is removed, and the cells are rinsed with PBS.
2) Cells are permeabilised by adding 0.5 ml of PERM buffer (Invitrogen, ThermoFisher Scientific, USA, catalog number: 00-8333-56) and kept at 4˚C for 4 mins.
3) Blocking is performed using 1 ml BSA and incubated at 37˚C for 5 mins.

4) Cells are then incubated with 100 µl of phalloidin (Invitrogen, ThermoFisher Scientific, USA, catalog number: A12381) to visualise actin filaments (F-actin) (1:1000 dilution) for 60 mins. After staining, cells are washed thrice with wash buffer (PBS + 0.5% (v/v) Tween© 20 (Sigma-Aldrich, catalog number: P1379).
5) Next, 10 µl of DAPI (Invitrogen, ThermoFisher Scientific, USA, catalog number: 62248) is added for nuclear counter staining, and cells are imaged using a fluorescence microscope **(Figure 5-4a)**.
6) Lastly, Alizarin Red S staining is performed to assess calcium deposition and confirm bone matrix mineralisation. Cells are incubated with Alizarin Red S solution (2% w/v, pH 4.2) (Sigma-Aldrich, catalog number: A5533) at room temperature for 20–30 minutes, followed by multiple washes with PBS to remove excess dye. Calcium deposits appear as red staining under the microscope **(Figure 5-4b)**.



(a) **(b)**

Figure 5-4:(a) Fluorescent microscopy (EVOS M7000, ThermoFisher). Cells stained with Actin and DAPI. The actin staining targets the filamentous actin, which is shown in red. This highlights the cell shape and morphology. DAPI stains the nuclei of the cells, which are shown in bright blue (Scale bar = 50µm) **(b)** Alizarin red S stain is used to detect calcium deposits and test for bone mineralisation (20x mag).

5.3.6 Long-term storage and reconstitution

The cells are cryopreserved in liquid nitrogen for long-term storage. This is done to maintain their viability, preserve the original cell phenotype. They can be thawed as needed.

1) Once the cells reach confluency, they are subcultured using trypsin-EDTA (Sigma-Aldrich, catalog number: T4049).
2) After 2-3 passages, the cells are trypsinised and centrifuged (4 min at 1400g). The supernatant is discarded, and the cell pellet is dissolved in the cryogenic media (containing 10% DMSO (ThermoFisher Scientific, catalog number: D-4120-PB08, 60% FBS and 30% DMEM).
3) Next, the cell suspension is aliquoted into 1 ml cryogenic tubes, transferred into Mr. Frosty and stored at -80˚C overnight. Later, these tubes are immersed in liquid nitrogen for indefinite storage (slow-freezing method).
4) To thaw the cells, the cryogenic tubes are removed from the liquid nitrogen and rapidly thawed at 37˚C in a water bath (quick thawing). Once the cryogenic media containing the cells have turned into liquid, the tubes are disinfected with 70% ethanol, and the cells are transferred into a six-well plate containing fresh DMEM.

5.3 Conclusion

Further research on bone structure, function and physiology is required to enhance our comprehension of bone biology. This should not only pertain to clinical research but must also extend towards ecological and evolutionary studies. The traditional understanding of the critical role of osteocytes in orchestrating bone formation and remodelling is being re-evaluated (Shahar & Dean, 2013; Atkins et al., 2015; Davesne et al., 2019). This is because of the way the anoesteocytic teleosts exhibit bone remodelling in the absence of the osteocytes, challenging the notion that osteocytes have an indispensable role in this. Studies have suggested that osteocytes play a fundamental role in mineral homeostasis (via osteocytic osteolysis), and their significance is less pronounced in detecting mechanical loading and bone reshaping. To better our understanding of anosteocytic bones, more studies on teleosts should be conducted, as they are the largest group representing anoesteocytic bone cells. Investigating the bone structures of African cichlids could be promising, as this group of teleost species has high evolutionary relevance and exhibits bone remodelling responses when subject to mechanical stress. This will provide valuable insights into the evolutionary and functional aspects of bone adaptation, which may have broader implications for understanding bone biology across vertebrates, including humans.

Chapter 6: General discussion

6.1 Summary of thesis

In this thesis, I have investigated various sources of variation that are central to evolutionary diversification. This included phenotypic plasticity, which is the ability of a developmental system to produce multiple phenotypes in response to environmental conditions. The extent to which plasticity generates evolutionary variation depends on the nature of the underlying reaction norm, while flexible and environmentally sensitive reaction norms can introduce novel phenotypic variation that may facilitate adaptive divergence, more canalised reaction norms may instead constrain phenotypic outcomes. Accordingly, phenotypic plasticity can act either as a generator of variation or as a source of constraint, depending on how developmental responses are structured (West-Eberhard, 2003; Pfennig et al., 2010). Plasticity itself is a trait with an underlying genetic architecture that can respond to selection. Next, I examined how a specific mutation in a gene (*zeb1*) contributes to variation in craniofacial morphology. Lastly, I considered both types of variation and investigated genotype-by-environment (GxE) interactions in craniofacial traits. Although statistical support for G×E interactions was limited, the analyses suggested patterns of variation in reaction norms for craniofacial shape, indicating that these traits may have the potential to evolve under selection. However, given the lack of strong statistical support, these findings should be viewed as indicative rather than conclusive evidence linking G×E to the evolution of plasticity (Via & Lande, 1985; Pigliucci, 2001).

Chapter 2 demonstrates plastic changes in the craniofacial region of eight representative species of Lake Malawi cichlids. The findings reveal higher phenotypic plasticity in ecological generalists occupying intermediate positions along the biting–suction ecomorphological axis, which decreases towards the extremes where obligate suction or biting specialists are placed. This pattern is consistent with the prediction that canalisation of a trait occurs with an increase in specialisation (Waddington, 1953; West-Eberhard, 2003; Levis & Pfennig, 2019). At a molecular level, RNA-seq highlights that gene-expression plasticity also generally decreases with an increase in specialisation.

Chapter 3 identifies the transcription factor *zeb1* as a key modulator of mandibular shape linked to trophic diversification and ecological specialisation. The results show that a

mutational change in the *zeb1* gene causes variation in gene expression and craniofacial variation.

Chapter 4 examines a candidate gene for morphological plasticity, *wnt7ba* and elucidates that a specific genotype-by-environment interaction may contribute to variation in the craniofacial morphology leading to adaptive divergence.

Chapter 5 introduces a novel protocol for establishing primary bone cell cultures from African cichlids. This working protocol fills a significant gap in bone biology by examining the structure and function of bones in species lacking osteocytes. Conducting direct *in vitro* cultures of cichlid bone cells will provide new opportunities for future research to study cellular plasticity in adaptive radiation and understand the mechanisms underlying this plasticity.

6.2 Exploring mechanisms of adaptive variation

6.2.1 Phenotypic plasticity

The environment is known to play a significant role in the evolution of adaptive variation (Laland et al., 2014; Parsons et al., 2016). In this context, phenotypic plasticity has emerged as a key phenomenon and is recognised as a progenitor of variation that allows populations to develop adaptive phenotypes in different environments. This leads to the emergence of new, complex traits and ecological niches (Pfennig et al., 2010; Moczek et al., 2011; Moczek et al., 2015). Adaptive divergence is traditionally studied through a genetic perspective, focusing on how selection acts on standing genetic variation (Schluter, 1996; Schluter & Conte, 2009; Seehausen et al., 2014). However, phenotypic plasticity can enable populations to explore diverse ecological niches, potentially directing evolutionary paths by influencing which genetic differences are expressed through the phenotype to become fixed (Levis & Pfennig, 2019). Therefore, including plasticity in models of adaptive divergence may offer valuable insights into rapid adaptation and the potential for evolutionary novelty (Pfennig et al., 2010). A significant concept here is genetic assimilation (Waddington, 1953; West-Eberhard, 2003; West-Eberhard, 2005), which predicts that phenotypes initially induced by environmental cues can become canalised. This evolutionary process allows initially plastic traits to stabilise and be refined through natural selection. A trait may evolve to become either more or less sensitive to environmental changes, enabling organisms to optimise trait variation in response to environmental fluctuations (Ghalambor et al., 2007). However,

plasticity does not invariably promote evolutionary divergence. A contrasting prediction holds that plasticity can buffer genetic variation through phenotypic expression. When plastic responses are sufficient to maintain fitness under environmental variation, the genetic variance available for selection to act upon is reduced, diminishing the opportunity for evolution (Ancel, 2000; Price et al., 2003). Paenke et al. (2007) demonstrated that while moderate plasticity may facilitate genetic assimilation, high plasticity consistently slows the rate of genetic evolution by returning phenotypes toward optima through non-genetic means (Lande, 2009). Furthermore, when plastic responses buffer individuals against environmental variation, selective pressures on underlying genetic variants are reduced, meaning that environmentally induced phenotypes can mask genetic variation, slowing or preventing genetic divergence and potentially constraining evolutionary responses (Crispo, 2008; Ghalambor et al., 2007). Non-adaptive plasticity, where plastic responses move phenotypes away from fitness optima, selective pressures may instead increase, generating mismatches between phenotype and environment (Ghalambor et al., 2007). Together, these findings highlight that plasticity can both facilitate and constrain adaptation, depending on whether plastic responses are adaptive or non-adaptive, and the degree to which they align with prevailing selective pressures. These contrasting predictions that plasticity as a progenitor of divergence versus plasticity as a buffer against it are not mutually exclusive, and the conditions under which each predominates remain an active area of investigation (Paenke et al., 2007; Levis & Pfennig, 2019).

In chapter 2, I found evidence showing that plasticity decreases with specialisation (Skulason et al., 2019). Plasticity in craniofacial morphology was greater in generalist species occupying intermediate positions along the biting–suction ecomorphological axis, and declined towards both ends of the continuum, where the specialist biters and suckers are found (Schluter, 2000; Pfennig et al., 2010). These patterns support the prediction that generalists maintain higher phenotypic plasticity to cope with variable environments, whilst specialists display canalised morphologies adapted to stable, narrow ecological niches via genetic assimilation (Pigliucci & Murren, 2003; Schneider & Meyer, 2017). This trend was consistent in both the major ecologically diverged groups- rock and sand dwellers, which makes a compelling case that the results robustly support the theoretical prediction. By linking ecological specialisation to the loss of plasticity, this chapter contributes to a growing body of work emphasising the role of developmental processes, such as plasticity and genetic assimilation, in shaping adaptive radiation (West-Eberhard, 2005; Aubret & Shine, 2009; Levis & Pfennig, 2019).

6.2.2 Genetic basis of phenotypic plasticity

While phenotypic plasticity is now widely recognised as a central component of the evolutionary process, we still have very little understanding of the genetic underpinnings of plasticity in natural systems undergoing rapid adaptive diversification (Gibert, 2017). The genotype is a key predictor of phenotypic variation and thus the form-function relationships that are essential for adaptive divergence.

In chapter 2, I address this gap by examining the genetic basis of phenotypic plasticity within one of the largest known adaptive radiations, the Lake Malawi cichlids. Although there is literature on the genetic basis for diet-induced morphological traits in cichlids (Albertson et al., 2005; Cooper et al., 2011; Parnell et al., 2012; Parsons et al., 2015, 2016; Hu & Albertson, 2017; Navon et al., 2020), there are not many studies that have explicitly examined how these factors contribute to plasticity in an adaptive radiation. My work extends beyond studying external morphological phenotypes to include molecular phenotypes, aiming to gain a better understanding of canalisation and the mechanisms by which variation occurs and responds to selection. The transcriptomic analyses from RNA sequencing revealed variation in diet-induced gene expression plasticity, which were in correspondence with the patterns observed in the outward craniofacial plasticity. The results revealed a high number of differentially expressed genes in the generalist species, which decreased in the specialised species. These findings support the view that plasticity at the molecular level can be an important mechanism facilitating genetic assimilation. Here, I speculate that several gene pathways respond to diet-induced mechanical stress and contribute to craniofacial plasticity, which is essential for varied feeding strategies in these species.

Chapter 4 also explores the genetic basis of adaptive plasticity by studying a candidate gene, *wnt7ba*. The Wnt/β-catenin signalling pathway is a key regulator of craniofacial development in rapidly diversifying cichlids (Parsons et al., 2014). Building on QTL mapping in Lake Malawi cichlids that revealed *wnt7ba* near a genomic region of strong allelic divergence, I used zebrafish mutants to examine how *wnt7ba* influences craniofacial morphology under different feeding regimes. Cichlids and zebrafish belong to distinct orders of teleosts that diverged roughly 300 million years ago (Near et al., 2012), yet both exhibit a remarkably conserved mode of craniofacial form mediated by Wnt signalling (Parsons et al., 2014). By linking a candidate gene to diet-dependent morphological outcomes, this work advances beyond merely studying phenotypic variation to identify a molecular mechanism

underlying adaptive plastic responses, thus providing a deeper understanding of the genetic architecture that can evolve and promote diversification. I studied the wild type and mutant genotypes and analysed the genotype-by-environment interaction using diet-induced plasticity experiments. The results showed no significant genotype by diet effect on shape, but this should not be taken as conclusive evidence of no effect, as the sample size for this experimental setup was small. Trajectory analysis indicated a greater magnitude of diet-induced shape differences among mutants, consistent with evidence in the deformation grids, suggesting regulation of plasticity by the *wnt7ba* gene. Building on this foundation, a logical next step would be to increase the sample size and explore the subtle effects of *wnt7ba* in contributing to ecologically relevant craniofacial variation.

6.2.3 Variation driven by mutational change

Chapter 3 builds on a previous QTL study that found evidence of divergence in *zeb1* by examining fine-scale phenotypic variations between two ecologically similar cichlid species (McWhinnie, 2020). To further evaluate *zeb1* as a candidate gene, I compared its expression in *Labeotropheus fuelleborni* (LF) and *Tropheops* sp. 'red cheek' (TRC). My qPCR results revealed a significant difference in *zeb1* expression. This pattern indicates that *zeb1* plays a key role in shaping species-specific mandible formation and is a potential driver of morphological divergence linked to trophic diversification and ecological specialisation. Although earlier studies have investigated cichlid mandible variation using a QTL approach (Albertson et al., 2005; Parsons & Albertson, 2009), focusing on a single, functionally plausible candidate gene provides a more precise understanding of how specific regulatory changes contribute to craniofacial evolution (McWhinnie, 2020).

The second part of this chapter looked at wild-type and *zeb1* zebrafish mutants. This integrative approach was performed to get a better understanding of the influence of *zeb1* on mandible shape variation. Although the overall shape variation did not differ significantly between genotypes, deformation grids showed more warping in the anterior region (including the mandible) of the *zeb1* mutant zebrafish, pointing towards higher variation in that region. Mutations are a source of novel genetic variation leading to evolutionary change (Eyre-Walker & Keightley, 2007). Although most mutations are deleterious, some confer advantageous effects that natural selection can act upon, enabling populations to adapt and evolve (Barrett & Schluter, 2008). This chapter provides evidence of how *zeb1* correlates with jaw-shape variation and trophic specialisation, illustrating how a specific regulatory mutation can influence ecologically relevant morphology. Furthermore, craniofacial skeletal

development is highly conserved across vertebrates, so insights gained from cichlids and other adaptive radiations are likely relevant and broadly applicable to other vertebrates as well (Powder & Albertson, 2016).

6.2.4 Plasticity and its genetic basis in cellular mechanisms

Beyond morphological plasticity, this thesis also explores bone cellular plasticity as a mechanism of craniofacial variation. Cichlids are an exemplary model of bone remodelling as their craniofacial structures respond to mechanical stimuli (Bouton et al., 2002; Parsons et al., 2016; Navon et al., 2020; McWhinnie et al., 2022). This considerable variation supports evolutionary divergence between biting and suction modes of feeding (Kocher, 2004; Cooper et al., 2010). This adaptability offers extensive evolutionary advantages by creating significant biomechanical variations in feeding, leading to crucial shifts in trophic ecology.

Despite this clear in vivo responsiveness, it remains uncertain whether these changes are driven by cellular mechanotransduction or primarily by the fish's morphology. Culturing bone cells under controlled *in vitro* conditions offers a way to determine whether isolated mechanical stress has a different impact on the species. In the fish overall, other factors like nutritional profile, bone density, muscle loading, and jaw speed might influence the response, whereas in the cellular study, mechanical stress will be the sole factor responsible for the observed changes. While cichlids clearly respond to mechanical stress, inherent *in vivo* conditions may play a role in overall responses. This makes it difficult to directly assess the role of mechanical stress on bone tissue. As craniofacial structures remodel in response to mechanical loading, they serve as an excellent model to investigate plasticity and genetic assimilation at a cellular level. Examining genetic assimilation and specialisation in cichlid osteogenic cultures will offer a robust, explicit test of whether the mechanical stress-induced phenotypes are underpinned by in vitro mechanisms.

In **chapter 05**, I introduce a novel, cost-effective protocol for establishing primary bone cell cultures from cichlids. By isolating primary osteogenic cells from species chosen along the biting-sucking ecomorphological axis, this approach enables characterisation of their mechanotransduction pathways and comparison of osteogenic responses among species with differing trophic ecologies and plasticities. This working protocol fills a significant gap in bone biology by facilitating the study of osteocyte-independent mechanisms of bone formation and remodelling, and provides a valuable framework for linking cellular responses

to mechanical stress with evolutionary patterns of craniofacial variation in both generalist and specialist cichlids.

6.3 Future research directions

The work in **chapter 2** can be broadened in the future by adding additional species from the Malawi ecomorphological axis to get a better understanding of how phenotypic plasticity and the tendency toward genetic assimilation change from the centre of the axis toward its extremes. Expanding the RNA-seq dataset would help better understand how molecular plasticity moves toward genetic assimilation. Furthermore, incorporating analyses of RNA splicing could be another step to gain insights into coding-region variation and how post-transcriptional regulation contributes to plastic responses and their specialisation across species.

Chapter 3 research can be expanded by exploring additional candidate genes (including *zeb1*) using small molecule experiments, where the normal development of the mandible is perturbed by a chemical agonist/antagonist (Parsons et al. 2014). This study has looked at a relevant small-scale adaptive divergence between TRC and LF, but this work could be expanded to include other species from the other end of the Malawi feeding axis, like *Rhamphochromis chilingali* (a specialist suction feeder) or *Maylandia zebra* (a suction-feeding generalist), to comparatively explore *zeb1* further. By expanding and looking at additional species, this would help us further understand how the mandible and craniofacial morphology have diversified (Irschick et al. 2013). *zeb1* is known to induce the expression of BMP inhibitors, like *follistatin (fst)* and *chordin-like (chrdl)* and influence bone formation (Postigo et al., 2003; Liu et al., 2008; Mock et al., 2015). Future work could examine additional BMP antagonists or interacting genes to uncover potential compensatory networks that may have masked expression differences in the current study (Wang et al., 2004; O'Connell et al., 2012).

The findings in **chapter 4** should be regarded as a pilot investigation of *wnt7ba* in diet-induced craniofacial plasticity. The next step could be to increase the sample size to detect genotype–environment interaction and to examine other Wnt pathway components that might interact with *wnt7ba* to mediate plastic craniofacial responses.

Building on the bone cell culture protocol established in **chapter 5**, future work should focus on the response of *in vitro* bone cells isolated from both highly plastic

generalists and specialist biters or suction feeders to mechanical stress to test whether they display a plastic response at the cellular level. If such a response is detected, the next step would be to investigate the genetic basis of cellular plasticity by selecting a panel of genes involved in bone growth and formation and quantifying their expression across species. Finally, future research could extend beyond craniofacial traits to other plastic systems like exploring variation in fin morphology, scale structure, visual system, performance or mate choice to build a more comprehensive picture of how diverse plastic traits and their genetic bases contribute to the remarkable adaptive radiation of Lake Malawi cichlids.

6.4 Conclusion

A central focus for the field of evo-devo is understanding the mechanisms underlying adaptive phenotypes. With a wide range of craniofacial variation that has evolved in the Lake Malawi cichlids over a relatively short time frame, they are an excellent system with which to test and explore this. By using multiple methods, including morphometrics, genetics and cellular work, this thesis has adopted an integrative approach to answer evolutionary questions about adaptive variation.

Appendix: Chapter 2 supplementary materials

Supplementary table 1: Pairwise differences in magnitude of plasticity (Procrustes distance) among species in response to diet treatments ($p < 0.05$ is significant). LF- *Labeotropheus fuelleborni,* TRC- *Tropheops 'red cheek',* MZ- *Maylandia zebra,* LC- *Labidochromis caeruleus*, CB- *Copadichromis borleyi*, AS- *Aulonocara stuartgranti,* DC- *Dimidiochromis compressiceps,* RC- *Rhamphochromis chilingali*

Comparison	d	UCL (95%)	Z	P value
AS: CB	0.00367127	0.09999922	-1.74967073	0.943
AS: DC	0.045592905	0.0883157	0.43376475	0.361
AS: LC	0.146405013	0.16731847	1.28967535	0.096
AS: LF	0.065362845	0.09197182	0.94074054	0.182
AS: MZ	0.061704509	0.1264399	-0.35332261	0.642
AS: RC	0.04036172	0.10430927	-0.06304224	0.547
AS: TRC	0.050166583	0.08943169	0.6852954	0.256
CB: DC	0.041921635	0.05462374	1.12183727	0.145
CB: LC	0.142733742	0.21176063	0.60433476	0.257
CB: LF	0.069034115	0.06371575	1.75805534	0.038
CB: MZ	0.065375779	0.0757041	1.26424507	0.095

LC: LF	0.211767858	0.20101533	1.76006323	0.041
LC: MZ	0.208109522	0.24220358	1.18377874	0.113
LC: RC	0.186766732	0.20607121	1.27331959	0.091
LC: TRC	0.196571596	0.19878798	1.63354625	0.054
LF: MZ	0.003658336	0.09171596	-1.95641951	0.966
LF: RC	0.025001125	0.06756654	0.20326513	0.428
LF: TRC	0.015196262	0.06876473	-0.36496129	0.653
MZ: RC	0.02134279	0.07171952	-0.31312348	0.63
MZ: TRC	0.011537926	0.0935162	-1.69194729	0.949
RC: TRC	0.009804863	0.06909275	-0.84375232	0.798
CB: RC	0.04403299	0.05935214	1.16725189	0.122
CB:TRC	0.053837853	0.06997288	1.23471256	0.106
DC: LC	0.100812107	0.19892235	-0.03489371	0.529
DC: LF	0.11095575	0.06254109	2.67754517	0.002
DC: MZ	0.107297414	0.08093373	2.48110397	0.01
DC: RC	0.085954625	0.05791183	2.28754883	0.01
DC: TRC	0.095759488	0.06272815	2.38708665	0.003

Supplementary table 2: List of DE genes identified in *Labidochromis caeruleus, Copadichromis borleyi, Rhamphochromis chilingali*, and their potential functions.

Gene	Function	Reference
	Labidochromis caeruleus	
nwd1	It is a member of the STAND (signal transduction ATPases with numerous domains) family known to be potentially involved in neurogenesis and neurite outgrowth. It also has a role in lipid accumulation.	Bao et al., 2024 Yamada et al., 2025
	Rhamphochromis chilingali	
aplnra	Has been found to play a role in early morphogenetic cell movements and contributes to cardiovascular development.	Nornes et al., 2009
nmrk1	Is involved in cellular metabolism and energy production.	Fletcher & Lavery, 2018
prr5a	Has been observed in cell proliferation and growth.	Woo et al., 2007
	Copadichromis borleyi	
smc4, smc2	Part of the condensin genes, which play a role in development and cell proliferation.	Mönnich et al., 2009
tacc3	Aids in the alignment of chromosomes during mitosis and is responsible for microtubule outgrowth.	Booth et al., 2011
hlfa	Encodes a transcription factor involved in circadian rhythms.	Idda et al., 2012; Zhao & Fent, 2016
ncapd2	Role in cell division, involving mitotic chromosome condensation and segregation.	Seipold et al., 2009
qdpra	Plays a role in melanin synthesis and neurotransmission.	Breuer et al., 2019
dnajc4	It is a member of the DnaJ heat shock protein (Hsp40) family, which plays a role in protein folding and cellular stress regulation.	Song et al., 2014; Yan et al., 2021
hunk	Enables protein serine/threonine kinase activity and is involved in intracellular signal transduction.	Kritikos et al., 2025
mylk5	Encodes for a group of enzymes called myosin light chain kinases (MLCKs), crucial for muscle contraction and other cellular processes.	Stull et al., 2011 ; Martinsen et al., 2014
rfc5	Involved in DNA replication and repair.	Cai et al., 1996 ; Park et al., 2024
kif15	Plays a role during cell division and microtubule spindle formation.	Tanenbaum et al., 2009; Brouwers et al., 2017
lmo7a	Regulates actin cytoskeletal dynamics and Wnt signalling during neural crest cell migration.	Ott et al., 2008 ; Tatarakis et al., 2020
inab	Plays a role in neuron development and regeneration.	Van Ryswyk et al., 2014

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