



## Molecular investigation of mechanical strain-induced phenotypic plasticity in the ecologically important pharyngeal jaws of cichlid fish

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### Summary

Phenotypic plasticity in the form of alterations to teleost skeletons can result from a range of environmental factors, such as the hardness of the prey, particularly when exposure occurs early during development. Determining the molecular underpinnings of teleost skeletal plasticity is hampered by a limited understanding of the molecular basis of bone remodeling in derived teleost fish, whose bones are acellular, lacking the cell type known to orchestrate bone remodeling in mammals. We are using a fitting molecular model for phenotypic plasticity research: the East African cichlid *Astatoreochromis alluaudi*, with the aim to shed light on the molecular basis of phenotypic plasticity and on the remodeling of acellular bones. For this fish, sustained ingestion of a hard diet induces a ‘molariform’ lower pharyngeal jaw (LPJ), with molar-like teeth set in an enlarged, relatively dense jaw, while a softer diet results in a smaller, finer ‘papilliform’ LPJ morphology, representing the ‘ground state’ for this species. Through comparing genome-wide transcription in molariform and papilliform LPJs, our previous research has shed light on the molecular basis of phenotypic plasticity in the teleost skeleton and by extension, on acellular bone remodeling. In this manuscript we construct a model for the molecular basis of mechanically induced skeletal plasticity in teleosts, which involves iterative cycles of strain and compensatory cellular proliferation. Furthermore, we propose a framework for testing the potential influence of phenotypic plasticity and genetic assimilation on adaptive radiations.

### Mechanically-mediated phenotypic plasticity in the teleost skeleton

The myriad skeletal forms displayed by teleost fish is the product of their evolutionary past and ecological present, through interactions between both genetic and epigenetic factors that act in concert to orchestrate ontogeny. An inherent property of vertebrate skeletons is their ability to respond to mechanical strain, which results in a better match between the strength and size of skeletal elements, to the physical forces that act upon them. An idea first

posited by Julius Wolff in 1892, the law of bone remodeling, has been subsequently supported by a considerable amount of evidence, both empirical and theoretical in nature (Wolff, 1892; Chamay and Tschantz, 1972; Frost, 1990; Mullender and Huiskes, 1995; Vieira et al., 2013) but also see Pearson and Lieberman (2004). Numerous instances of phenotypic plasticity of the teleost skeleton, both adaptive and maladaptive, have been attributed to the action of mechanical strain. Maladaptive deformities known to cause economic losses in aquaculture include spinal bending (lordosis and kyphosis), which results from neuromuscular influences (Gorman and Breden, 2007) and excessive swimming activity (Kihara et al., 2002), and various fusions and malformations associated with mechanical overload and accelerated growth (Witten et al., 2009). On the other hand, putatively adaptive characteristics such as directional mouth asymmetry (Van Dooren et al., 2010) and pharyngeal jaw robustness and tooth size and shape in cichlids (Muschick et al., 2011; Gunter et al., 2013) result from mechanical strain due to food ingestion. Additionally, water velocity and exercise, which also exert mechanical stress on the skeleton, influence overall body shape in fish such as salmon, trout (Pakkasmaa and Piironen, 2001) and pumpkinseed sunfish (Robinson and Wilson, 1996; Yavno and Fox, 2013), in addition to influencing the rate of ossification in the skeleton (Pakkasmaa and Piironen, 2001; Grünbaum et al., 2012).

Despite the abounding evidence that mechanical strain remodels the teleost skeleton, similar to mammals, little is known of its cellular and molecular mechanisms (Witten and Huysseune, 2009; Fiaz et al., 2010). Specifically, fundamental cellular differences exist between the bones of neoteleosts and mammals – neoteleosts lack osteocytes, the cell type that evidently co-ordinates bone remodeling amongst tetrapods (Witten and Huysseune, 2009). Thus, through understanding the molecular basis of strain-mediated bone remodeling in higher teleosts we can gain insight into the basis of phenotypic plasticity that is of both adaptive and economic importance. Additionally our research provides vital information on the role of osteocytes in bone development, as neoteleosts clearly achieve bone remodeling in the absence of this cell type, which is supposedly indispensable for efficient bone remodeling in mammals (Bonewald, 2011).

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### Remodeling cellular vs acellular bone

Mammalian bones, far from being static or dead tissues, are peppered with osteocytes: living cells that are housed in canaliculi, microscopic fluid-filled canals that perforate bone (Bonewald, 2011). At localised areas of bones that have been exposed to mechanical strain, fluid is forced through the canaliculi causing shear stress on the membranes of osteocytes inducing a molecular cascade that increases the proliferation of osteoblasts (Thompson et al., 2012). The increased activity of osteoblasts causes a local increase in bone deposition in the regions that receive the highest amount of strain (Robling et al., 2006), at the expense of regions that receive less strain where bone may be resorbed by osteoclasts. This localised deposition and resorption of bone through the combined activity of osteoblasts and osteoclasts leading to alterations in bone shape, is termed *bone modeling*. If the strain magnitude exceeds the bone's resistance, damage such as microcracks can occur inducing *bone remodeling*, a process that involves concurrent bone resorption and deposition in discrete bone regions through the co-ordinated action of osteoclasts and osteoblasts (Currey, 2002; Dean and Shahar, 2012).

As molecular signals that are critical to bone modeling and remodeling originate from osteocytes and higher teleosts lack this cell type, they must use an alternative mechanism to sense mechanical strain and induce osteoblast and osteoclast proliferation (Dean and Shahar, 2012; Shahar and Dean, 2013). While it has been clear for some time that acellular teleost bones respond to mechanical strain (Meyer, 1987; Huyseune et al., 1994; Day and McPhail, 1996; Hegrenes, 2001; Kranenbarg et al., 2005), it was only recently recognised that this is in part due to remodeling rather than purely modeling (Currey and Shahar, 2013). Additionally, while it was previously considered that only osteocytes extend their cell membranes into bone, it was subsequently demonstrated that the bone-lining osteoblasts can have cytoplasmic extensions that permeate deep into the bone (Sire and Meunier, 1994). This provides a putative mechanism for sensing associated changes in fluid flow and suggests that while neoteleosts specifically lack osteocytes, they may use alternative cell types and achieve similar remodeling outcomes as species with cellular bones.

### Transcriptional basis of skeletal remodeling in a modern teleost

In spite of the economic and evolutionary importance of skeletal plasticity in neoteleosts, little is known of its molecular basis. Our research has recently addressed this topic in the pharyngeal jaws of the cichlid fish, *Astatoreochromis alluaudi*: a model for phenotypic plasticity research for the last 50 years (Greenwood, 1965; Hoogerhoud, 1986a,b,c; Huyseune et al., 1994; Huyseune, 1995; Smits, 1996; Smits et al., 1996; Gunter et al., 2013). Notably, this species displays two morphs in the wild (Hoogerhoud, 1986a; Huyseune et al., 1994; Huyseune, 1995), a molariform morph with hypertrophied pharyngeal jaws with large, molar-like teeth, adapted to crushing hard-shelled molluscs (Greenwood, 1965; Witte, 1981) and a papilliform morph with more slender LPJ better adapted to chewing softer food items such as insects (Meyer,

1989; Sloomweg et al., 1994) (Fig. 1). These two morphs are largely the product of diet-induced phenotypic plasticity, where the sustained ingestion of a hard diet (from ~30 mm SL) induces a molariform LPJ, while a soft diet results in a papilliform LPJ (Greenwood, 1959, 1965; Hoogerhoud, 1986a,b; Smits, 1996; Smits et al., 1996) (Table 1). The molariform morph displays increased trabecular bone density (Huyseune et al., 1994) and progressively larger generations of replacement teeth (Huyseune, 1995), leading to the hypothesis that the molariform morph develops through increased mechanical strain on the jaws as a result of the different diets. Further support for this hypothesis came from finite element analysis of the LPJs of a neotropical cichlid, which demonstrated that the region receiving the highest mechanical strain during food intake was also the densest and bore the largest teeth (Hulseay et al., 2008).

While the morphological implications of diet-induced LPJ plasticity have been well characterised, its molecular basis was only recently investigated. To achieve this objective, we conducted a transcriptome analysis of phenotypic plasticity in the *A. alluaudi* LPJ, comparing genome-wide gene expression in molariform and papilliform LPJs (Gunter et al., 2013). Our investigation identified 187 differentially expressed transcripts, which shed light on teleost skeletal plasticity and by extension the molecular basis of acellular bone remodeling. Here we have used these results to construct a model of the molecular basis of mechanically induced skeletal plastic-

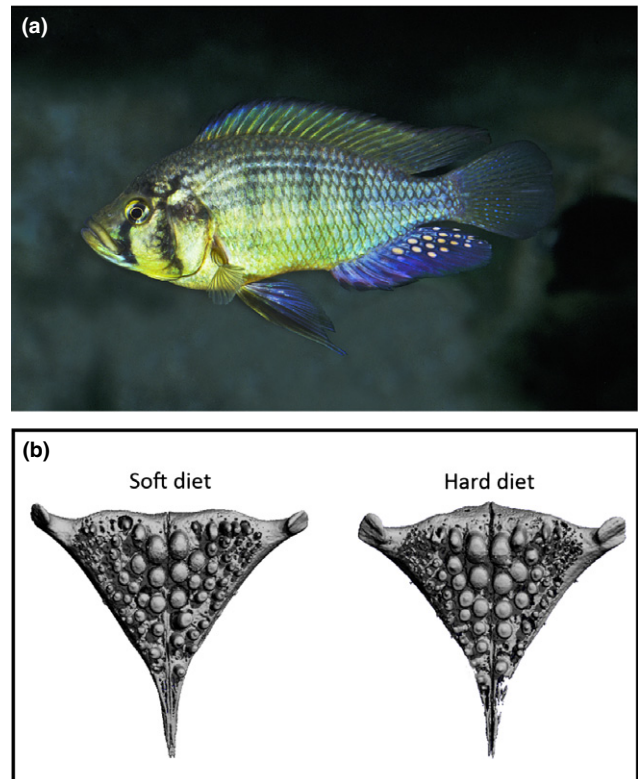


Fig. 1. Diet-induced adaptive plasticity in the lower pharyngeal jaws (LPJ) of the cichlid *Astatoreochromis alluaudi*. (a) *A. alluaudi* mature male. (b) Ingestion of either hard-shelled snails, or soft, pulverised snails induces the development of alternative LPJ morphs, characterised by differences in tooth size, jaw size and jaw shape

Table 1  
Mechano-responsive pathways expressed in mammalian bones, compared to our analyses on the LPJ of a cichlid fish

Gene Class	Mammalian	Cichlid
AP-1	Mantila Roosa et al. (2011b) Xing et al. (2005)	Gunter et al. (2013)
Apoptosis	Mantila Roosa et al. (2011b), Xing et al. (2005)	Gunter et al. (2013)
Calcium signalling	Mantila Roosa et al. (2011b), Xing et al. (2005)	Gunter et al. (2013)
Cell cycle	Mantila Roosa et al. (2011b)	Gunter et al. (2013)
Chemokine	Mantila Roosa et al. (2011b), Xing et al. (2005)	Gunter et al. (2013)
Cytokine	Mantila Roosa et al. (2011b), Xing et al. (2005)	Gunter et al. (2013)
Cytoskeleton	Xing et al. (2005)	Gunter et al. (2013)
Growth factors	Mantila Roosa et al. (2011b), Xing et al. (2005)	–
Heat shock proteins	Xing et al. (2005)	Gunter et al. (2013)
Ion Channel	Mantila Roosa et al. (2011b)	Gunter et al. (2013)
Matrix	Mantila Roosa et al. (2011b), Xing et al. (2005)	Gunter et al. (2013)
Muscle	Mantila Roosa et al. (2011b), Xing et al. (2005)	Gunter et al. (2013)
Neurotransmitter Signal transduction	Mantila Roosa et al. (2011b) Mantila Roosa et al. (2011b), Xing et al. (2005)	– Gunter et al. (2013)
Solute carrier	Mantila Roosa et al. (2011b), Xing et al. (2005)	Gunter et al. (2013) <sup>a</sup>
Tgf- $\beta$ signalling	Mantila Roosa et al. (2011b)	Gunter et al. (2013) <sup>b</sup>
Wnt/ $\beta$ -catenin signalling	Mantila Roosa et al. (2011b)	–

<sup>a</sup>Differential expression of solute carriers was detected in less stringent RNA-seq statistical analyses.

<sup>b</sup>Differential expression of *bmp2*, a member of the Tgf- $\beta$  family, was detected using qRT-PCR but not RNA-seq.

ity and to make predictions about the role of phenotypic plasticity in evolution.

#### Transcription during remodeling of teleost acellular bones

Importantly, our study has shed light on the molecular processes that underlie mechanical strain induced remodeling of teleost acellular bones, which are strikingly similar to those that remodel mammalian cellular bones (Xing et al., 2005; Mantila Roosa et al., 2011a,b). For example molariform LPJs showed an increased expression of several immediate early genes (*c-fos*, *ier2*) and calcium pathway genes (*ryanodine* and *annexin*). Overexpression of *c-fos* may indicate an enhanced proliferation of osteoclasts, as this gene is a key osteoclast determinant in mammals (Grigoriadis et al., 1994). In concert with this, we observed the overexpression of genes involved with osteoblast proliferation and differentiation (*osx* and *runx2b*), which in light of the putatively increased proliferation of osteoclasts suggests that development of molariform LPJs involves active bone remodeling. Cross-talk is likely to have occurred between osteoblast and osteoclast

differentiation pathways as, for example, *runx2* and *c-fos* functionally interact during mechanical strain enabling co-ordinated activity of osteoblasts and osteoclasts (D'Alonzo et al., 2002). Moreover, we detected the upregulation of *cx43*, a gap junction gene that regulates communication between osteocytes and osteoblasts in mammalian bones (Su et al., 1997; Taylor et al., 2007), demonstrating the importance of gap junction communication in the remodeling of acellular bones. It should be noted that *cx43* is also associated with tooth development (About et al., 2002) and response to tooth damage (Mitsiadis and Rahiotis, 2004), so confirming its precise role requires further spatial investigations. Last, various lipid pathway genes were downregulated in molariform LPJs, suggesting that increased numbers of mesenchymal cells may be recruited at a cost to adipose cells (Beresford et al., 1992) that fill the medullary cavities of teleost bones (Witten and Huysseune, 2009).

Several genes involved in the immune response were significantly downregulated in molariform LPJs, as shown by our functional annotation analyses, which indicated the overrepresentation of terms such as immune response (7 of 27 genes), response to wounding (5 of 27) and inflammation (4 of 27). This observation is consistent with the proposal that the inflammatory response influences teleost skeletal deformities (Gil-Martens, 2010) and with Vieira et al. (2013), who detected the expression of various immune-related genes in the bones of gilthead sea bream in response to starvation stress. Moreover, human studies have demonstrated the altered expression of inflammatory genes in response to aerobic exercise (Bruunsgaard, 2005). As immune cell lineages such as macrophages and osteoclasts share a common precursor (hematopoietic stem cells), our observation suggests that molariform LPJs may display a shift in stem cell differentiation that favours osteoclasts over macrophages (Yin and Li, 2006). This may be of particular importance for the eruption of larger molariform teeth in the hard diet individuals, as the process of tooth eruption requires extensive bone remodeling and teeth are replaced approximately every month for this species (Huysseune, 1995). The proposed shift in hematopoietic stem cell differentiation may be driven in part by the overexpression of *c-fos*, as mouse *c-fos* knock-outs display an overabundance of macrophages and a dramatic reduction in osteoclasts (Yang and Karsenty, 2002).

#### A model for transcriptional basis of plasticity in the LPJ of a cichlid fish

The pathways identified by our study have enabled the construction of a model for the molecular basis of strain-mediated remodeling of the LPJ of *A. alluaudi* (Fig. 2), which may be relevant to the remodeling of teleost bones more generally. We predict that the genes identified by our study form an integrated network that both responds to mechanical strain and subsequently induces a downstream morphological response (Gunter et al., 2013; Young, 2013). For example, *runx2* is known to induce expression of collagen genes (Zheng et al., 2003) and *c-fos* is known to induce *periostin*, which is involved in bone and tooth repair (Kashima et al., 2009). Additionally, as was noted earlier, *runx2* and *c-fos* functionally interact in

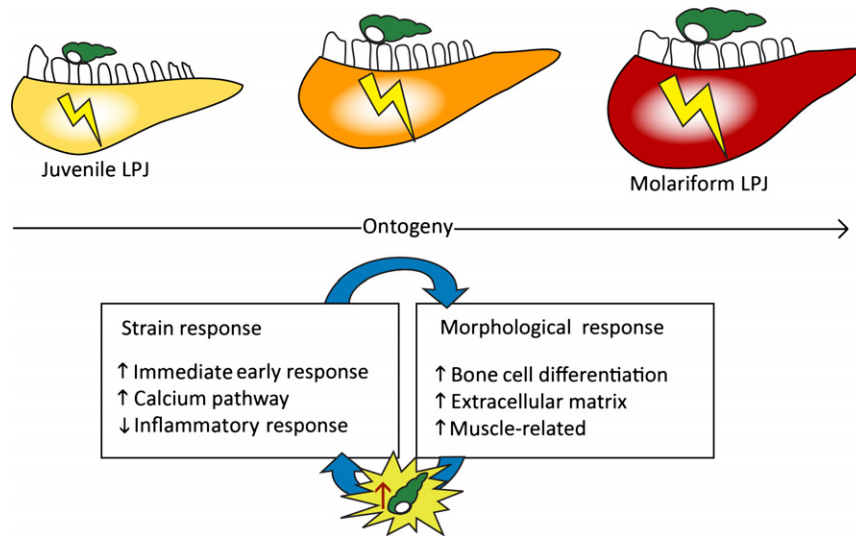


Fig. 2. Model of the transcriptional basis of phenotypic plasticity in the LPJ of a cichlid. The act of breaking hard-shelled snails between the upper and lower pharyngeal jaws invokes a multi-stage transcriptional response that leads to gradual increases in jaw density and tooth size over time. Mechanical strain induces an immediate response, which secondarily induces morphological pathways that are predicted to alter the size and shape of the LPJ. Through offering our fish progressively larger snails throughout the experimental period, strain responses of ever increasing magnitude were induced, leading to exaggerated expression of morphological pathways, leading the LPJ to become progressively larger over time to resist the increased forces exerted by the hard-shelled snails.

response to mechanical strain (D'Alonzo et al., 2002). Wolff's law predicts that compensatory growth in response to the application of mechanical strain (from snail crushing) would ultimately attenuate the strain-induced transcriptional response. However, we consider that this growth only affords the fish to crush progressively harder prey items inducing a strain response of ever-higher magnitude, bounded only by the maximal hardness of the ingested food items and the architectonic constraints that the other cranial bones impose on LPJ growth (Smits et al., 1996).

There is an evolutionary arms race going on between the hardness of the snail shells and the cracking force that the fish can muster. This results in broken teeth in quite a few individual cichlid fish that we have examined (A. Meyer, pers. obs.). Indeed, our experimental design took this into account, by offering snails of ever increasing size throughout the 18-month treatment period. Our data support the hypothesis that the molariform morph develops through the iterative action of mechanical strain cycles on the pharyngeal jaw apparatus during growth of this and other species of cichlids, including fish in the neotropical Midas cichlid species flock (*Amphilophus cf. citrinellus*) in which we have done similar experiments (Meyer, 1990, 1993; Muschick et al., 2011). The entire pharyngeal mill performs as a single functional unit and not only the bones and teeth of the lower pharyngeal jaw (the fifth ceratobranchial bones) are affected, but also the upper pharyngeal jaw (formed by the infrapharyngobranchials) and the apophysis on the ventral side of the neurocranium (the functional joint against which the upper pharyngeal jaw abuts) are enlarged in molariform fish compared to papiliform fish. This is consistent with previous observations on the *A. alluaudi* LPJ, which showed that progressively larger generations of teeth developed in molariform jaws (Huyssene, 1995) and that plasticity in LPJ size cannot be induced

in aquarium-raised adult *A. alluaudi*, which do not grow during the treatment period (Smits, 1996).

### Role of phenotypic plasticity in teleost evolution

In addition to providing insight into the molecular basis of acellular bone remodeling, our study has provided a basis upon which to test the role of phenotypic plasticity in the evolution of East African cichlid fishes, a lineage that has undergone rapid and explosive speciation (Meyer et al., 1990; Salzburger et al., 2005; Elmer et al., 2009). Due to the rapidity of their speciation and limited genetic variability between species (Meyer et al., 1990; Elmer et al., 2009), it has been hypothesised that phenotypic plasticity and subsequent genetic assimilation has played a key role in cichlid evolution (Meyer, 1987, 1993; Wimberger, 1994; Stauffer and Gray, 2004). Namely, a hypothetical 'plastic' ancestor colonized the lakes of East Africa rapidly filling diverse trophic niches through developing alternate morphologies that facilitated efficient niche exploitation. Secondly, these initially plastic phenotypes are thought to have become genetically fixed through the process of genetic assimilation. Indeed, *A. alluaudi* belongs to a relatively basal lineage amongst cichlids (Salzburger et al., 2005) and experiments on other cichlid species suggest that basal lineages harbour plasticity for traits that are fixed in more derived lineages (Meyer, 1987, 1990; Muschick et al., 2011). Our future investigations will test the hypothesis that genetic assimilation contributed to cichlid evolution, through investigating the evolution of regulatory sequences from the 'plasticity genes' identified by our study – specifically focusing on the so-called shear-stress responsive elements (SSREs). We postulate that derived lineages from the lacustrine adaptive radiations will display a reduced degree of plasticity in response to a mechanically stimulating

diet, matched by a reduction in SSREs in the promoter regions of the 'plasticity genes'. Our planned investigations will utilise a mechanistic knowledge of the molecular basis of acellular bone remodeling to test the role of adaptive phenotypic plasticity and subsequent genetic assimilation in a rapidly evolving lineage.

### Summary

We are entering an exciting time, where new methods such as next-generation DNA sequencing allow us to gain an understanding of the molecular basis of phenotypic plasticity in neoteleost acellular bones, which has been long assumed to occur through different mechanisms than the remodeling of cellular bones (Moss, 1962). Two recently established medaka reporter lines enable the *in vivo* visualisation of osteoblasts (Renn and Winkler, 2009) and osteoclasts (To et al., 2012), which offers the possibility to determine the cellular and molecular bases of altered bone remodeling in response to changes in mechanical environments (Wagner et al., 2003) and nutritional status (Vieira et al., 2013). Our research demonstrates an unprecedented level of similarity between the molecular pathways involved in the remodeling of neoteleost acellular bones and mammalian cellular bones. We identified a multitude of molecular pathways that are sure to instruct future research on the ecologically and economically important topic of acellular bone remodeling.

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