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Regulatory gene networks that shape the development of adaptive phenotypic plasticity in a cichlid fish

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Abstract

Phenotypic plasticity is the ability of organisms with a given genotype to develop different phenotypes according to environmental stimuli, resulting in individuals that are better adapted to local conditions. In spite of their ecological importance, the developmental regulatory networks underlying plastic phenotypes often remain uncharacterized. We examined the regulatory basis of diet-induced plasticity in the lower pharyngeal jaw (LPJ) of the cichlid fish *Astatoreochromis alluaudi*, a model species in the study of adaptive plasticity. Through raising juvenile *A. alluaudi* on either a hard or soft diet (hard-shelled or pulverized snails) for between 1 and 8 months, we gained insight into the temporal regulation of 19 previously identified candidate genes during the early stages of plasticity development. Plasticity in LPJ morphology was first detected between 3 and 5 months of diet treatment. The candidate genes, belonging to various functional categories, displayed dynamic expression patterns that consistently preceded the onset of morphological divergence and putatively contribute to the initiation of the plastic phenotypes. Within functional categories, we observed striking co-expression, and transcription factor binding site analysis was used to examine the prospective basis of their coregulation. We propose a regulatory network of LPJ plasticity in cichlids, presenting evidence for regulatory crosstalk between bone and muscle tissues, which putatively facilitates the development of this highly integrated trait. Through incorporating a developmental time-course into a phenotypic plasticity study, we have identified an interconnected, environmentally responsive regulatory network that shapes the development of plasticity in a key innovation of East African cichlids.

Keywords: *Astatoreochromis alluaudi*, bone remodelling, cichlid, developmental time-course, evo-devo, pharyngeal jaw

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Introduction

Traditionally, research on adaptive morphological variation has focused predominantly on genetic variability (e.g. Clausen *et al.* 1941; Morgan 1947; Lewontin 1974). However, throughout recent decades, investigations have demonstrated that a broad range of environmental variables, both internal and external to the organism, play an instructive role in the development of adaptive phenotypes (Adler & Harvell 1990; Janzen & Paukstis 1991; West-Eberhard 2004; Gilbert 2005; Beldade *et al.* 2011). Phenotypic variability can arise when environmental cues modulate the developmental trajectories encoded by a single genotype through a process termed phenotypic plasticity (Schlichting & Pigliucci 1998; Pfennig *et al.* 2010). Integrative approaches such as 'ecological developmental biology', which incorporate evolutionary biology, developmental biology and ecol-

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ogy, have the power to shed new light on the role of phenotypic plasticity in evolution (Gilbert 2001; West-Eberhard 2004; Gilbert & Epel 2009). While a historical lack of strong molecular evidence has hampered the acceptance that phenotypic plasticity would promote (rather than impede) speciation, logically it stands to reason that it should promote speciation under certain ecological circumstances (Price *et al.* 2003; Nicotra *et al.* 2010). Indeed, one of the basic concepts of evolutionary biology is that selection acts on an organism's phenotype (Mayr 1963; Lande & Arnold 1983; Schwander & Leimar 2011; Scheiner & Holt 2012) regardless of whether this phenotype is genetically encoded, opening the possibility that plastic phenotypes might represent an important intermediate step in speciation. Moreover, it has been recognized that phenotypic plasticity itself can be the target of selection (Pigliucci 2007; Wund *et al.* 2008).

In spite of its potential importance in generating adaptive phenotypes, understanding the molecular mechanisms underlying phenotypic plasticity has only become technically possible in recent years. To date, several ground-breaking studies have shown that alternative phenotypes may be induced through the alteration of gene expression by epigenetic means, as well as alterations of hormonal and enzymatic activity (Denver 1997; Gilbert 2005; Aubin-Horth & Renn 2009; Jablonka & Raz 2009; Snell-Rood *et al.* 2010; Beldade *et al.* 2011; Sommer & Ogawa 2011). Such studies typically focus on the molecular bases of polyphenisms, discrete alternative phenotypes that arise through environmentally mediated switches in developmental pathways (Abouheif & Wray 2002). Nonetheless, trait values for environmentally induced phenotypes often form a continuous distribution, the reaction norm (Woltereck 1913).

To determine the molecular bases of phenotypic plasticity, as a crucial step to evaluating its effects on species evolution, it is essential to investigate the temporal expression dynamics of environmentally responsive genes that mediate the plastic response, for which developmental time-course experiments are a powerful tool (Aubin-Horth & Renn 2009). For organisms that respond plastically to an environmental cue, genes that are upstream in a regulatory cascade underlying a plastic trait are expected to alter their expression earlier in the presence of this inductive cue compared to genes that are more downstream (Aubin-Horth & Renn 2009). Thus, it is necessary to interpret changes in gene expression in a plastic trait throughout development, to be able to delineate the roles of specific genes throughout the plastic response.

In a previously published analysis, we characterized the molecular basis of diet-induced phenotypic plasticity in the lower pharyngeal jaw (LPJ) in an East African fish, *Astatoreochromis alluaudi* (Gunter *et al.* 2013). The LPJ forms part of the pharyngeal jaw apparatus (PJA), a key innovation of cichlid fishes that contributed significantly to their explosive adaptive radiation within <2 Myr (Liem 1974; Meyer *et al.* 1990; Elmer *et al.* 2009). Through evolving highly specialized feeding morphologies and behaviours, many East African cichlids have been able to exploit narrow and creative trophic niches, such as algae scraping, Aufwuchs plucking and insect picking, often in parallel across the three main African lakes (Meyer *et al.* 1990; Kocher *et al.* 1993; Meyer 1993a; Stiassny & Meyer 1999; Schön & Martens 2004; Young *et al.* 2009). In contrast, *A. alluaudi* is a generalist species, basal to the modern haplochromines, which inhabits Lake Victoria, its satellite lakes and associated river systems (Greenwood 1964; Hoogerhoud 1984; Salzburger *et al.* 2005). Its' plastic LPJ allows this cichlid to exploit the available food resources efficiently across varying habitats: if their preferred diet of soft food (such as insects) is sufficiently available, they develop a slender, 'papilliform' LPJ, bearing numerous fine teeth (Slootweg *et al.* 1994). However, if soft food is scarce, individuals feed on hard-shelled molluscs that induce the formation of a robust molariform LPJ, bearing fewer, more molar-like teeth (Fig. 1) (Greenwood 1964; Huysseune *et al.* 1994; Slootweg *et al.* 1994; Huysseune 1995). Further cases of trophic plasticity have been investigated amongst Neotropical cichlids, leading to the hypothesis that these might be more phenotypically plastic than most African cichlids (Meyer 1987, 1989, 1990, 1993b; Muschick *et al.* 2011). However, the general relationship between phenotypic plasticity and speciation rates is still unclear and remains hotly debated (Pfennig *et al.* 2010; Thibert-Plante & Hendry 2011; Landry & Aubin-Horth 2014).

In this study, we investigate the molecular basis of environmentally induced morphological divergence that results in the papilliform and molariform LPJ phenotypes in *A. alluaudi*. A time-course experiment was employed, exposing naïve juvenile fish to experimental diets for variable time periods, allowing us to analyse gene expression across 8 months of plastic development in *A. alluaudi*. We investigated the expression of 19 candidate genes that were previously found to be associated with induced plasticity in the LPJs of *A. alluaudi* (Gunter *et al.* 2013). Of these 19 candidates, 16 belong to six main functional categories, including 'immediate early genes', 'haem pathway genes', 'matrix-related genes', 'bone-related genes', 'muscle-related genes' and 'calcium pathway genes '. We present evidence that most of these genes are not only associated with the plastic phenotypes, but that they also contributed to the early and on-going development of LPJ plasticity in *A. alluaudi*. Finally, we shed light on the putative

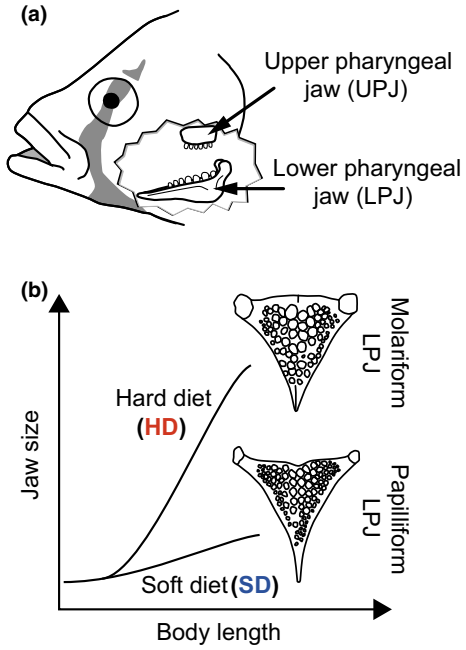

Fig. 1 Cichlid fishes possess a mechanically robust pharyngeal jaw apparatus. (a) The PJA is comprised of a pair of upper pharyngeal jaws (UPJ) that articulate directly with the neurocranium and the lower pharyngeal jaw (LPJ), which is formed by the suturing of the 5th ceratobranchial arches. (b) For the cichlid *Astatoreochromis alluaudi*, the mechanical properties of the diet influence LPJ development, whereby individuals fed a soft diet develop a smaller, more slender papilliform LPJ, the baseline condition, while individuals fed a hard diet develop a larger, more robust molariform LPJ that withstands increased biting forces. Image modified from Hoogerhoud (1984).

regulatory network underlying LPJ plasticity by utilizing an analysis of transcription factor binding sites on the genome of a closely related cichlid.

Materials and methods

Fish husbandry and experimental set-up

A developmental time-course experiment was conducted on an inbred strain of *Astatoreochromis alluaudi* using modifications to the methods described in Gunter *et al.* (2013), assuring minimal genetic variation between individuals. Two broods (which share the same grandparents) comprising a total of ~40 individuals were raised on *Artemia salina* nauplii and Tetramin flake food until they reached a standard length (SL) of ~30 mm. These individuals were split randomly in two equally sized groups, and each group was raised in a substrate-free 100-l tank for up to 8 months. Tanks were set up in a temperature-controlled room set to 25 °C with artificial illumination, regular water changes and weekly water quality assessments ensuring minimal heterogeneity between the tanks. Comparisons to an independent study with a similar experimental design (H. M. Gunter unpublished results) demonstrated that there were no systemic tank effects (Fig. S1, Supporting Information). Each of the two groups was fed *Melanoides* spp. snails, with one group receiving whole snails, which the fish had to crack with their pharyngeal jaw apparatus (PJA) (the hard diet (HD) group), and the second group receiving an equivalent amount of snails that had been finely pulverized (the soft diet (SD) group). After 1, 3, 5 and 8 months of treatment, fish from each of the two groups were randomly selected and sacrificed within 30 min of feeding using a lethal dose of Tricaine (MS222). These time points were chosen to evenly cover the treatment period, which was predicted to include size of plastic divergence, previously determined to be 55 mm SL (Huysseune *et al.* 1994). Mean SL did not differ between the two diet groups for any of the four developmental time points (Fig. S2, Supporting Information). A total of 33 individuals were sampled across all developmental stages. Sex was not taken into account as previous investigations indicated that diet and SL are far greater determinants of LPJ plasticity in *A. alluaudi* (Gunter *et al.* 2013). The fish's SLs were measured, LPJs were dissected and stored in RNA*later* (Qiagen) at −20 °C.

Morphometric measurements

To determine the stage at which LPJ plasticity could first be detected, the size of each LPJ was analysed according to Gunter *et al.* (2013). These analyses were made from digital images, which were captured after the LPJs had been cleaned of surrounding connective tissue. Ten linear morphometric measurements of all LPJs were obtained following Gunter *et al.* (2013) (Fig. S3, Supporting Information). ANCOVAS were performed using R (RDC-Team 2005), utilizing each linear morphometric measure as a dependent variable, diet as a factor and SL as a covariate (Fig. S4, Supporting Information). SL was plotted against each linear measurement to evaluate the relationship between size and morphological divergence. As there was a significant linear relationship between SL and all other linear morphometric measurements (for linear regressions all $P < 0.05$), only the residuals of the respective variables were used for further analyses. The morphological differences amongst the diet groups were evaluated for each time point by performing Wilcoxon signed-rank tests in R. Multiple testing correction was not performed, but we chose rather to interpret our results with caution. All *P*-values are listed in the Supporting Information (Tables S2,S3, Supporting information).

RNA extraction and cDNA synthesis

RNA was extracted from the LPJ samples using the RNA Mini kit (Qiagen). In addition to the steps recommended by the manufacturer, LPJs were ground to a fine powder while submerged in liquid nitrogen and homogenized in a FastPrep (MP Biomedicals). Polyacryl carrier (MRC) was added to increase RNA yield, and samples were treated with an optional on-column DNase treatment (Qiagen). Furthermore, additional wash steps with 80% ethanol were included to remove all traces of salt. RNA quantity was assessed using a fluorometer (Qbit 2.0), and its integrity was confirmed using a Bioanalyzer 2100 (Agilent). RNA was determined to be free of gDNA contamination according to noRT controls, and cDNA was synthesized using Invitrogen SuperscriptIII and oligo dT primer, according to Gunter *et al.* (2013).

qRT–PCR

We examined the expression of 19 candidate genes that were previously shown to be either up- or downregulated in the LPJs of *A. alluaudi* individuals that received a hard diet (HD), vs. the control, which received a soft diet (SD), for a period of 18 months (Gunter *et al.* 2013). The upregulated genes include: *cfos*, *ier2* and *rgs2*, termed immediate early genes (Nose & Shibanuma 1994; Versele *et al.* 1999; Ott *et al.* 2009); *klf4*, a pleiotropic transcription factor involved in differentiation of stem cells including osteoblasts and osteoclasts (Nose & Shibanuma 1994; Michikami *et al.* 2012); *ryr1*, *anxa6* and *srl*, termed calcium pathway genes (Leberer *et al.* 1989; Meissner 1994; Song *et al.* 2002; Treves *et al.* 2005); *tnnt*, *tpm4* and *des*, termed muscle-related genes (Perry 1998; Mantila Roosa *et al.* 2011); *col6*, *col12* and *thbs3*, termed matrix-related genes (Tucker *et al.* 1997; Gelse *et al.* 2003) and *runx2b* and *osx*, which orchestrate osteoblast proliferation and differentiation, termed bone-related genes (Nakashima *et al.* 2002; Li *et al.* 2009b). Downregulated genes include *alas1* and *c1ql*, which are putatively associated with haem biosynthesis and haematopoiesis, termed haem pathway genes (Sadlon *et al.* 1999; Mei *et al.* 2008); *abcb3* and *gif*, which are associated with MHC function (Edidin 1983; Karttunen *et al.* 2001; Nonaka & Nonaka 2010) and Vitamin B12 binding and transport, respectively (Greibe *et al.* 2012) (Table S1, Supporting Information).

Primers used in this study were either described previously in Gunter *et al.* (2013) or were derived from RNA-seq contigs generated by the same study, and their efficiency was tested using standard curves using a Bio-Rad CFX96 cycler (efficiencies ranged between 92% and 108%; Table S1, Supporting Information). As in Gunter *et al.* (2013), *actinr* and *twinfilin* were used as housekeeping genes, which were selected from an RNA-seq data set, on the basis of showing the lowest variability between diet treatments and individuals. Primer concentrations and annealing temperatures were optimized to ensure negligible dimer amplification, based on assessment of the melt curve and comparison to no template controls. All primers were used at a concentration of 0.3 pmol/μL, and annealing temperatures were 60 °C for *ier2*, *gif* and *klf4* and 55 °C for all other genes. For each gene, all samples were run in duplicate on a single 96-well plate, alongside a no template control. For each run, we ensured that negligible/no dimer was produced based on assessment of the melt curve and comparison to the no template controls. Gene expression values were calculated for each sample and gene using the respective primer efficiencies. Relative quantification values (RQ-values) were calculated by scaling to the maximum expression value observed for each gene. Relative quantification values were further normalized to a normalization factor, calculated from the expression of the aforementioned housekeeping genes (Vandesompele *et al.* 2002).

Statistical analysis of qRT–PCR

Wilcoxon signed-rank tests were performed to analyse differences in candidate gene expression between diet groups across the four time points as data distributions were nonparametric. We did not perform correction for multiple testing (see above) and report all *P*-values in the Supporting Information (Table S4, Supporting Information). A PCA was performed on a covariation matrix to explore patterns of co-expression within our gene expression data set. A hierarchical cluster analysis was then conducted using a Spearman rank correlation based matrix for specimen clustering and Euclidean distances for gene expression clustering in R, to independently examine the robustness of any identified clusters and to explore the clustering of specimens across treatment and diet groups (following Haas *et al.* (2013)). Throughout the study, we applied a significance level of $\alpha = 0.05$.

Analysis of transcription factor binding sites

To gain insight into the regulatory network of LPJ plasticity, we investigated the distribution of transcription factor binding sites (TFBSs) in the promoter regions of the candidate genes. As the genome of *A. alluaudi* has not been sequenced, the genome of the closely related cichlid *Astatotilapia burtoni* was chosen for this purpose. Both species are generalist feeders, inhabiting comparable trophic niches, and phenotypic plasticity can be induced by diet in the LPJs of each, although to a lesser

extent in *A. burtoni* (H.M. Gunter, unpublished). Moreover, they display a high degree of genetic similarity, computed to be 99.13% based on exon similarity calculations performed using samtools. To calculate this similarity, we divided the total number of SNPs between the two species by the total number of aligned nucleotides (indels were masked – samtools-0.1.18; Li *et al.* 2009a).

We identified all 19 candidate genes in the genome of *A. burtoni* and used Jaspar to investigate the distribution of TFBSs in their promoters (version 5.0; http://129.177.120.189/cgi-bin/jaspar2010/jaspar_db.pl). The investigated interval spanned from 1 kb upstream from each gene's translation start site to the end of the first exon, encompassing both the putative promoter region and the 5′-UTR (similar to Lerch *et al.* (2012)). In some cases (*cfos*, *rgs2*, *runx2b*, *alas1* and *anxa6*), more than one translation start site was identified, and we analysed the promoters of each separately, accounting for potential overlaps. TFBSs were selected above a conservative threshold of 0.85 (Kwon *et al.* 2012) of the relative matrix score, and results for threshold levels of 0.9 and 0.95 are also reported as an indicator of the distribution of TFBS qualities. Moreover, we focused on the distribution of binding sites for two transcription factors that have been shown to be mechanically responsive: AP1 (a heterodimer comprising a JUN and a FOS protein unit, referred to as 'JUN::FOS' in Jaspar) and CREB1 (cAMP response element-binding protein 1) (Davidovitch *et al.* 1984; Nomura & Takano-Yamamoto 2000). Finally, we examined TFBSs for RUNX2B ('RUNX2' in Jaspar) and KLF4 to identify further potential regulatory pathways amongst our candidate genes.

Results

Diet influences morphological divergence in Astatoreochromis alluaudi LPJ development

Using a split brood experiment, we investigated the regulatory networks underlying the development of diet-induced adaptive plasticity in the LPJ of *Astatoreochromis alluaudi*. Our analyses indicated the on-going divergence of LPJ size between diet groups across the developmental time-course, with the majority of significant differences being detected after 5 and 8 months of treatment (Fig. 2, Table S2, Fig. S3, Supporting Information). At these time points, HD fish already displayed the typical molariform LPJ phenotype, with relative increases in jaw weight, average tooth size, centroid size and size of muscle attachment horns, in comparison with SD fish.

The ANCOVA analyses on the linear morphometric data indicated a significant factor effect and a significant interaction for the log of jaw weight and suture width, suggesting morphological divergence between the two diet groups. In addition, average tooth size and centroid size also showed increased values in the HD treatment, and although they did not attain statistical significance, they displayed a trend towards morphological divergence ($0.05 < P < 0.1$) between diet treatments (Fig. 2). Indeed, this trend was observed for most linear measurements and was supported by nonoverlapping confidence intervals (Table S3, Fig. S4, Supporting Information).

Our linear morphometric data show that morphological divergence in the LPJ of *A. alluaudi* was first detectable at 5 months of treatment. The confidence intervals of our scatterplots suggest that significant morphological divergence is likely to have occurred at a SL of 55–60 mm (which coincides with a treatment duration of between 3 and 5 months, Fig. S2, Supporting Information) for variables such as LPJ weight, suture width and average horn width (Fig. 2, Fig. S4, Supporting Information). Such variables are indicative of a robust, functionally integrated molariform LPJ phenotype, where the LPJ is denser, the two 5th ceratobranchials are united by a stronger suture, and movement of the LPJ is controlled by larger, stronger muscles (e.g. Huysseune *et al.* 1994; Smits *et al.* 1996; Hulsey 2006). This divergence lies in the middle of the developmental time-course employed by our study, enabling gene expression to be compared for stages prior to and after the onset of observable morphological divergence.

Diet influences gene expression during Astatoreochromis alluaudi LPJ development

Quantitative reverse transcription–PCR (qRT–PCR) was used to analyse the expression of previously identified candidate plasticity genes in a developmental time-course of diet-manipulated *A. alluaudi*. As determined by our morphological analyses, this time-course captured the period prior to and shortly following the development of observable morphological differences that arose due to the plastic response. We observed significant gene expression differences between treatments after 3, 5 or 8 months of treatment (*rgs2*, *ryr1*, *anxa6* and *tnnt*; *osx* and *alas1*; *col12*, respectively) (Fig. 3, Table S4, Supporting Information). Further trends of differential expression ($0.1 > P > 0.05$) were observed after 3, 5 or 8 months of treatment (*srl*, *tpm4* and *des*; *tpm4* and *gif*; *srl* and *osx*, respectively). After 1 month of treatment, mean expression levels were higher in SD than HD individuals for 17 of the 19 candidate genes (albeit not significantly); however, this pattern was inverted for most genes after 3 months of treatment (Fig. 3, Table S4, Supporting Information). After treat-

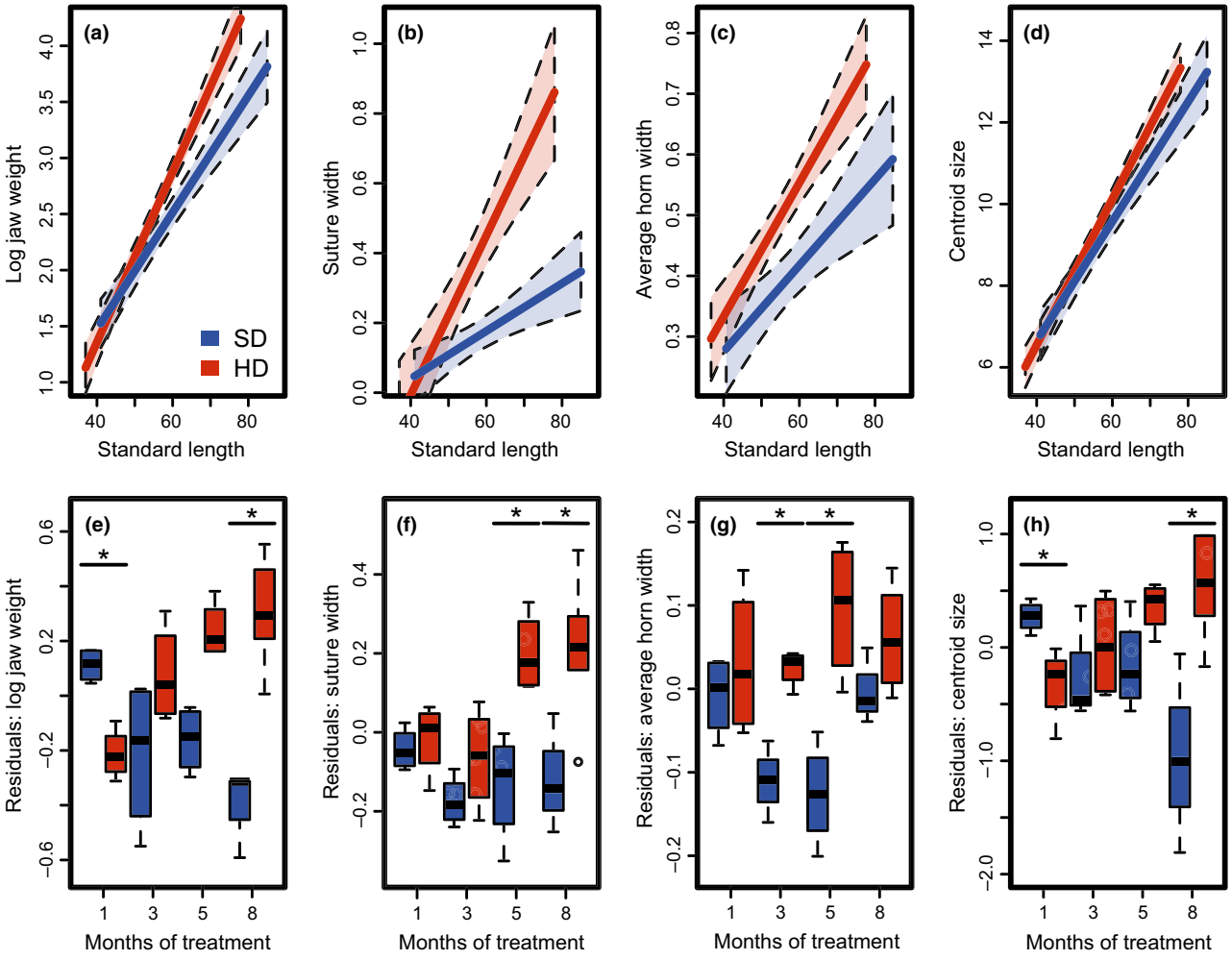

Fig. 2 Developmental plasticity in LPJ size for *Astatoreochromis alluaudi* fed either a hard diet (HD) or soft diet (SD). Four linear morphometric measurements were plotted against (a-d) standard length and (e-h) months of treatment. Morphometric measures include (a,e) LPJ weight, (b,f) suture width, (c,g) horn width and (d,h) centroid size. (a-d) Different slopes were detected for each morphological measurement in each diet group, indicating that morphological divergence arose at ~55–60 mm. (e-h) Residuals of morphometric measurements tended to increase in HD compared with SD individuals across development (* = $P < 0.05$). Marked areas reflect 95% CIs (a-d) and boxplots show the median, the 1st and 3rd quartiles as hinges and upper and lower whiskers (e-h).

ment periods of 5 and 8 months, consistent trends were not observed amongst all genes, but we observed divergent gene expression patterns between the functional categories. Within functional categories, we observed a high degree of co-expression across most genes when diet-specific expression patterns were examined across all developmental time points. Additional analyses were thus employed to formally explore the degree of co-expression within the functional categories, including a principal component analysis (PCA) and hierarchical clustering.

Genes within functional categories display marked co-expression

PCA was used to analyse co-expression between the candidate genes, comparing gene expression values from HD and SD treatments across all developmental time points. Groups of co-expressed genes were identified through visual examination of the PCA plots (Fig. 4, Table S6, Supporting Information). Three groups were clearly identifiable in each of the first three principle components (PCs) of the analysis, namely (i) the muscle-related genes and *srl* clustered on PC1; (ii) immediate early genes and *klf4* aligned on PC2; and (iii) matrix-related genes aligned on PC3. Additional genes grouped more loosely on the first three PCs. Specifically, *anxa6* and *ryr1* were associated with both the muscle-related genes on PC1, and the matrix-related genes on PC3. Also, the bone-related genes *runx2b* and *osx* aligned with the matrix-related genes on PC3, albeit less of their total variance was explained by this PC

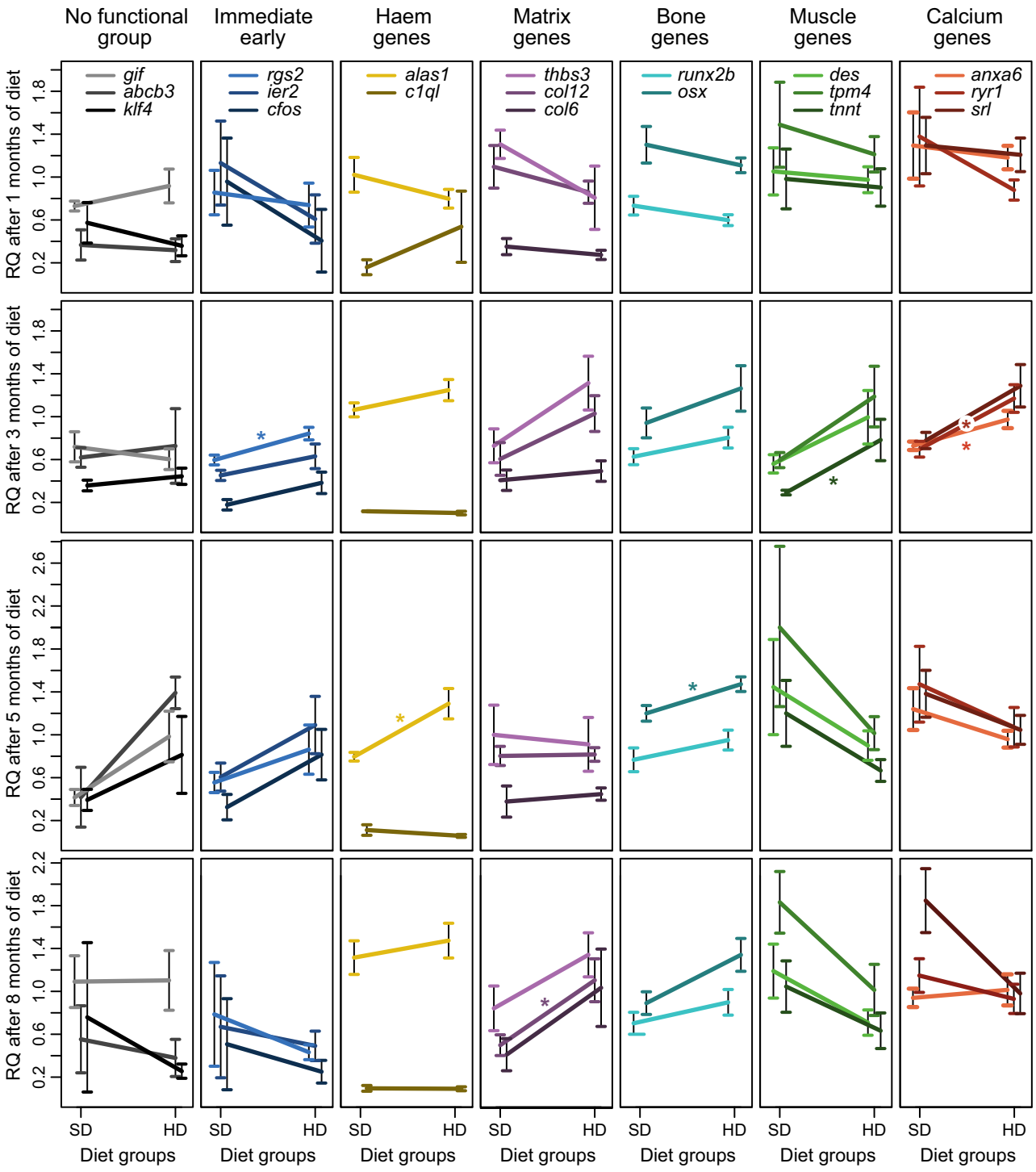

Fig. 3 Expression of candidate genes displays a dynamic pattern during the development of diet-induced plasticity in the LPJ. Reaction norms of gene expression for SD vs. HD treatments, with expression plotted separately for treatment periods of 1, 3, 5 and 8 months, and for genes of each functional category. Genes within functional categories tend to display similar patterns of expression within each time point, while expression is highly differential between developmental stages, and between functional categories (* = $P < 0.05$). Shared line coloration hues reflect the respective functional categories.

than for the matrix-related genes. Moreover, a few genes did not display detectable co-expression, namely the haem pathway genes, in addition to *abcb3* and *gif*. No further meaningful co-expression groups could be identified (Table S6, Supporting Information).

A hierarchical cluster analysis was then conducted, using only the genes that displayed clustered co-expression according to the PCA (Fig. 5, Fig. S1, Fig. S6, Supporting Information). Similar to the PCA, three distinct clusters were detected, with gene compositions that concurred with results of the PCA. Immediate early genes and *klf4* formed one cluster, calcium pathway genes clustered with the muscle-related genes and the bone-related genes clustered with the matrix-related genes, suggesting that these clusters form three putative regulatory modules. When all expression patterns were considered, specimens clustered neither according to their treatment group nor the treatment duration, suggesting that the treatment effects were rather subtle in our samples, consistent with our observation of subtle morphological differences.

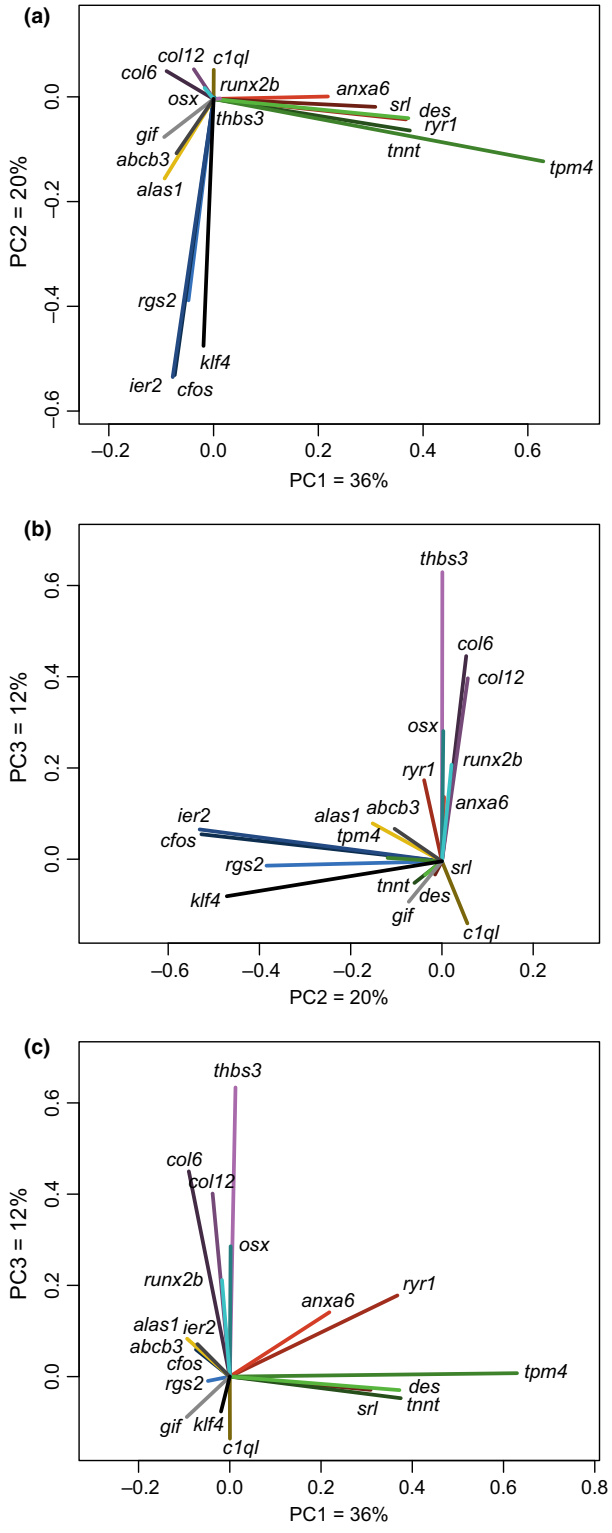

Fig. 4 PCA on candidate gene expression patterns indicates coregulation amongst functionally related genes. (a,c) Muscle-related and calcium pathway genes cluster on PC1, which explains 36% of the total variation. (a,b) Immediate early genes and *klf4* cluster on PC2, which explains 20% of the total variation. (b,c) Matrix- and bone-related genes cluster on PC3, which explains 12% of the total variation. Plot lines of the PCA are colour-coded according to the functional class of each gene: immediate early genes are blue, calcium pathway genes are red, muscle-related genes are green, matrix-related genes are violet, bone-related genes are cyan, haem pathway genes are ochre and others black (as in Fig. 3).

Transcription factor binding site analysis indicates regulatory basis of co-expression

A transcription factor binding site (TFBS) analysis was utilized to evaluate the regulatory basis of the identified patterns of co-expression amongst functional categories. Using the TFBS detection program Jaspar, a total of 10,741 TFBSs were identified in the promoter regions and 5′-UTRs of our 19 candidate genes. Of these, 244 were binding sites of four key TFs that are putatively involved in establishing the observed plastic phenotypes. These include two mechanically responsive TFs, AP1 and CREB1, in addition to two bone-related TFs KLF4 and RUNX2B (Fig. 6, Fig. 7, Table S7, Supporting Information).

We estimated the strength of regulatory relationships between the selected TFs and our candidate genes, based on the total number of predicted TFBSs in their promoters, and the predicted binding strength at each TFBS (based on the position weight matrix score) (Hallikas *et al.* 2006). Based on this, the strongest putative regulatory targets of AP1 were estimated to be the immediate early genes *cfos*, *rgs2* and *ier2*, both collagens and *runx2b* and *des*. Moreover, AP1 was estimated to have a more moderate regulatory influence on *abcb3*, *alas1*, *c1ql*, *gif*, *osx* and the remaining muscle-related and calcium pathway genes. We estimated the strongest putative regulatory target of CREB1 to be *runx2b*, while having a more moderate influence on *cfos*, *rgs2*, *klf4*, *srl*

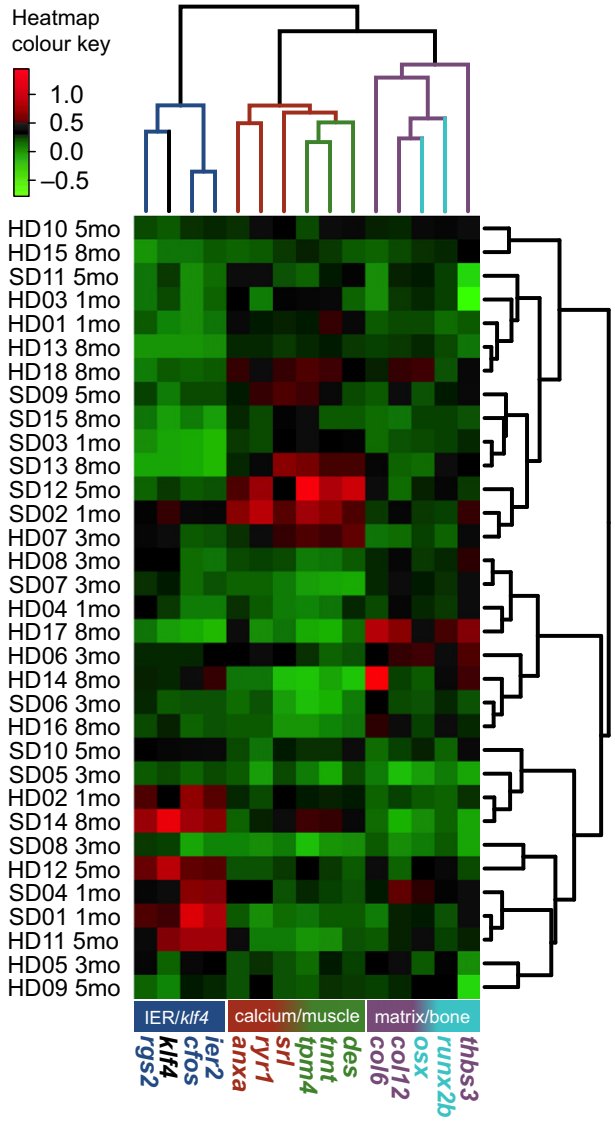

Fig. 5 Hierarchical cluster analysis on candidate gene expression patterns indicates coregulation amongst functionally related genes. The analysis shows three main clusters: (i) *klf4* and the immediate early genes, (ii) bone-related genes and matrix-related genes and (iii) calcium pathway genes and muscle-related genes. HD = hard diet, mo = months of treatment, SD = soft diet. Coloration of the gene names refers to their respective functional class: immediate early genes are blue, calcium pathway genes are red, muscle-related genes are green, matrix-related genes are violet, bone-related genes are cyan and others black (as in Fig. 3 & 4).

and *tnnt*. RUNX2B was estimated to have a moderate regulatory influence on *gif*, *runx2b* and *osx*. Finally, KLF4 was estimated to have the strongest regulatory influence on *alas1* and a more moderate influence on *c1ql*, *rgs2*, *klf4*, *osx*, *anxa6*, *ryr1*, *tnnt* and *des*.

Of the 170 TFs for which binding sites were detected, twelve were shared amongst all candidate genes.

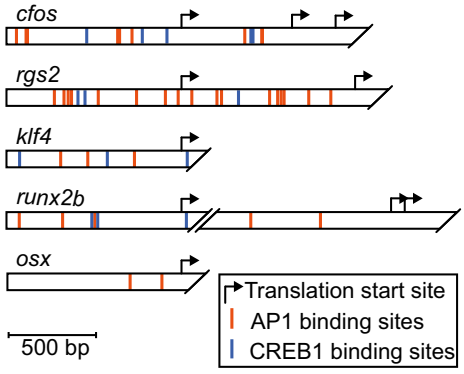

Fig. 6 The regulatory regions of our candidate genes contain numerous predicted binding sites for mechanically responsive TFs. Coloured lines indicate predicted binding sites for the mechanically responsive transcription factors AP1 and CREB1 (cAMP response element-binding protein 1) in the regulatory regions of *cfos*, *rgs2*, *klf4*, *runx2b* and *osx*. Immediate early genes *cfos* and *rgs2* include the highest density of mechanically responsive TFBSs.

Within functional categories, we detected characteristic TFBSs that may contribute to the observed co-expression. Amongst all immediate early genes and *klf4*, binding sites for 14 TFs were detected that were not shared by all members of any other functional group. These include the mechanically responsive TFs JUN, GATA4 and CEBPβ (Sumpio *et al.* 1994; Swynghedauw 2006; Sen *et al.* 2009). For all matrix and bone-related genes, binding sites for three TFs were detected. For all muscle-related and calcium pathway genes, binding sites for two TFs were detected, including the muscle-specific TF MYOG that contributes to myogenic cell differentiation in mesenchymal stem cells (Wright *et al.* 1989). Binding sites for two TFs (MYCN and FOS) were detected in the calcium pathway genes *ryr1* and *anxa6*, but not in any other muscle-related gene or *srl*, potentially explaining their relatively distinct expression in comparison with the other genes within their co-expression cluster.

Interestingly, amongst TFs with the most abundant binding sites in the promoter regions of our candidate genes, there are two that are known to be involved in development and plasticity, which thus may have contributed to the observed plastic phenotypes (Table S7, Supporting Information). These include ARID3A, which is involved in the regulation of developmental plasticity in mouse and human cells (An *et al.* 2010) and PRRX2, which is involved in craniofacial bone development in mice (Lu *et al.* 1999) as well as matrix alterations of the vascular system and smooth muscles (Bergwerff *et al.* 1998). Binding sites for both TFs were found to be present in high numbers in the promoter of *osx*, a major regulator of bone development.

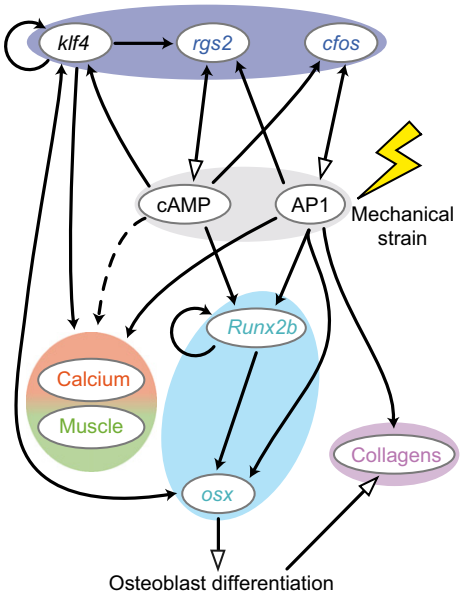

Fig. 7 Major regulatory pathways of LPJ phenotypic plasticity as suggested by the TFBS analysis. Closed arrowheads indicate the direction of regulatory influence inferred from the analysis, while open heads indicate regulatory relationships described in previous publications (see text for details). The dashed arrow targeting 'calcium pathway' and 'muscle-related' genes indicates that only some, but not all of these genes exhibit cAMP (mediated by CREB1) TFBSs. The strain responsive transcription factor AP1 appears to be of central role in the upstream regulation of LPJ phenotypic plastic response to mechanical strain, regulating collagens, calcium and muscle-related genes, immediate early genes and *runx2b*. *runx2b* expression may also be regulated via the strain responsive second messenger cAMP (mediated by CREB1), which also influences *klf4*, *cfos*, calcium pathway and muscle-related genes and *rgs2* expression. RUNX2B appears as an intermediate TF that regulates expression of a key osteoblast regulator, *osx*, together with KLF4 and AP1.

Discussion

Unravelling the molecular basis of phenotypic plasticity is a powerful first step in empirically evaluating the potential role of plasticity in evolution (West-Eberhard 2004, 2005; Bell & Aubin-Horth 2010; Beldade *et al.* 2011). Indeed, molecular tools are increasingly incorporated into studies of plasticity in phenotypic traits such as melanization in *Daphnia* (Scoville & Pfrender 2010), phase change in locusts (Wang & Kang 2014), hypoxia tolerance in killifish (Whitehead *et al.* 2011) and changing reproductive phenotypes in cichlids (Maruska *et al.* 2011). Our study examines gene expression across a developmental time-course, shedding light on the transcriptional network involved with the initiation of phenotypic plasticity in the LPJ of the cichlid *Astatoreochromis alluaudi*. Drawing upon our previous transcriptome study (Gunter *et al.* 2013), 19 candidate genes were selected, with putative functions that span various organizational levels within the molecular network that orchestrates LPJ development. By examining the expression of these candidate genes in LPJ samples taken during the early divergence of diet-induced phenotypes, we gained insight into the complex molecular network that drives the development of a famous example of adaptive phenotypic plasticity.

This study is, to our knowledge, the first to use controlled experimental conditions to examine the developmental onset of diet-induced plasticity in the LPJs of *A. alluaudi*, identifying the period between 3 and 5 months of diet treatment as a critical period for the first appearance of measurable plasticity. At this stage, the SLs of both treatment and control fish were ~55–60 mm, consistent with previous observations on aquarium-raised *A. alluaudi* (Huysseune *et al.* 1994). This size at divergence is relatively delayed compared with the wild populations that inhabit Lake Victoria and its surrounding satellite lakes, for which LPJ divergence already occurs at an SL of ~40 mm (Hoogerhoud 1986b). Our observation suggests that the applied treatment was less intensive than that provided by natural conditions, where juvenile fish may ingest hard food items at an earlier age and where their diet is likely to be more diverse than what we have provided (Cosandey-Godin *et al.* 2008). It is also plausible that selection may have acted upon the norm of reaction of this laboratory line, which has been bred in captivity for more than 25 years.

The candidate gene expression analyses indicated that major transcriptional changes coincide with the morphological transition period between 3 and 5 months of treatment. The first significant expression differences were detected between diet groups after 3 months of treatment, notably an upregulation of calcium pathway genes and immediate early genes amongst HD fish. Calcium channels are known to be rapidly activated in osteoblasts in response to mechanical strain (Walker *et al.* 2000), which in turn initiates the upregulation of immediate early genes (Chen *et al.* 2000). The combined upregulation of both calcium and immediate early genes after a treatment period of 3 months is consistent with our hypothesis that intense mechanical strain precedes the appearance of plastic phenotypes in *A. alluaudi*. A treatment period of 5 months was associated with a relative increase in expression of *osx*, a major regulator of osteoblast differentiation, in HD fish. Notably, 5 months of treatment coincides with the first period of detectable morphological divergence, most probably involving enhanced proliferation of osteoblasts. Finally, we identified *col12* to be significantly upregulated in HD fish after 8 months of treatment, which is likely to reflect alterations to the tensile properties of the extracellular matrix

(Izu *et al.* 2011), concomitant with an overall increase in size of the LPJ. The majority of gene categories were relatively upregulated in HD at 3 months and beyond, albeit they infrequently attained statistical significance, likely due, in part, to our relatively low sample size. Nonetheless, it is interesting to note that statistically significant gene expression differences were observed in an order that reflects our hypothesis that mechanically responsive genes are initiated first, followed by osteoblast differentiation genes, then matrix-related genes (Gunter & Meyer 2014, in press).

The cluster analysis indicated a high degree of co-expression amongst genes of related functional categories, suggesting that they form regulatory modules that act in concert, generating the observed phenotypic plasticity. Three major regulatory clusters were identified by our analyses, including one comprised of the immediate early genes and *klf4*, a second that includes matrix- and bone-related genes, and a third that includes muscle-related and calcium pathway genes. The identified clusters comprise genes of multiple functional categories; however, it is likely that in this context, they are functionally related. For example, while *klf4* is a pleiotropic transcription factor (Dang *et al.* 2000), its clustering with immediate early genes suggests that *klf4* expression follows an immediate early pattern of induction in the *A. alluaudi* LPJ, similar to previous observations of its response to fluid shear stress (Peters *et al.* 2003). Moreover, the coregulation of bone- and matrix-related genes, as well as muscle-related and calcium pathway genes could be due to the fact that osteoblasts secrete bone matrix, and calcium flux is integral to muscle function.

Intriguingly, we observed differential expression of muscle genes in HD and SD LPJs, despite being cleaned of muscle prior to RNA extraction. This pattern is potentially due to the crosstalk between bone and muscle pathways (Mo *et al.* 2012; Bonewald *et al.* 2013). It has been demonstrated that muscle damage induces the differentiation of myoblastic precursors in the bone marrow cavities of mice, which migrate into the muscles and assist with their repair (Ferrari *et al.* 1998; LaBarge & Blau 2002). Thus, the observed upregulation of muscle genes in HD jaws at 3 months may be induced by damage to the LPJ adductor muscles during chewing. The detected co-expression of calcium and muscle genes further suggests that myocytes undergo differentiation in the LPJ, as calcium homeostasis is integral to myogenesis and muscle contractile function (Davies 1963; Hauser *et al.* 2008).

In order to further characterize the regulatory relationships amongst our candidate genes, the transcription factor binding sites in their promoter regions were analysed. Specifically, we sought to identify genes whose expression is likely to respond to our diet treatments, first focusing on two mechanically responsive transcription factors, AP1 and CREB1 (cAMP response element-binding protein 1) (Davidovitch *et al.* 1984; Nomura & Takano-Yamamoto 2000), as mechanical strain is the environmental stimulus that induces plasticity in *A. alluaudi* (Gunter *et al.* 2013). AP1 binding sites were detected in the promoter regions of all 19 candidate genes, and CREB1 binding sites were detected in 12 of the 19 candidates. This abundance of TFBSs suggests that immediate early genes such as AP1 are major regulators of phenotypic plasticity in the cichlid LPJ, influencing gene expression at various levels of the regulatory cascade (Fig. 7). Together, these observations and the detection of divergence in expression patterns prior to the onset of detectable morphological plasticity suggest that all genes in our analysis contribute to the establishment of the plastic phenotypes. However, constructing a structured regulatory hierarchy was beyond the scope of this project.

While we identified informative patterns of expression for most of our candidate genes, several genes displayed unexpected expression patterns, including some that were inverted in comparison with the results of our previous study (Gunter *et al.* 2013). Specifically, these include representatives from the immediate early, muscle-related and calcium pathway genes. We suggest that the dynamic expression of these genes reflects heterologous cycles of bone remodelling and tooth replacement that occur in the LPJ. In response to microdamage caused by mechanical strain, bones are locally resorbed by osteoclasts, and new, stronger bone is subsequently secreted by osteoblasts, improving the mechanical robustness of the bone (Hadjidakis & Androulakis 2006). Indeed, many expression patterns induced in bones by mechanical strain vary considerably with time (Mantila Roosa *et al.* 2011). Remodelling is also an essential process in tooth replacement, which occurs approximately once per month in *A. alluaudi* (Huysseune 1995). Such dynamic gene expression patterns highlight the importance of including multiple developmental time points when trying to determine the molecular basis of phenotypic plasticity, as gene expression at a specific time point within a gene expression cycle may vary considerably. Moreover, we detected different expression patterns after 1 month of treatment, in comparison with 3, 5 or 8 months, with the majority of candidate genes being upregulated in SD (albeit not significantly). We hypothesize that this may be due to a stressful period of adjustment to the mechanically stimulating diet that caused HD individuals to receive a lower level of nutrition than SD. It is also possible that gene expression after 1 month of treatment reflects an early stage of the plastic response,

characterized by an overall decrease in gene expression, potentially representing an innate cost of plasticity. However, further experiments that include a pretreatment sample and denser sampling earlier in the development of plasticity are required to investigate this phenomenon in more detail.

Our study demonstrates that environmental inputs can act on a developmental pathway at various hierarchical levels, generating an adaptive and functionally integrated trait. Nineteen candidate plasticity genes, which encode a combination of transcription factors, signalling and structural proteins, were found to contain putative binding sites for mechanically responsive TFs in their promoter regions. Moreover, trends towards differential expression of these genes were observed prior to the appearance of plastic phenotypes. In particular, muscle and calcium genes displayed tight co-expression, suggesting that mechanical strain induces the differentiation of myocytes in the LPJ, integrating the development of these two tissue types. Together, these results suggest that diet-induced mechanical strain directly influences gene expression across various pathways, which together result in the development of a functionally integrated phenotype, such as the PJA of cichlid fishes. This finding extends the hypothesis of Young (2013) that the molecular pathways underlying phenotypic plasticity involve environmental stimulation of 'upstream genes', which direct the acquisition of a plastic phenotype, further altering the expression of 'downstream genes'. Specifically, our results indicate that the mechanically responsive TFs AP1 and CREB1 can regulate the expression of a suite of LPJ development genes without signalling via intermediates. Similarly, in the sea urchin, thermal stress was shown to influence the expression of genes distributed throughout the endomesodermal and ectomesodermal developmental network in a complex pattern that was not consistent with sequential gene regulation (Runcie *et al*. 2012).

The molariform phenotype of durophagous cichlids such as *A. alluaudi* is complex, involving coordinated alterations in the size of the pharyngeal jaws and their adductor muscles alongside an increase in tooth size and decrease in tooth number (Hoogerhoud 1986a; Huysseune *et al.* 1994; Huysseune 1995). Mismatch in any of these elements would render individuals less able to process hard food items, particularly in the light of the tight architectonic constraints within the oral cavity of teleosts (Meyer 1989; Smits *et al.* 1996; Chapman *et al*. 2001; Binning *et al.* 2010). Our previous investigation proposed that pleiotropic genes are likely to guide the development of both teeth and jaws (Gunter *et al*. 2013). Here, we propose a further molecular explanation for integrated development of muscles and bones of the PJA, namely through coordinated signalling between both tissues, which induces the differentiation of muscle precursor cells in the medullary cavity. Plasticity favours the development of complex, functionally integrated phenotypes without relying on multiple interdependent mutations, pleiotropy or linkage disequilibrium (West-Eberhard 2004, 2005). These investigations provide strong molecular evidence for the integration of different developmental modules during the establishment of adaptive phenotypes, which has previously been demonstrated on a morphological level (Badyaev *et al.* 2005).

In conclusion, through examining the expression of putative plasticity genes in a developmental context, we have identified that a strongly environmentally responsive network underlies adaptive plasticity in a key innovation of a cichlid fish. Our analysis identified the coordinated coexpression of functionally related genes, which we consider to represent regulatory modules. As mechanical strain directs the expression of each of these modules, it promotes the development of an integrated, complex phenotype, the molariform PJA, which enables the efficient exploitation of an alternative trophic niche in *A. alluaudi*. This study forms a platform to empirically assess the molecular trajectories that underlie adaptive phenotypes across a phylogenetic context in cichlid fishes. Ultimately, it will help to evaluate the importance of phenotypic plasticity in cichlid evolution, specifically in the light of evolutionary mechanisms such as genetic assimilation and genetic accommodation (Waddington 1953; West-Eberhard 2004).

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Data accessibility

Linear morphometric measurements: doi:10.5061/dryad.rc638

Normalized relative quantification (RQ) of candidate genes: doi:10.5061/dryad.rc638

Distance matrices of the cluster analyses: doi:10.5061/dryad.rc638

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Gene clusters found in hierarchical cluster analyses of candidate genes coincide strongly for this and a previous study.

Fig. S2 Standard length of specimens according to treatment group and time in treatment.

Fig. S3 Residuals of linear morphometric measurements across time points and between diet groups.

Fig. S4 Linear morphometric measurements across SL of specimens in the two diet groups.

Fig. S5 PCA on linear morphometric measurements suggests phenotypic divergence between diet groups.

Fig. S6 Mean gene expression between diet groups across the developmental time-course. Solid and dashed lines reflect SD and HD fish, respectively. Error bars reflect 95% CIs.

Fig. S7 Individual hierarchical cluster analyses for each of the three major gene cluster.

Table S1 Gene abbreviations and primers used in this study.

Table S2 Results of linear morphometric measurement comparisons between diet groups.

Table S3 ANCOVAs on linear morphometric measures considering diet groups, time point and SL of the fish.

Table S4 Comparisons of RQ values between diet groups for each time point.

Table S5 Loadings and importance of components for the PCA on morphometric measurements (Fig. S5, Supporting Information).

Table S6 Loadings and importance of components for the PCA on gene expression.

Table S7 Distribution of TFBSs in the promoters of candidate genes.