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Molecular investigation of genetic assimilation during the rapid adaptive radiations of East African cichlid fishes

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Abstract

Adaptive radiations are characterized by adaptive diversification intertwined with rapid speciation within a lineage resulting in many ecologically specialized, phenotypically diverse species. It has been proposed that adaptive radiations can originate from ancestral lineages with pronounced phenotypic plasticity in adaptive traits, facilitating ecologically driven phenotypic diversification that is ultimately fixed through genetic assimilation of gene regulatory regions. This study aimed to investigate how phenotypic plasticity is reflected in gene expression patterns in the trophic apparatus of several lineages of East African cichlid fishes, and whether the observed patterns support genetic assimilation. This investigation used a split brood experimental design to compare adaptive plasticity in species from within and outside of adaptive radiations. The plastic response was induced in the crushing pharyngeal jaws through feeding individuals either a hard or soft diet. We find that nonradiating, basal lineages show higher levels of adaptive morphological plasticity than the derived, radiated lineages, suggesting that these differences have become partially genetically fixed during the formation of the adaptive radiations. Two candidate genes that may have undergone genetic assimilation, *gif* and *alas1*, were identified, in addition to alterations in the wiring of LPJ patterning networks. Taken together, our results suggest that genetic assimilation may have dampened the inducibility of plasticity related genes during the adaptive radiations of East African cichlids, flattening the reaction norms and canalizing their feeding phenotypes, driving adaptation to progressively more narrow ecological niches.

KEYWORDS
adaptive radiation, *alas1*, *Astatoreochromis alluaudi*, *Astatotilapia burtoni*, Cichlidae, flexible stem, *gif*, *Haplochromis ishmaeli*, pharyngeal jaw apparatus, phenotypic plasticity, *Pseudocrenilabrus multicolor*, *Tropheus moorii*

1 | INTRODUCTION

Adaptive radiations often involve either the colonization of novel habitats or movement into vacant niche space that can arise after the eradication of existing diversity (Losos, 2010). The formation of such species flocks is characterized by an explosive increase in species number within a lineage, typically via specialization to a multitude of ecological niches (Schluter, 2000). While intense competition between species for limited resources often results in extinction (competitive displacement) (Sepkoski, 1996), lineages that undergo adaptive radiation respond, more often, through diversification via the de novo occupation of vacant ecological niches (ecological

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opportunity) (Schluter, 2000). Thus, identifying the unique genetic, epigenetic, morphological and ecological features of lineages that undergo adaptive radiations provides a first step in understanding the mechanisms that promote speciation (Brawand et al., 2014; Henning & Meyer, 2014). In spite of the potential value of stem lineage characterization, few studies on adaptive radiation have focused on this aspect (Schluter, 2000; West-Eberhard, 2003).

It has been hypothesized that adaptive radiation may be initiated by lineages that have recently evolved key innovations (Simpson 1953), or display high levels of variation that is either genetic (Hedrick, 2013), plastic (Pfennig et al., 2010) or a combination of the two (Gomez-Mestre & Jovani, 2013). In spite of this, current research into the basis of adaptive radiations predominantly focuses on ultimate causes, namely natural selection of standing genetic variation (Mayr, 1961; Schluter, 2000), with fewer studies focusing on proximate causes such as phenotypic plasticity and developmental flexibility in stem lineages (West-Eberhard, 2003). Both proximate and ultimate experimental approaches are useful in explaining evolutionary phenomena; however, it is increasingly clear that proximate and ultimate causes can interact to drive evolution, so studies that incorporate both have higher explanatory power (Laland, Odling-Smee, Hoppitt, & Uller, 2013; Laland, Sterelny, Odling-Smee, Hoppitt, & Uller, 2011). Thus, studies that incorporate factors such as phenotypic plasticity have the potential to deepen our understanding of adaptive radiation, as it is likely to play an underappreciated role. Indeed, many of the model adaptive radiations contain at least some "basal" phenotypic plasticity in key ecological traits (West-Eberhard, 2003), such as sticklebacks (Lucek, Sivasundar, & Seehausen, 2014; Morris et al., 2014; Oke et al., 2016; Wund, Baker, Clancy, Golub, & Fosterk, 2008; Wund, Valena, Wood, & Baker, 2012), Anolis lizards (Kolbe & Losos, 2005; Losos et al., 2000), Darwin's finches (Tebbich, Sterelny, & Teschke, 2010), Hawaiian spiders (Yim, Brewer, Miller, & Gillespie, 2014) and cichlid fishes (Bouton, Witte, & Van Alphen, 2002; Meyer, 1987b; Muschick, Barluenga, Salzburger, & Meyer, 2011; Stauffer & van Snick Gray 2004; Wimberger, 1991). Moreover, a recent study even linked phenotypic plasticity to a major evolutionary transition—the origin of tetrapods (Standen, Du, & Larsson, 2014). In spite of this, phenotypic plasticity is frequently not considered both in discussions of mechanisms leading to adaptive radiations and in evolution more generally (Pigliucci, 2007; West-Eberhard, 2003).

During the initial phases of adaptive radiations, phenotypic plasticity can provide an inherent competitive advantage during the colonization of new or heterogeneous habitats (Baldwin, 1896; Ghalambor, McKay, Carroll, & Reznick, 2007; Lande, 2009; Morris et al., 2014; Richards, Bossdorf, Muth, Gurevitch, & Pigliucci, 2006). Phenotypically plastic species, which are commonly generalists (Van Tienderen, 1997), are also more likely to meet their resource requirements during the colonization of novel environments (Baldwin, 1896). Additionally, phenotypic plasticity enhances trait variability within stem lineages by generating multiple phenotypes from single genotypes (West-Eberhard, 2003) by exaggerating trait differences between lineages through ecological displacement (Schluter, 1994), and through increasing the accumulation of standing genetic variation (Gomez-Mestre & Jovani, 2013). Together, this would be expected to enhance the probability of rapid and parallel colonization of open niches, as is observed in adaptive radiations (Pfennig et al., 2010). Although phenotypic plasticity is likely to contribute to lineage diversification, the degree to which it contributes to this process remains unresolved (Hendry, 2016) and is an ongoing topic of debate (De Jong, 2005; Schneider & Meyer, 2017).

The concept of genetic assimilation shows how novel phenotypes (phenotypic diversification) can originate from phenotypic plasticity (Crispo, 2007; Pfennig et al., 2010; Schneider & Meyer, 2017). How might phenotypic plasticity contribute to the origin of new phenotypes (phenotypic diversification) and potentially to speciation (lineage splitting)? Genetic assimilation can contribute to speciation when phenotypic plasticity generates alternative phenotypes (West-Eberhard, 1989, 2003) that are later fixed through reproductive isolation. In this scenario, when specific plastic phenotypes are consistently induced over many generations through sustained exposure to stable environmental stimuli, natural selection acts only on these phenotypes and the associated quantitative genetic variation, while alternative phenotypes remain non-induced and are thus "released" from directional or stabilizing effects of selection (Waddington, 1953, 1961; West-Eberhard, 1989). Subsequently, the environmental sensitivity of these alternative phenotypes declines when natural selection acts either positively (DeWitt, Robinson, & Wilson, 2000) or negatively (Ghalambor et al., 2015) to the direction of plastic change, or through neutral processes (Masel, King, & Maughan, 2006). Cases that involve a reduction in phenotypic plasticity are termed genetic assimilation, while any shift in reaction norm after exposure to a novel environmental stimulus can be termed genetic accommodation (Crispo, 2007). For the purposes of this manuscript, we use the term genetic accommodation for changes that cannot be classed as genetic assimilation.

Genetic assimilation was first demonstrated empirically in fruit flies by Conrad H. Waddington (1953), and a putative molecular mechanism was proposed much later (Rutherford & Lindquist, 1998; Waddington, 1961). Although genetic assimilation has been demonstrated by common garden experiments (Carroll, Dingle, & Klassen, 1997; Carroll, Klassen, & Dingle, 1998; Chapman, Galis, & Shinn, 2000; Cook & Johnson, 1968; Ghalambor et al., 2007; Parsons & Robinson, 2006; Reznick & Ghalambor, 2005; Robinson & Wilson, 1996), the loci that may underlie this have rarely been studied in natural populations (Ledón-Rettig, Pfennig, Chunco, & Dworkin, 2014; Parsons et al., 2016). Therefore, their importance in natural evolutionary processes is difficult to evaluate. The genetic variation that contributes to genetic assimilation can arise through accumulation of standing genetic variation in the stem lineage (Jarosz & Lindquist, 2010), through epigenetic alterations (Sollars et al., 2003; Zhao et al., 2005) or through de novo mutation. Then, selection (Snell-Rood, Van Dyken, Cruickshank, Wade, & Moczek, 2010) or drift (Masel et al., 2006) influences the degree of plasticity of ecologically relevant traits in subsequent generations. For example, genetically assimilated loci in a derived, specialist lineage may show a lower magnitude expression response to a plastic stimulus (i.e., a

shallower reaction norm) in comparison with that of the stem lineage. In the case of adaptive radiations, the restriction of plasticity would make particular trait values increasingly heritable thus potentially allowing natural selection to further fine-tune the phenotypes to the specific niche requirements. Thus, less plastic specialist lineages would be expected to outcompete more plastic, generalist lineages when they inhabit a stable environment.

To test the role of phenotypic plasticity and possible subsequent genetic assimilation in adaptive radiations, we selected East African cichlid fishes as our models. As one of the most species-rich families of vertebrates, and with exceptionally large and diverse adaptive radiations that arose independently in East Africa and the New World, cichlid fishes represent a particularly suitable model for investigating speciation and adaptive radiation (Albertson, Markert, Danley, & Kocher, 1999; Brawand et al., 2014; Friedman et al., 2013; Genner et al., 2007; Salzburger, 2009; Schluter, 2000; Seehausen, 2006; Seehausen et al., 2008; Turner, 2007; Wagner et al., 2013). The adaptive radiations of the East African lakes are particularly impressive, with around 500 species in Lake Victoria alone, most of which evolved in less than 100,000 years (Genner et al., 2007; Meyer, Kocher, Basasibwaki, & Wilson, 1990; Salzburger, Mack, Verheyen, & Meyer, 2005; Stager & Johnson, 2008). Phenotypic plasticity may well have contributed to these dramatic radiations, as it plays a role in generating alternative feeding phenotypes that fine-tune the exploitation of specialized niches (Machado-Schiaffino, Henning, & Meyer, 2014; Meyer, 1987b, 1990a,b; Muschick et al., 2011; Stauffer & van Snick Gray, 2004; Van Dooren, Van Goor, & Van Putten, 2010; Wimberger, 1991). Moreover, phenotypic plasticity has been demonstrated in the feeding apparatus' of cichlid species from within (Bouton et al., 2002; Kerschbaumer, Postl, Koch, Wiedl, & Sturmbauer, 2011; Machado-Schiaffino et al., 2014; Muschick et al., 2011) and outside of (Stauffer & van Snick Gray, 2004; Wimberger, 1991) the cichlid adaptive radiations; however, until now, it was not compared in a single experiment.

Arguably the best-studied cichlid example of adaptive phenotypic plasticity is the pharyngeal jaw of *Astatoreochromis alluaudi* (Greenwood, 1965; Gunter et al., 2013; Hoogerhoud, 1986a,b; Huysseune, 1995; Huysseune, Sire, & Meunier, 1994; Schneider, Xiong, Li, Meyer, & Gunter, 2014; Smits, 1996; Smits, Witte, & VanVeen, 1996). This species modulates the development of its pharyngeal jaws (a second pair of "crushing" jaws in the throat), in response to the robustness of the available diet. Ingesting a soft diet (SD; e.g., insect larvae) induces a slender "papilliform" jaw with numerous fine teeth, while a hard diet (HD; hard-shelled snails) induces a

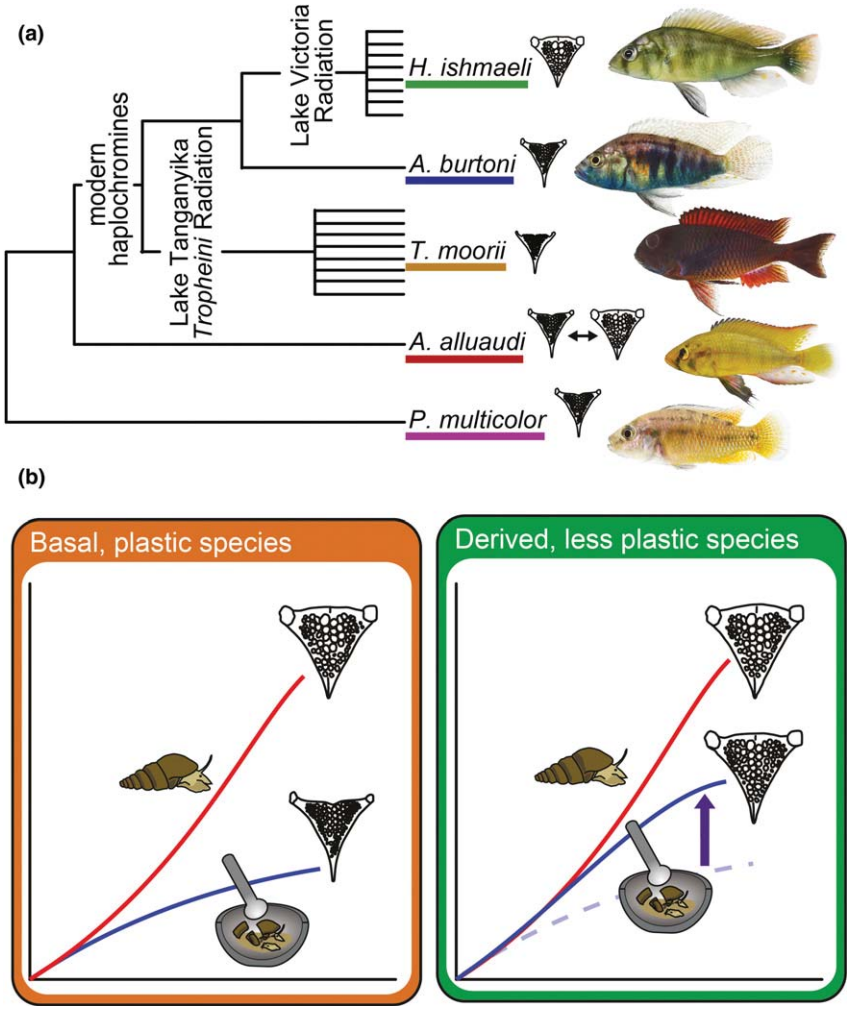

FIGURE 1 Evolutionary relationships and lower pharyngeal jaw (LPJ) morphology sketches of cichlid species included in this study. (a) Representatives of lineages basal to adaptive radiations include the riverine generalist species *Astatoreochromis alluaudi*, *Pseudocrenilabrus multicolor* and *Astatotilapia burtoni*. More "derived" species from adaptive radiations include *Tropheus moorii*, a specialist representative of the Lake Tanganyika Tropheini radiation, and *Haplochromis ishmaeli*, a specialist representative of the Lake Victoria radiation. (b) Hypothesis for the direction of change in morphology and gene expression under the assumption of genetic assimilation. Here, the baseline condition (soft diet) of more derived species should approach the induced condition (hard diet) for plastic species, and there should be overall a more limited degree of plasticity

hypertrophied "molariform" jaw with larger, molar-like teeth, most notably in the Lower Pharyngeal Jaw (LPJ) (Figure 1a). Thus, the resulting adults were suggested to more efficiently exploit the two alternative diet niches and avoid competition with other more specialized species (Cosandey-Godin, Binning, & Chapman, 2008; Greenwood, 1959, 1965; Hulsey, Hendrickson, & de León, 2005; Slootweg, Malek, & Mccullough, 1994). In addition to numerous morphological studies, recent molecular investigations of adaptive plasticity in the pharyngeal jaws of *A. alluaudi* have shed light on its transcriptional underpinnings (Gunter et al., 2013; Schneider et al., 2014). Specifically, this research has identified a number of genes that display dynamic patterns of expression throughout development of molariform and papilliform jaws. These genes are putatively involved in the development of bone, tooth and muscle, as well as the response to mechanical strain, the putative plasticity stimulus (Gunter et al., 2013; Schneider et al., 2014).

The goal of this research was to investigate whether diet-induced adaptive phenotypic plasticity is likely to have contributed to adaptive radiations in East African cichlid fishes. To examine this, we tested whether (i) proxies of the founders of the adaptive radiation are highly plastic in an adaptive trait (LPJ molariform/papilliform morphologies) and (ii) whether plasticity at that trait is lost during the adaptive radiation because of genetic assimilation. Our taxa included two specialist species from two independent adaptive radiations in Lakes Victoria (*Haplochromis ishmaeli*) and Tanganyika (*Tropheus moorii*) and two riverine generalist species branching more basally—that are phylogenetically and geographically outside these adaptive radiations. These include *A. alluaudi*, and *Astatotilapia burtoni*, plus a member of a more basally branching ancestral lineage, *Pseudocrenilabrus multicolor* (Figure 1a; for phylogenetic relationships see Salzburger et al., 2005 and Brawand et al. 2014). The relatively basal, nonradiating riverine lineages are hypothesized to fill a similar ecological niche to the common ancestor(s) of the radiations that colonized the lakes (Clabaut, Bunje, Salzburger, & Meyer, 2007; Genner et al., 2007; Meyer, 1993; Stager & Johnson, 2008) and the two others occupy more specialized trophic niches (Greenwood, 1965; Yamakoa, 1983). Diet manipulation experiments allowed us to test whether the cichlid adaptive radiations are likely to have evolved from a phenotypically plastic ancestral lineage that specialized and radiated to fill an array of narrow ecological (trophic) niches. The LPJs of diet-manipulated fish were then examined for morphology and gene expression through qRT-PCR of mechanically responsive genes identified by our previous study, with the goal of testing for genetic assimilation and its putative transcriptional basis.

2 | MATERIALS AND METHODS

2.1 | Cichlid diet experiments

Five cichlid species were selected for diet manipulation experiments based on previous evidence of diet-induced phenotypic plasticity, phylogenetic position and the diets of natural populations (Figure 1a). These included two rather basal species: *Astatoreochromis alluaudi* and *Pseudocrenilabrus multicolor*. *A. alluaudi* is known to have a highly plastic pharyngeal jaw that becomes larger and more robust in response to a hard diet such as hard-shelled snails. It is geographically widespread with populations that inhabit both riverine and lacustrine environments, exploiting both insects and algae (soft diet) and molluscs (hard diet) (Cosandey-Godin et al., 2008; Greenwood, 1965; Slootweg et al., 1994; Witte, 1981). We used a laboratory population that was obtained from the Mwanza Gulf in Lake Victoria in 1984 and, since then, was kept at Leiden University and the University of Konstanz and has been used in numerous plasticity experiments (Gunter et al., 2013; Hoogerhoud, 1986b; Huysseune, 1995; Huysseune et al., 1994; Schneider et al., 2014; Smits, 1996; Smits et al., 1996). As a second out-group, we used the generalist riverine species *P. multicolor*, which branches basally to *A. alluaudi* (Binning & Chapman, 2008; Salzburger et al., 2005) and has no previous record of plasticity in the pharyngeal jaws, albeit its gills (which are adjacent to the LPJ) are known to be plastic (Chapman et al., 2000; Crispo & Chapman, 2008, 2010). Additionally, more phylogenetically derived species were included: the riverine species *Astatotilapia burtoni*, a generalist that feeds mostly on insects, but also plants and algae (Salzburger et al., 2005; Sturmbauer, Hainz, Baric, Verheyen, & Salzburger, 2003; Theis, Ronco, Indermaur, Salzburger, & Egger, 2014), *Haplochromis ishmaeli*, a snail-cracking specialist endemic to Lake Victoria (Greenwood, 1965; Hoogerhoud, 1986a; Slootweg, 1987), and *Tropheus moorii*, an algae browsing specialist that is endemic to Lake Tanganyika (Sturmbauer, Mark, & Dallinger, 1992; Sturmbauer & Meyer, 1992; Sturmbauer et al., 2003; Yamakoa, 1983). All species had been bred in captivity for multiple generations and were obtained from laboratory stocks (*A. alluaudi* [Witte, Leiden] *A. burtoni* [Hoffman, Austin] and *T. moorii* [Sturmbauer, Graz]) or the aquarium trade (*H. ishmaeli* and *P. multicolor*). These species are likely to display similar patterns of development, as this is the case for cichlid species separated by greater phylogenetic distance (Holden & Bruton, 1994).

Diet manipulation experiments were conducted using methods modified from Gunter et al. (2013) and Meyer (1990a,b). Briefly, 1–2 clutches of ~6-month-old fish from each species (all bred at University of Konstanz) were raised separately then split into two groups. Each group was raised in a single tank and fed diets that differed only in the mechanical strain required for processing, but not in their nutritional content. One group ingested a hard diet (HD) composed of hard-shelled snails that had to be cracked with their pharyngeal jaws, and the second group ingested a soft diet (SD) of an equivalent quantity of crushed snails. Both were also fed ad libitum with commercial flake food each morning. The experiment was terminated after approximately 8 months, a period of time known to induce significant plasticity in *A. alluaudi* (Schneider et al., 2014). Our previous work showed that plasticity-driven size and shape differences develop gradually (Schneider et al., 2014), with statistically significant adaptive divergence after ~5 months of treatment. For all species, HD individuals were observed to take the snails into their mouths during the experimental period (H. Gunter pers. obs.). The standard length for each individual was noted, and the lower pharyngeal jaws (LPJ) were

dissected and stored in RNA-later at −20°C for further processing. All statistical analyses were performed with R (RC-Team 2012).

2.2 | Analytical workflow

Three main datasets were analysed in this study: LPJ linear morphometric measurements, LPJ geometric morphometric measurements and LPJ candidate gene expression measurements (details below). Each of the three raw data sets was processed, either for between-species comparisons or for within species diet group comparisons (HD vs. SD). For all six data subsets, the following analyses were performed: (i) a principal component analysis (PCA), in which all principal components (PCs) are considered until their cumulative explained variance exceeds 95% of the total variance; (ii) an analysis of variance (ANOVA) or multivariate ANOVA (MANOVA), testing for differences in PCs by species or diet (selection was depended on the number of considered PCs); (iii) pairwise comparisons among species or between diet groups, performed separately for each considered PC (when species were compared, Tukey-HSD post hoc correction was performed). Additional data set-specific analyses were performed as outlined below. Finally, we integrated gene expression and linear morphometric measurements using multiple linear regressions with species-specific PCA components as dependent and gene expression variables as independent variables.

2.3 | Linear morphometric analyses

LPJs were cleaned and photographed, and linear and geometric morphometric measurements were made according to Gunter et al. (2013). Final sample sizes for linear morphometric measurements were (n = SD, HD): *A. alluaudi* n = 9, 11; *A. burtoni* n = 15, 11; *H. ishmaeli* n = 8, 9; *P. multicolor* n = 5, 9; and *T. moorii* n = 7, 6. Briefly, linear measurements included length, width and depth of the LPJ, width of the muscle attachment horns, various tooth measurements and LPJ weight (Fig. S1). For species comparisons, data were first standardized through dividing morphometric measurements by standard lengths and then centring each variable (through subtracting the mean value from each data point). For diet group comparisons, first a linear regression was fitted separately for each species and measurement, using the morphometric variable as a dependent length and standard length as an independent variable to obtain size-corrected residuals. These fit residuals were then scaled and centred, that is, the mean for each gene was subtracted from individual values and was then used for within-species diet group comparisons. In addition to the aforementioned PCA and downstream analyses, pairwise comparisons of diets were conducted on all measurement variables using t tests for unequal variances and false discovery rate (FDR) post hoc p-value corrections.

2.4 | Geometric morphometric analyses

The geometric morphometrics analyses used two landmarks and 14 semi-landmarks that outlined the LPJs (Fig. S1D). Standardization for species comparisons was carried out by aligning all species' landmarks together using the R geomorph package procrustes fit (Adams & Otárola-Castillo, 2013). For diet group comparisons, LPJ landmarks were aligned for each species separately. As the LPJ is considered a bilaterally symmetrical structure, variation between the left and right side was removed: landmark coordinates from the left side of the LPJ were mirrored onto the right side along the LPJs' anterior–posterior axes and average coordinates for each landmark pair per LPJ were calculated. Then, left-side-averaged landmarks were mirrored back to the right side. No allometric effect of standard length on shape variation was detected within species. Final sample sizes were (n = SD, HD): *A. alluaudi* n = 9, 11; *A. burtoni* n = 11, 9; *H. ishmaeli* n = 8, 9; *P. multicolor* n = 5, 9; and *T. moorii* n = 6, 6.

2.5 | Gene expression analyses

RNA was extracted from the LPJs using a modified protocol, suitable for extractions from bones (Gunter et al., 2013). Thirteen candidate genes were selected from our previous publications (Gunter et al., 2013; Schneider et al., 2014), based on having putative functions in driving plastic development in the LPJs. These fall into different categories and are likely to influence different aspects of the development of plasticity in the LPJ, including the immediate response to mechanical strain, bone modelling and remodelling, and the development of larger teeth and muscles. Specifically, these include the "immediate early" genes *c-fos* and *rgs2*; genes that influence the osteoblast lineage *runx2b* and *osx*; extracellular matrix genes *col12* and *col6*; calcium pathway genes *ryr* and *srl*, and the muscle-related genes *tpm4* and *des*. Moreover, we included *gif*, *alas1* and *c1q-like*, genes with putative roles in the inflammatory response, which were observed to be repressed or induced in response to a strain-inducing diet in *A. alluaudi* (Gunter et al., 2013; Schneider et al., 2014). These genes showed a dynamic expression pattern during plastic development (Schneider et al., 2014), and the analysis of gene expression in the LPJs at ~8 months represents a snapshot of this pattern.

RNA extraction and gene expression analyses were performed only for *A. alluaudi*, *A. burtoni* and *H. ishmaeli* as morphometric measurements indicated no significant diet-induced divergence in *P. multicolor* and *T. moorii*, and we did not expect to see a difference in expression in these jaws (see Results section). Synthesis of cDNA and qRT-PCR was performed according to Schneider et al. (2014). Briefly, after confirming the quality of total extracted RNA (Bioanalyzer RIN values above 6.0 and A260:280 > 1.8), cDNA was synthesized using Superscript III, primed by oligo dTs. Primers of candidate genes were designed using sequence from *A. alluaudi* RNA-seq contigs (Table S1; Gunter et al., 2013), and their efficiencies were optimized for *A. alluaudi*, *A. burtoni* and *H. ishmaeli* through analyses of standard curves. Specifically, each primer pair was tested on a dilution series generated from pooled cDNAs from each species. In the cases where E < 1.85 or >2.15, new primers were designed, based on genome traces for *A. burtoni* (*osx*, *rgs2* and *gif*) (Brawand et al., 2014), or alternative regions of the *A. alluaudi* gene sequence for *H. ishmaeli* (*actinR*, *twinfilin*, *osx*, *runx2* and *gif*). Final sample sizes

were (n = SD, HD): *A. alluaudi* n = 9, 11; *A. burtoni* n = 11, 9; *H. ishmaeli* n = 8, 9.

qRT-PCR was used to analyse candidate gene expression in two technical replicates for each individual, where expression levels were averaged (Gunter et al., 2013). For among-species comparisons, gene expression levels were calculated using the formula: $\text{norm.exp}_{g\,i} = \frac{E_{g\,sp}{}^{-Ct_{g\,i}}}{\left(E_{act\,sp}{}^{-Ct_{act\,i}} + E_{twin\,sp}{}^{-Ct_{twin\,i}}\right)/2}$, which enabled between-species comparisons. Parameters are individual (i), mean C_t values (C_t) and its species' (sp) and gene's (g) respective efficiency value (E). Values were then normalized by dividing them by the mean absolute expression values of the two housekeeping genes (*act* and *twin*). Although our e-values were calculated using best practice methods, any biases in their calculation would more strongly affect the species comparisons than the between diet, within species comparisons. Across the three species, gene expression was then scaled and centred, that is, the mean for each gene was subtracted from individual values. For diet group comparisons, we calculated standardized and normalized gene expression levels according to reference gene expression levels following Gunter et al. (2013). These gene expression values were then scaled and centred species-wise.

In addition to the aforementioned analyses, diet-induced differences in candidate gene expression were analysed using pairwise t tests (for unequal variances). To correct for multiple testing, false discovery rate corrections were applied to all p-values for each species. Two-way ANOVAs on the gene expression data set were conducted (one per gene) using "gene expression" as the dependent variable and "diet group" and "species" as factors. To determine which species' reaction norm differs from which, the interaction of the two factors was also included and all ANOVAs were performed as pairwise comparisons, that is, each including only two of the three species. Notably, as gene expression was standardized gene-wise, gene effects are not informative so we focus our interpretation on the interaction term. To explore gene co-expression patterns, hierarchical clustering analyses were performed for each species (Haas et al., 2013). Finally, linear discriminant function analyses (lDFA) were conducted for *H. ishmaeli*, *A. burtoni* and *A. alluaudi* to predict species and diet group memberships, respectively, using gene expression data (using the lda() function from the R "MASS" package). Accuracy and leave-one-out (LOO) accuracy was calculated using the same dataset that was used to calculate the predictor to estimate predictor quality.

Reported effect sizes across analyses represent Cohen's d for ANOVAs and t tests, and η^2 for MANOVAs. The number of asterisks in plots indicates varying levels of significance (*.05 > $p \geq$ .01; **0.01 > $p \geq$ .001; ***.001 > p).

2.6 | Correction for sample-size effects by subsampling

To determine the levels of plasticity among plastic species (*H. ishmaeli*, *A. burtoni* and *A. alluaudi*, see Results) and facilitate comparisons among the plastic species, species-wise PCAs were also performed on randomly subsampled datasets (to equalize sample sizes of n = 17) for the linear morphometric, geometric and gene expression data sets. After each subsampling, MANOVAs were performed for each species on all considered PCs. This procedure was repeated 1,000 times for the linear morphometric data set (as sample sizes were more uneven) and 100 times for the other two data sets (which had more even sample sizes). Mean p-values, mean considered PCs and mean percentages of explained variation on PC1 are reported in Table S2.

2.7 | Integrating gene expression and morphology

To integrate gene expression measurements and morphometric measurements, multiple regression analyses were performed for *H. ishmaeli*, *A. burtoni* and *A. alluaudi* separately. For each analysis, one principal component of the species-wise PCAs on linear morphometric measurements was used as dependent variable, while the scaled and centred gene expression values were used as independent variables. Model selection was then performed by removing and/or adding individual genes (i.e., independent variables) from/to the model (using stepAIC() functions from the R "MASS" package). The model with the lowest AIC value was considered as the final model. These regressions were individually performed for PCs1-3, and thus, covering the largest proportion of variation found in the linear morphometric datasets.

3 | RESULTS

3.1 | Lower pharyngeal jaws of the focal species have distinct sizes, shapes and gene expression patterns

After conducting controlled feeding trials on the five focal cichlid species, LPJs were dissected, enabling both linear and geometric morphometric analyses to be performed. Our among-species PCA analysis of linear morphometric measurements confirmed that each of the five investigated species has a unique LPJ morphology, putatively reflecting their ecological niches (Figures 1a, 2a, Fig. S2). The first PC, which was predominantly loaded with LPJ weight, explained >95% of the total variation (Figure 2b; Fig. S2). An ANOVA indicated that the focal species differ significantly across PC1 (df = 4, F = 56.5, p < .001).

Similarly, our PCA of geometric morphometric measurements indicates moderate separation between the focal species, albeit to a lesser degree than the linear morphometric analyses (compare Figure 2a–c). Here, PC1 explains 51% of the variation, and PC2 explains 25% of the variation, with three further PCs required to explain 95% of the variation (Fig. S3). Jaw length and the shape of the posterior margin of the LPJ contribute most significantly to PC1 (Figure 2c,d), measures that are associated with relative molariformity (Gunter et al., 2013; Muschick et al., 2011). The different species clustered in a similar order to the linear morphometrics PCA, with *Astatoreochromis alluaudi* showing the most strongly molariform phenotype according to its position on PC1, and *Tropheus moorii* showing the most papilliform phenotype. A MANOVA confirms that species differ according to the considered PCs (df = 4, *Pillai* = 1.6, F = 10.2, p < .001).

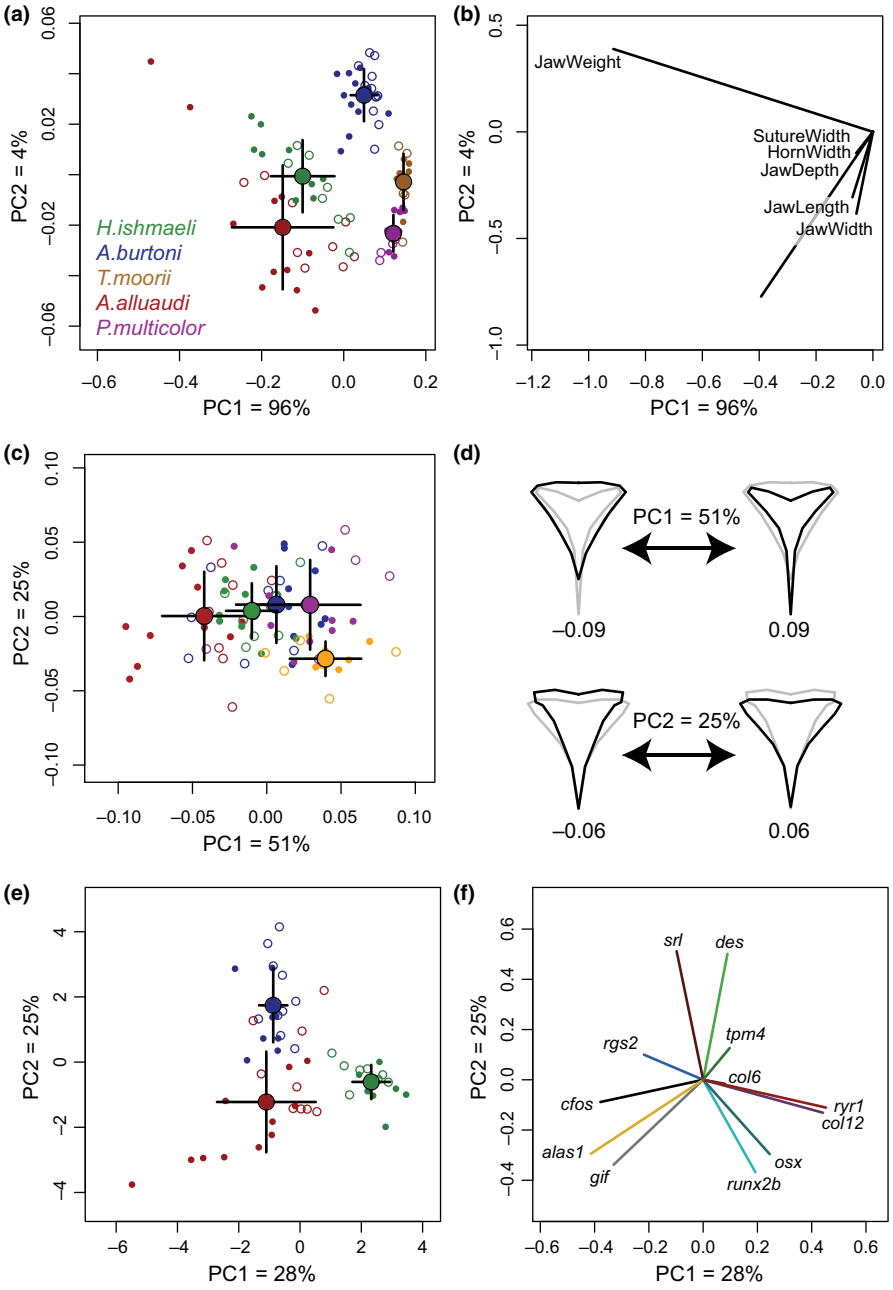

FIGURE 2 The lower pharyngeal jaws (LPJs) of five focal cichlid species occupy different morphospace, as per linear and geometric morphometric, and gene expression. (a, b) Principal component analyses were prepared for all linear LPJ measurements (absolute measurements divided by standard length), (c, d) geometric morphometric measurements and (e, f) gene expression values (normalized to two housekeeping genes). (a) The five focal cichlid species can be separated based on PC1 and PC2. The cross-hairs show mean and standard deviations within each species. (b) PC loadings indicate that JawWeight is the most significant contributor to PC1. (c) The five focal cichlid species are moderately well separated on PC1 and PC2. The cross-hairs show mean and standard deviations within each species. (d) Black shapes indicate the most extreme shape of the respective axis end, while grey shapes indicates the extreme of the other end. (e) The three cichlid species used for gene expression analysis can be separated based on PC1 and PC2. The cross-hairs show mean and standard deviations within each species. (f) The 12 candidate genes contributed variably to PC1 and PC2

A PCA on candidate gene expression was performed for the three species to visualize transcriptional differences (Figure 2e,f, Fig. S4) that may underlie species-level differences in LPJ morphology (Figure 2a–d, Figs. S2 and S3). All species could be separated on the first two PCs, whereby *Haplochromis ishmaeli* was significantly separated from *Astatotilapia burtoni* and *A. alluaudi* on PC1, and *A. burtoni* was significantly separated from *H. ishmaeli* and *A. alluaudi* on PC2 (Figure 2e, Fig. S2). PC1 explains 28% of the variation between individuals, and PC2 explains a further 25%. A further six PCs are required to explain 95% of the variation (Figs. S2 and S4). Different genes contribute to each of the PCs, with no single gene explaining a substantial portion of the variation (Figure 2e). Our MANOVA confirms species-specific gene expression ($df = 2$, *Pillai* = 1.8, $F = 147.4$, $p < .001$).

3.2 | Specialists of radiating lineages have shallower reaction norms than generalist, nonradiating lineages

Similar to our previous experiments (Schneider et al., 2014), we confirmed that hard diet (HD) and soft diet (SD) treatments cause *A. alluaudi* to develop divergent LPJ morphologies after a feeding period of 8 months (Fig. S5; Table S3). For this species, we observed strong differences in all linear morphometric measurements examined (Fig. S5, Table S3). Interestingly, significant morphological plasticity was also detected in the more slender-jawed species, *A. burtoni*, where LPJ plasticity has not previously been demonstrated (Fig. S5; Table S3). For this species, the largest differences were observed in LPJ depth, largest and average tooth size ($p < .001$) and jaw area ($p < .01$). Morphological divergence was also demonstrated for *H. ishmaeli*, albeit to a lesser

extent than for *A. alluaudi* or *A. burtoni* (Fig. S5, Table S3). Significant differences were observed in LPJ area and depth, centroid size, weight, horn width and suture width ($p < .05$). No significant differences were observed in *T. moorii* or *Pseudocrenilabrus multicolor* (Fig. S5, Table S3).

We conducted correlation-matrix-based PCAs of linear morphometric measurements for each species to explore patterns of diet-induced size variation (Figure 3). For *A. alluaudi*, PC1 explains 63% of the identified variation, and the two diet groups are clearly separable on this axis ($p < .001$, effect size Cohen's d = 2.61) (Figure 3m–p). For PCAs generated for *A. burtoni* and *H. ishmaeli*, PC1 explains 59% and 45% of the total variation, respectively, with both species showing significant divergence between the two diet treatments ($p < .001$ & Cohen's $d = -2.16$ for *A. burtoni* and $p < .05$ & Cohen's $d = 1.41$ for *H. ishmaeli*) (Figure 3a–h). In contrast, only 39% and 34% of the variation is explained by PC1 in *P. multicolor* and *T. moorii*, respectively, and diet-induced divergence is not statistically significant (Cohen's $d = 0.41$ and -0.23 for *P. multicolor* and *T. moori*, respectively, Figure 3i–l; q–t). MANOVA analyses on all considered PCs confirmed these patterns (Table S4). Subsampling of *A. alluaudi* and *A. burtoni* to match the sample size of *H. ishmaeli* confirmed that *A. alluaudi* is the most plastic species according to the linear morphometrics, closely followed by *A. burtoni*, while *H. ishmaeli* is considerably less (but still significantly) plastic (Table S5).

Further PCAs were conducted on geometric morphometric measurements for each species to explore patterns of diet-induced shape variation (Figs. S6 and S7). Hard and soft diets induced significant differences on at least one PC for *A. alluaudi*, *A. burtoni* and *H. ishmaeli* (PCs 2, 1 and 3, respectively; Fig. S7). No significant shape differences were observed for *P. multicolor* or *T. moorii* on any of the PCs that cumulatively explain >95% of the variation (Figs. S6 and S7). Furthermore, MANOVA analyses suggest that although *A. burtoni* is the only species with significant diet group differences on PC1 (which carries the highest proportion of variation), significance using multiple PCs is higher in *A. alluaudi* and *H. ishmaeli* (Table S6), as also suggested by the higher effect sizes ($\eta^2 = 0.38$, 0.63 and 0.59 for *A. burtoni*, *H. ishmaeli* and *A. alluaudi*, respectively). Subsampling procedures further suggest that the attained level of significance of *A. burtoni* on PC1 may be the result of having a slightly higher sample size than *H. ishmaeli* and *A. alluaudi* (Table S2). Nonetheless, *A. burtoni* still shows a slightly higher level of significance than *H. ishmaeli*.

3.3 | Species-specific patterns of gene expression associated with adaptive plasticity in basal vs. derived lineages

Expression of thirteen candidate genes was examined in the LPJs of HD and SD individuals for *A. alluaudi*, *A. burtoni* and *H. ishmaeli*, using qRT-PCR (Fig. S8, Table S7). These were categorized according to functional annotations that include immediate early genes, heme genes, matrix genes, bone genes, muscle genes and calcium genes (Gunter et al., 2013). A few genes showed statistically significant differences in their expression between SD and HD groups. Among them were *gif* and *alas1* for *A. alluaudi*, and *osx* and *col6* for *H. ishmaeli*. Moreover, an ANOVA predominantly supported these results, with significant interactions between species and diet for *osx*, *alas1*, *col6* and *col12* (Table 1), while marginal significance was detected for *gif*.

Species-wise PCA on gene expression revealed that in *H. ishmaeli*, *A. burtoni* and *A. alluaudi* the scores of PC1 and PC3; PC1 and PC2; and PC4 were significantly different between diet groups, respectively ($p < .05$, Fig. S9). Combined with significant MANOVAs using the considered PCs as dependent and diet as an independent variable ($df = 1, 1, 1$ Pillai = 0.85, 0.75, 0.79, $F = 5.5, 4.0, 6.58$, $p = .013, .018, .002$, $\eta^2 = 0.85, 0.75, 0.79$; for *H. ishmaeli*, *A. burtoni* and *A. alluaudi*, respectively), our data suggest that gene expression patterns differ between diet groups, although other factors may affect gene co-expression, as the scores of most heavily loaded PCs do not differ more strongly between diet groups. Our subsampling suggests that slightly higher significance levels in *A. burtoni* may be an artefact from slightly unbalanced sample sizes as subsampling reduces its significance level to lower than *A. alluaudi* or *H. ishmaeli* and effect sizes are similar among species (Table S2).

Linear discriminant function analysis was used to evaluate whether an individuals species classification could be correctly predicted based on its diet-induced gene expression patterns (Figure 4, Fig. S10). LD1 separated *H. ishmaeli* from *A. alluaudi* and *A. burtoni*, while LD2 separated all three species (Figure 4a). LOO cross-validation indicated a high accuracy, showing that all three species could be separated on the basis of gene expression (Figure 4b). Interestingly, genes relating to muscle structure and function (specifically calcium channels) on both LD 1 and LD 2 contributed most strongly to the predictors (*ryr1*, *srl* and *tpm4*), suggesting that they may dictate the observed species-level differences in the diet-induced plastic response (Fig. S10).

3.4 | Gene expression relates to principle components of morphological variation

We used multiple regression analysis to explore the relationship between gene expression and the first two principal components of linear morphological variation among *A. alluaudi*, *A. burtoni* and *H. ishmaeli* (Figure 5). Significance of selected multiple regression models was found for PC1 for *A. alluaudi*, PCs1 and 2 for *A. burtoni*, and PC2 for *H. ishmaeli*. Among these, the PCs showed significant

FIGURE 3 Influence of diet on linear morphometric measurements in the lower pharyngeal jaws (LPJs) of cichlid species from within and outside adaptive radiations. Principal component analyses were produced for all measurements, and (a, e, i, m, q) percentage of explained variance for each principal component, (b, f, j, n, r) variation on PC1 for soft diet (SD) and hard diet (HD) treatments, (c, g, k, o, s) variation at PC1 and PC2 and (d, h, l, p, t) loadings of each measurement on PC1–2 are displayed. Significant differentiation at PC1 was detected between HD and SD treatments for (b) *H. ishmaeli* ($p < .05$), (e) *A. burtoni* ($p < .01$), (m) *A. alluaudi* ($p < .001$); however, significant differentiation was not detected for (i) *T. moorii* ($p > .05$), or (q) *P. multicolor* ($p > .05$)

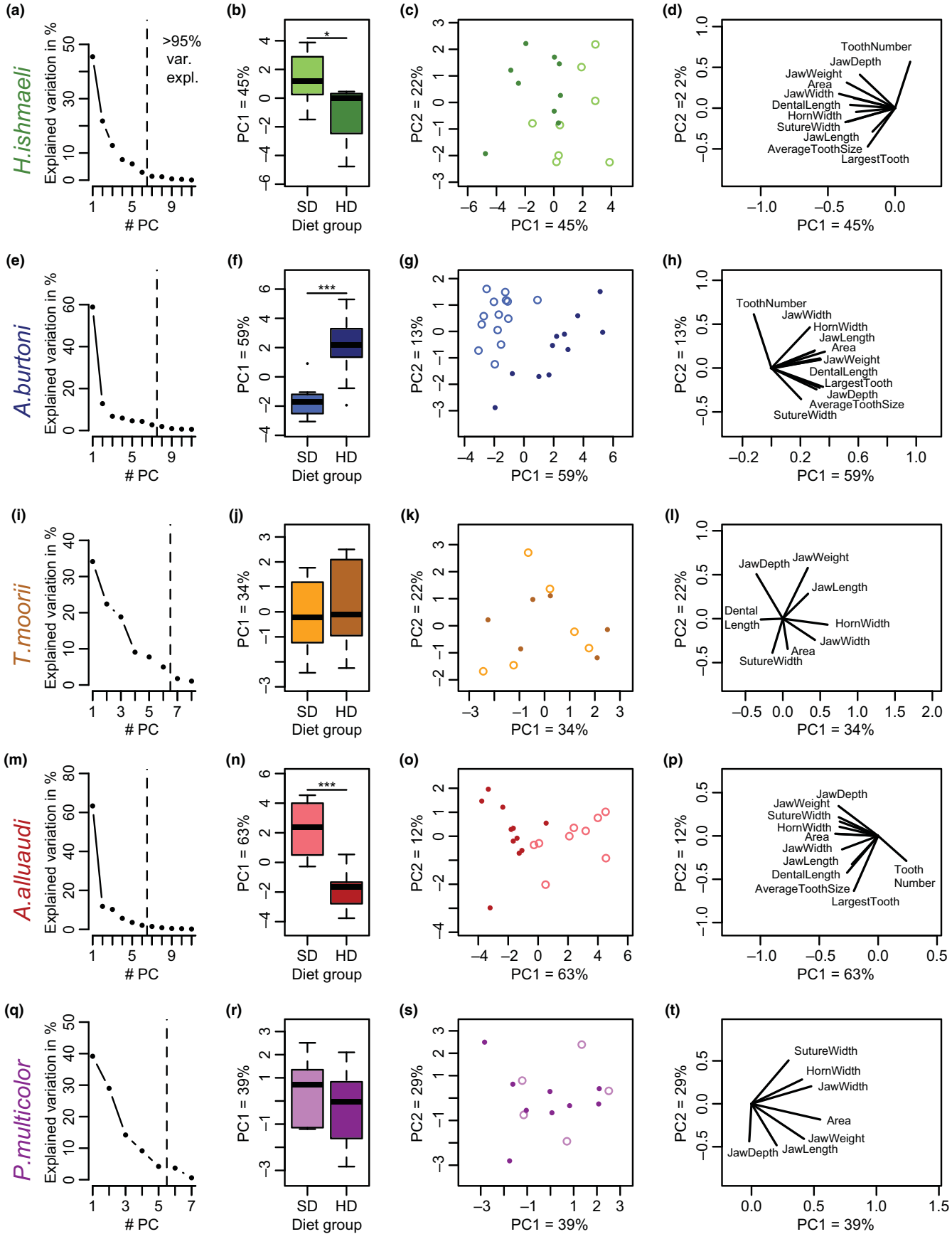
(a)
H.ishmaeli
Explained variation in %
>95% var. expl.
PC
(b)
PC1 = 45%
SD HD
Diet group
(c)
PC2 = 22%
PC1 = 45%
(d)
PC2 =2 2%
PC1 = 45%
ToothNumber
JawDepth
JawWeight
Area
JawWidth
DentalLength
HornWidth
SutureWidth
JawLength
AverageToothSize
LargestTooth
(e)
A.burtoni
Explained variation in %
PC
(f)
PC1 = 59%
SD HD
Diet group
(g)
PC2 = 13%
PC1 = 59%
(h)
PC2 = 13%
PC1 = 59%
ToothNumber
JawWidth
HornWidth
JawLength
Area
JawWeight
DentalLength
LargestTooth
JawDepth
AverageToothSize
SutureWidth
(i)
T.moorii
Explained variation in %
PC
(j)
PC1 = 34%
SD HD
Diet group
(k)
PC2 = 22%
PC1 = 34%
(l)
PC2 = 22%
PC1 = 34%
JawDepth
JawWeight
JawLength
Dental Length
HornWidth
JawWidth
Area
SutureWidth
(m)
A.alluaudi
Explained variation in %
PC
(n)
PC1 = 63%
SD HD
Diet group
(o)
PC2 = 12%
PC1 = 63%
(p)
PC2 = 12%
PC1 = 63%
JawDepth
JawWeight
SutureWidth
HornWidth
Area
JawWidth
JawLength
DentalLength
AverageToothSize
LargestTooth
Tooth Number
(q)
P.multicolor
Explained variation in %
PC
(r)
PC1 = 39%
SD HD
Diet group
(s)
PC2 = 29%
PC1 = 39%
(t)
PC2 = 29%
PC1 = 39%
SutureWidth
HornWidth
JawWidth
Area
JawWeight
JawDepth
JawLength

TABLE 1 Results from pair-wise ANOVAs using species as independent variable and gene expression as dependent variable

Gene	Species comparison	R^2	Est. diet	Est. species	Est. interact
runx2b	*alluaudi* vs. *burtoni*	.277	0.095	0.528**	−0.010
osx	*alluaudi* vs. *burtoni*	.577	−0.016	0.778***	−0.237
	alluaudi vs. *ishmaeli*	.172	−0.016	−0.062	0.247*
	burtoni vs. *ishmaeli*	.529	−0.254	−0.840***	0.484*
gif	*alluaudi* vs. *burtoni*	.406	0.691**	0.184	0.086
	alluaudi vs. *ishmaeli*	.503	0.691***	0.438**	−0.369 .
	burtoni vs. *ishmaeli*	.335	0.777***	0.254	−0.455 .
alas1	*alluaudi* vs. *burtoni*	.457	0.430**	0.343**	−0.014
	alluaudi vs. *ishmaeli*	.482	0.430***	0.518***	−0.349*
	burtoni vs. *ishmaeli*	.298	0.416***	0.175	−0.334*
col6	*alluaudi* vs. *ishmaeli*	.207	−0.412	−0.934**	0.864*
	burtoni vs. *ishmaeli*	.191	0.163	−0.266	0.288
col12	*alluaudi* vs. *burtoni*	.156	0.174	0.579**	−0.477
	burtoni vs. *ishmaeli*	.565	−0.303*	−0.858***	0.573**
ryr1	*burtoni* vs. *ishmaeli*	.142	0.079	0.341*	−0.089

Asterisks indicate the level of significance per model estimate.

diet-related differences for *A. alluaudi* and *A. burtoni*, but not *H. ishmaeli*, which showed a nonsignificant trend of diet separation on PC2. For *A. burtoni* and *A. alluaudi*, we identified genes significantly contributing to the above morphological PCs, with each species showing a unique pattern of gene regulation. Notably, for *A. alluaudi* and *A. burtoni*, genes such as *alas1* and *tmp4* are negatively and positively associated with the molariform phenotype, respectively (Figure 5c,e). Moreover, *alas1* and *gif* show opposite patterns of association for these two species, despite belonging to similar functional categories. For *H. ishmaeli*, *alas1* is positively associated with variation on PC2, while *tmp4* is negatively associated (Figure 5b). *c-fos* is positively associated with molariformity for *A. alluaudi*, *A. burtoni* and *H. ishmaeli*, potentially reflecting its role in remodelling of bone and eruption of new (larger) teeth (Figure 5b,c,e).

3.5 | Candidates for genetic assimilation identified in derived lineages

Our analysis of gene expression identified patterns that are suggestive of genetic assimilation or more specifically, a reduction in plasticity in derived lineages. First, gene expression patterns were compared to our model of genetic assimilation (Figures 1b and 6c–d). Expression of *gif* and *alas1* was consistent with our hypothesis of genetic assimilation, as both genes were significantly differentially expressed in *A. alluaudi* and *A. burtoni*, and their normalized expression levels in *H. ishmaeli* SD individuals were more similar to HD than for the other two species (Figure 6A). That is, the reaction norm for these genes was shallower for *H. ishmaeli* than for *A. alluaudi* or *A. burtoni*. Conversely, *osx* and *col6* showed expression patterns that represented a shift in the reaction norm in response to a novel environmental stimulus that differed from genetic assimilation (Figure 6b) and were categorized as genetic accommodation (Crispo, 2007). These were significantly differentially expressed in *H. ishmaeli*; however, they were not differentially expressed in *A. alluaudi* or *A. burtoni*.

To investigate any putative alterations in gene expression networks in our focal species, we conducted hierarchical clustering analyses (Figure 6e–g). As was observed in previous studies (Gunter et al., 2013; Schneider et al., 2014), genes from similar functional categories were co-expressed in *A. alluaudi*. For example, discrete clusters were generally observed for the matrix genes (*col6* and *col12*), the immediate early genes (*c-fos* and *rgs2*) and the bone genes (*osx* and *runx2b*). Also similar to Schneider et al. (2014), muscle-related genes (*tpm4* and *des*) clustered with calcium-related genes (*ryr1* and *srl*). The majority of genes analysed for *A. burtoni* and *H. ishmaeli* displayed patterns of co-expression that were similar to *A. alluaudi*; however, they were not identical (Figure 6e–g). Notably for *A. burtoni*, matrix genes *col6* and *col12* were not coregulated, but rather *col6* did not cluster with any other genes, while *col12* clustered with the bone genes (Figure 6f). Additionally, for *H. ishmaeli* the bone genes (*osx* and *runx2b*) clustered within the muscle/calcium

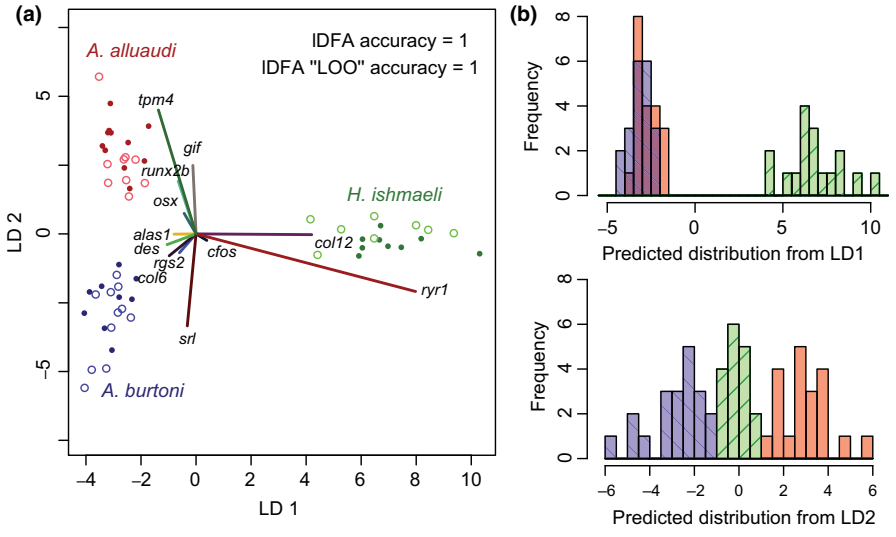

FIGURE 4 Linear discriminant function analysis (lDFA) shows that gene expression can be used as a reliable predictor of species membership. Here, species was the dependent variable and gene expression (absolute, normalized values) was the independent variable matrix. (a) The three focal species could be clearly differentiated and (b) standard as well as leave-one-out (LOO) cross-validation support the reliability of predictors

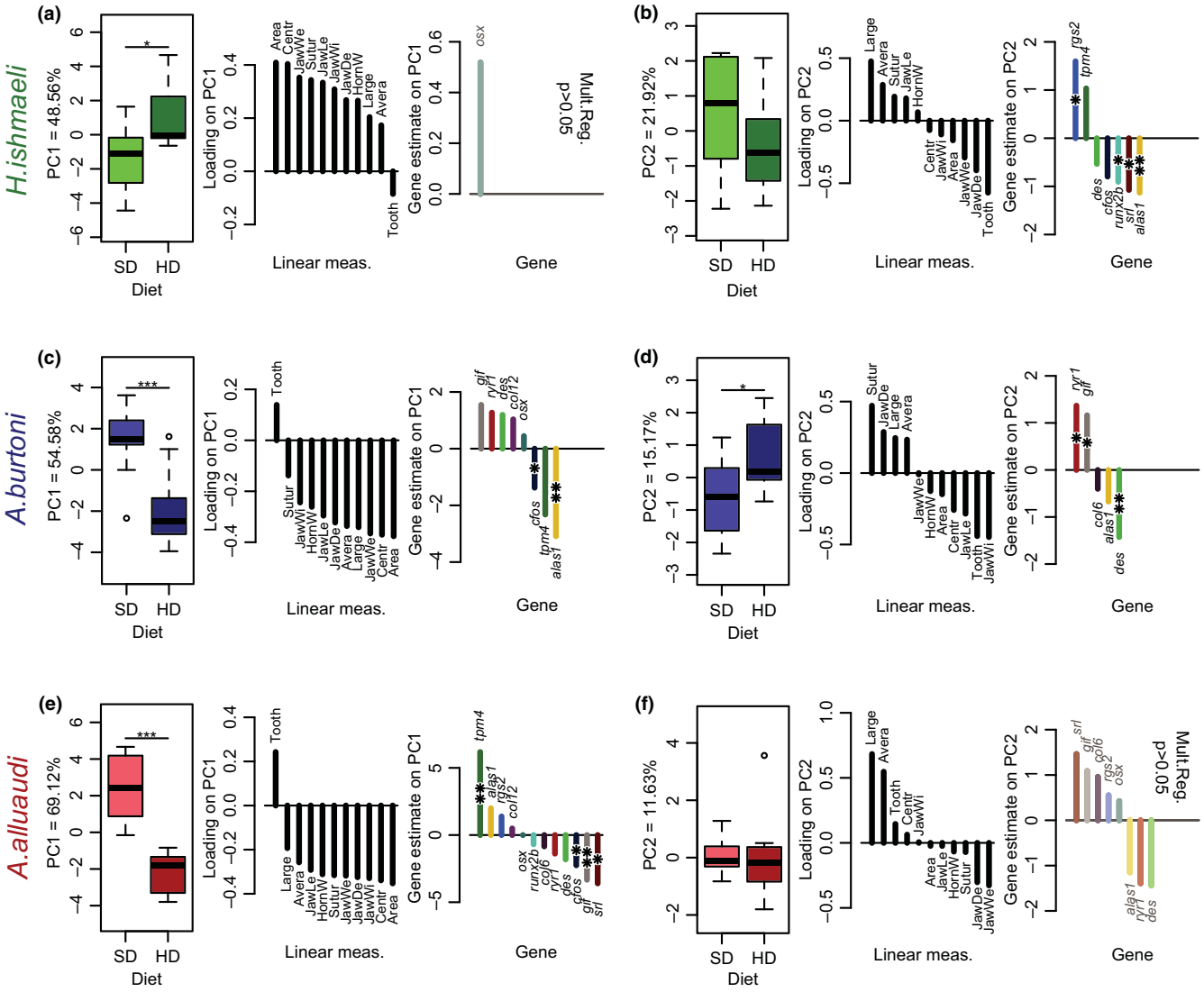

FIGURE 5 Linear regression shows that diet-induced morphology can be associated with gene expression. Here, the dependent variable was the PC1 scores of the PCA on linear morphometric measurements (Figure 2a) and gene expression was used as an independent matrix of variables. We report these for (a, c, e) PC1 and (b, d, f) PC2. Plots show (left) PC1 scores of linear morphometrics for the 3, (middle) the weighting of each morphometric variable on PC1 and (right) the contribution of each gene to the linear regression model. Area = dental area size, Avera = average tooth size, Centr = centroid size, HornW = horn width, JawDe = jaw depth, JawLe = jaw length, JawWe = jaw weight, JawWi = jaw width, Large = largest tooth size, Suture = suture width, Tooth = tooth number

genes, while the matrix genes (*col6* and *col12*) formed a discrete cluster (Figure 6e). Together, these results suggest potential differences in the mechanical properties of the bone matrix and the proliferation of bone and tooth cells that comprise the LPJs of these species. Moreover, three genes identified as being substantial for our lDFA predictor (*ryr1*, *srl* and *tpm4*) were also consistently co-expressed for all three species according to our hierarchical cluster analysis (Figures 5 and 6e–g).

4 | DISCUSSION

Adaptive radiations have been the focus of many research investigations; however, relatively few studies have focused on whether particular characteristics of founding lineages predispose them to forming adaptive radiations (Schluter, 2000; West-Eberhard, 2003). One proposal is that such lineages might be especially phenotypically plastic (i.e., flexible stem lineages), which might facilitate the repeated colonization of different replicated environments, such as lakes, where one of a set of previously evolved alternative or stage-specific phenotypes is advantageously expressed (West-Eberhard, 1989, 2003, 2005). This hypothesis has gained empirical support in recent years through research that examined inducible plasticity in lineages within a phylogenetic context (Kerschbaumer et al., 2011; Kolbe & Losos, 2005; Losos et al., 2000; Meyer, 1987b; Muschick, Indermaur, & Salzburger, 2012; Tebbich et al., 2010; Wund et al., 2008, 2012). Through comparing the impacts of diet manipulation (hard vs. soft diets)

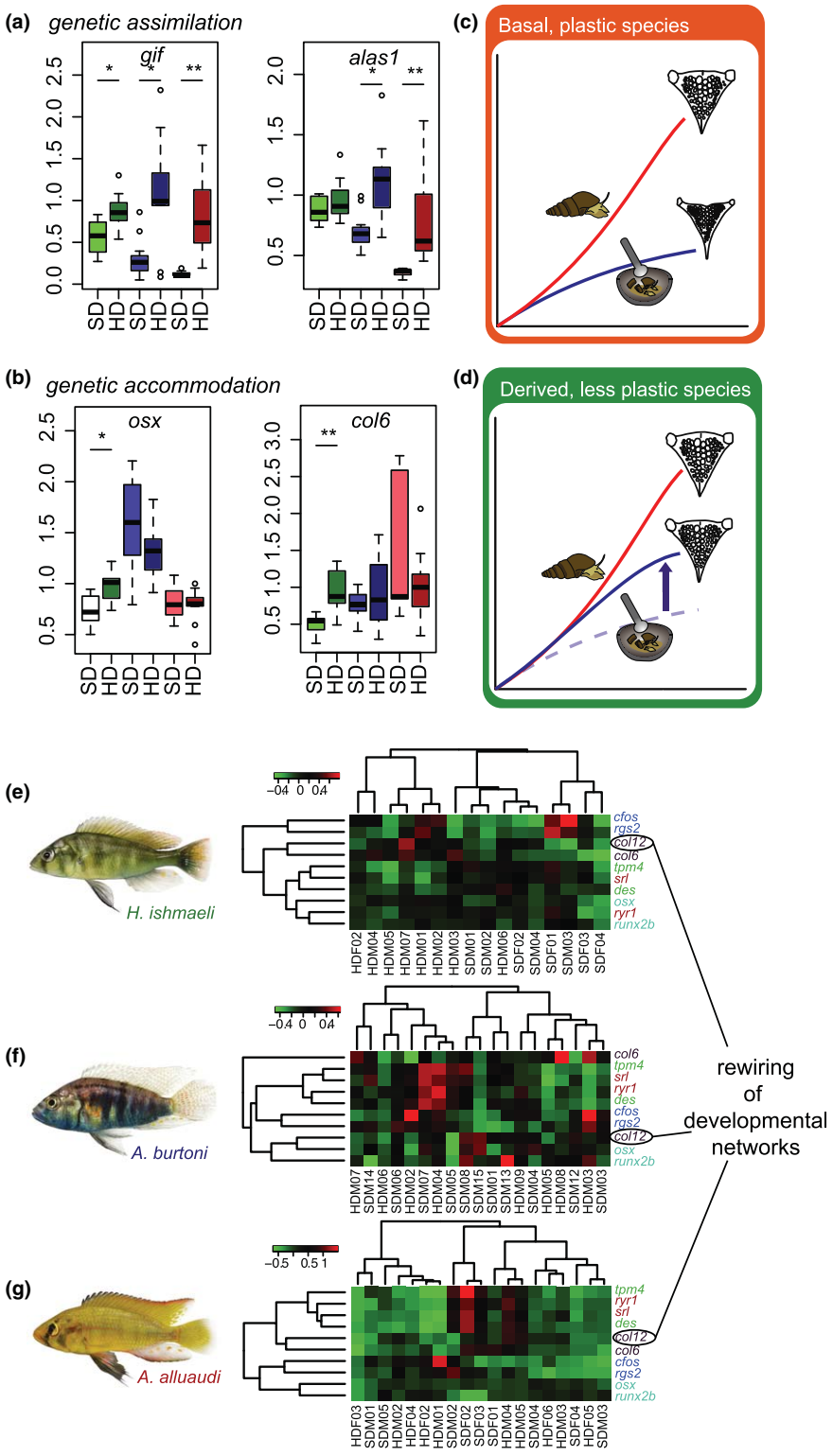

FIGURE 6 Analysis of gene expression and hierarchical cluster analysis on candidate gene expression suggest species-specific gene expression by rewiring of regulatory networks. Dampened norms of reaction in gene expression found in *gif* and *alas1* (a) suggest events of genetic assimilation in more specialized species (c, d) while steeper slopes in *osx* and *col6* in more specialized species (b) suggest rewiring of regulatory networks via genetic accommodation, potentially to induce altered new adaptive responses. In general, gene expression clusters by gene functional category for individuals fed a hard or soft diet for (e) *H. ishmaeli*, (f) *A. burtoni* and (g) *A. alluaudi*. HDM, hard diet male; HDF, hard diet female; SDM, soft diet female; SDF, soft diet female. Coloration of gene names refers to functional class: immediate early genes are blue, calcium pathway genes are red, muscle-related genes are green, matrix-related genes are violet, and bone-related genes are cyan. Deviations in clustering among species (e.g., *col12*) may indicate a rewiring of the underlying gene regulatory network in more specialized species

on morphology and gene expression in a range of East African cichlid species, we demonstrate that (i) proxies of the founders of the adaptive radiation are highly plastic in an adaptive trait (LPJ molariform/papilliform morphologies) and (ii) that plasticity at that trait is lower in the studied members of the adaptive radiations and (iii) we identify transcripts that have potentially undergone genetic assimilation.

It has been proposed that adaptive radiations should, in part, be driven by competitive interactions, as indicated by ecological character displacement (Schluter, 2000; but see [Stuart & Losos, 2013]). Indeed, the LPJs of the cichlid species included in our study predominantly occupy unique phenotype space, which is most likely caused by their distinct gene expression patterns. Such differences in LPJ shape tend to arise convergently among cichlids and are correlated

with differences in feeding efficiency on different diets (Elmer et al., 2014; Kusche, Recknagel, Elmer, & Meyer, 2014; Muschick et al., 2012). Interestingly, the sizes and shapes of *A. alluaudi* and *H. ishmaeli's* LPJs differ only subtly and they occupy what would appear to be the same ecological niche (both are pharyngeal mollusc crushers), albeit there may be fine-grained differences between them. Ecological separation of these two species is plausible in the wild, as they occupy slightly different spatial (Abila, 2011) and bathymetric environments (Witte, 1981), and may have a preference for different prey size classes (Slootweg, 1987). It is plausible that any of the >13 molluscivorous species in the Mwanza Gulf may show subtle, unappreciated differences in the micro-environment and niche inhabited (Witte, 1981). Such minor differences reduce direct competition between these species and may be important in enabling such explosive adaptive radiations. It should be noted, however, that *Astatoreochromis alluaudi* has a wide distribution in rivers and lakes (including Lake Victoria and its swampy satellite lakes), whereas *H. ishmaeli* is endemic to Lake Victoria (Slootweg, 1987; Witte, 1981). It is unknown whether their trophic niches would overlap completely if their geographic distributions were identical.

In addition to the observed species-level differences between LPJ morphologies, the phenotype space occupied by three of our five examined species was significantly expanded through diet-induced phenotypic plasticity. This reflects an adaptive response in *A. alluaudi* and likely, to a somewhat lesser extent, in *H. ishmaeli* and *A. burtoni*. Expanding the expressed phenotype to match available food resources is likely to confer a competitive advantage in terms of responding to seasonal and spatial variation in food availability, leading to local adaptation, albeit this was not explicitly tested in our study. Indeed, similar studies on different species have shown a putative advantage to adaptive phenotypic plasticity such as predator avoidance phenotypes in tadpoles (Pfennig & Murphy, 2000, 2002), drought tolerance in plants (Sultan, Barton, & Wilczek, 2009), and trophic polymorphisms in goodeid fish (Grudzien & Turner, 1984), arctic charr (Nordeng, 1983) and stickleback (Day, Pritchard, & Schluter, 1994; Svanbäck & Schluter, 2012; Wund et al., 2008). Although it is clear that phenotypic plasticity may increase niche width, the issue of whether adaptive plasticity promotes or hinders evolution remains a topic of debate (Hendry, 2016), perhaps because it is a question better treated on a case-by-case basis rather than as subject to a general rule.

In the few empirical studies that have directly assessed the role of phenotypic plasticity in evolution to date, some have indicated a role for plasticity (Schaum & Collins, 2014) and some have not (Torres-Dowdall, Handelsman, Reznick, & Ghalambor, 2012). It is likely that both responses might, under different ecological and evolutionary scenarios, lead to different outcomes. Thus, Hendry (2016) stresses that future research should aim to tease apart the conditions under which adaptive plasticity promotes or constrains speciation. Many studies have examined the temporal and spatial grain of environmental variability under which plasticity is expected to be favoured (Ghalambor et al., 2007; Scheiner, 1998; Snell-Rood et al., 2010; Stomp et al., 2008; Van Tienderen, 1997; Via & Lande, 1985; West-Eberhard, 1989).

Our data-set supports the hypothesis that ancestral plasticity may have been canalized during the formation of the cichlid adaptive radiations, in particular because the basal, generalist molluscivore *A. alluaudi* shows a higher level of inducible plasticity than the derived, specialist molluscivore, *H. ishmaeli*. Across the size and shape datasets, we observed the highest level of adaptive plasticity the LPJ of *A. alluaudi* (i.e., the highest level of statistical significance for the highest number of measured variables), an intermediate level in *A. burtoni*, a slightly lower level in *H. ishmaeli*, and almost none for *T. moorii* (an algal scraping specialist) or *P. multicolor*. Adaptive plasticity is well documented in *A. alluaudi* (Greenwood, 1965; Hoogerhoud, 1986a,b; Huysseune, 1995; Huysseune et al., 1994; Smits, 1996; Smits et al., 1996); however, diet-induced LPJ plasticity was not previously demonstrated in *H. ishmaeli* or *A. burtoni* to the best of our knowledge. The detection of lower plasticity in the LPJ of *H. ishmaeli* than *A. alluaudi* suggests that genetic assimilation has commenced in this lineage, but is incomplete. Moreover, our findings imply that LPJ plasticity may be more pronounced among the riverine, more generalist and more basal cichlid lineages—an intriguing hypothesis whose testing would require further, future studies. The observation of slightly higher plasticity in the LPJ of *A. burtoni* than of *H. ishmaeli* is of particular interest, as *H. ishmaeli* has a considerably more molariform jaw than *A. burtoni* (Hoogerhoud, 1986a), which is better suited to cracking snails, enabling a more effective exploitation of the experimental (hard) diet from the outset (Slootweg, 1987). The lack of plasticity in the LPJ of the basal generalist *P. multicolor* is most likely because this is a dwarf species with jaw muscles that are likely to be too small to crack hard snail shells. Indeed, its mean SL at the termination of the experiment was 44.6 mm—considerably less than the SL at which *A. alluaudi* first displayed significant LPJ plasticity (55–60 mm) in our previous developmental study on *A. alluaudi* (Schneider et al., 2014). The examination of additional basal representatives is especially important due to this unexpected result, keeping the caveat in mind that one would be investigating plasticity in extant lineages.

Our observation of generally higher plasticity in the more basal, riverine lineages, particularly in comparison with *H. ishmaeli*, provides support for the hypothesis that the cichlid radiations have been initiated by flexible stem lineages (Muschick et al., 2011; Parsons et al., 2016), as was suggested for other adaptive radiations (Tebbich et al., 2010; West-Eberhard, 2003; Wund et al., 2008). The potential that a flexible stem lineage has formed the East African cichlid radiations is particularly appealing as they have evolved multitudinous phenotypes in parallel, which may have involved hypervariable flexible stems (West-Eberhard, 2003), rather than the more limited polymorphic flexibility displayed by the other well studied lineages such as sticklebacks (Rundle, Nagel, Boughman, & Schluter, 2000). It is likely that adaptation to these multidimensional, specialized trophic niches has involved concomitant changes in learning and behaviour on top of, or instead of morphology (Meyer, 1986, 1987a), as behavioural changes have the potential to directly influence morphology through processes such as bone modelling and remodelling (Currey, 2002). Although a behavioural component was not incorporated into our study design, we observed that species such as *A. alluaudi*, *A. burtoni*

and *H. ishmaeli* reacted to the offered novel diets more readily and enthusiastically than the other species, particularly *T. moorii* (pers. obs. H. Gunter). It is plausible that behavioural differences between the species (particularly in *T. moorii*) may have contributed to the different levels of phenotypic plasticity in their LPJs.

In addition to interpreting our results in light of the flexible stem hypothesis, we are also able to make a broader comparison of generalist and specialist lineages. Our results show that generalist (basal) lineages (*A. alluaudi* and *A. burtoni*) display a higher level of plasticity than the more derived, specialist lineages (*H. ishmaeli* and *T. moorii*), in line with previous studies (Sultan et al., 2009; Svanbäck & Schluter, 2012), and with the niche variation hypothesis (Van Valen, 1965). Our results support the hypothesis that stem lineages leading to adaptive radiations contain generalist ancestral lineages, just as was found in earlier studies (Nosil, 2002) and models (Stomp et al., 2008; Van Tienderen, 1997); however, there are many exceptions, where specialists give rise to adaptive radiations, particularly among phytophagous insects such as Hawaiian drosophila (Schluter, 2000; West-Eberhard, 2003). Based on quantitative genetic models, phenotypic plasticity is likely to be lower in specialist species (Van Tienderen, 1997), and therefore, other factors such as standing genetic variation or de novo mutations may play a relatively larger role in these instances. However, West-Eberhard (2003) shows how flexible stem plasticity in behaviour and physiology could have played a role in the adaptive diversification of Hawaiian drosophila in addition to African cichlids and other fish taxa discussed in Schluter (2000). This is especially true for radiations seeded by the colonization of novel environments such as islands, where induced plasticity could unlock cryptic genetic variation (Ledón-Rettig et al., 2014; Queitsch, Sangster, & Lindquist, 2002; Rutherford & Lindquist, 1998) that would not otherwise have been exposed to natural selection (Schneider & Meyer, 2017).

While *A. alluaudi* and *A. burtoni* can be broadly described as generalist species, they show between population dietary differences, which would favour the maintenance of plasticity, given sufficient gene flow. *A. burtoni* shows lake-stream divergence that is determined through both genetic and plastic mechanisms, with lake fish consuming a plant/algae and zooplankton-biased diet, and stream fish consuming more snails, insects and plant seeds (Theis et al., 2014). Also, *A. alluaudi's* Lake Victoria populations are strictly molluscivorous (Bouton, Seehausen, & Alphen, 1997), while riverine and satellite lake species also feed on insects, algae and fish, whose proportions vary spatially (Abila et al., 2008; Binning & Chapman, 2008; Mbabazi, Ogutu-Ohwayo, Wandera, & Kiziito, 2004) and seasonally (Binning, Chapman, & Cosandey-Godin, 2009). It is possible that the broader distribution of these lineages has resulted in independent selection for higher plasticity, and basal plasticity is a more parsimonious explanation due to their phylogenetic positions. Although the Lake Victoria *A. alluaudi* populations are exclusively molluscivorous, this is not their preferred diet (Slootweg, 1987; Slootweg et al., 1994). Their occupation of this niche is most likely the result of intense competition (Bouton et al., 1997), whereby Lake Victoria's endemic, specialized cichlid species have a competitive advantage over *A. alluaudi*. So although LPJ plasticity is likely to benefit *A. alluaudi* by expanding its geographic distribution, it potentially comes at the expense of access to their preferred food items in regions of high competition. It should be noted that a single *A. alluaudi* population (from Lake Victoria) was used in this study, and we do not know how its plasticity compares to that of riverine and satellite lake populations. However, it seems likely that the other populations are plastic to an equal or greater degree than our experimental population, as riverine populations have been shown to ingest a more variable diet (Binning & Chapman, 2008; Binning, Chapman, & Dumont, 2010; Binning et al., 2009; Cosandey-Godin et al., 2008), so contemporary plasticity may be more beneficial.

Our study identified putative candidates for genetic assimilation. Two of these show higher inducibility in response to the plastic stimulus in the basal species *A. alluaudi* but not in the specialist, *H. ishmaeli* potentially explaining the lower plasticity of this species. These genes are *gif* and *alas1* and are involved in vitamin B12 absorption in the gut (Booth & Mollin, 1959; Greibe, Fedosov, & Nexo, 2012) and heme biosynthesis (Sadlon, Dell'Oso, Surinya, & May, 1999), respectively. Their roles in cichlid LPJ plasticity are not yet known. Interestingly, these genes were also identified as being significant in the multiple regression analysis, which shows that they are also likely to relate to species-level differences in the plastic response. Additionally, we identified two genes that showed a pattern opposite to genetic assimilation (*osx* and *col6*), which are involved in the differentiation of osteoblasts (Nakashima & de Crombrugghe, 2003; Nakashima et al., 2002) and form part of the bone matrix, respectively (Christensen et al., 2012). This suggests that our candidate genes display multidirectional patterns of regulatory evolution. Moreover, we identified potential genetic assimilation in patterns of gene co-expression. Specifically, *col6* was co-expressed with *col12* in *A. alluaudi* and *H. ishmaeli*, but no other genes in *A. burtoni*. These differences may have modified the stiffness of the extracellular matrix, leading to altered patterns of bone cell differentiation (Engler, Sen, Sweeney, & Discher, 2006; McBeath, Pirone, Nelson, Bhadriraju, & Chen, 2004) and/or altered mineralization (Wang et al., 2012), which may impact the mechanical properties of the bone (Nair, Gautieri, Chang, & Buehler, 2013). Interestingly, *col6*, *col12* and the IER genes are predicted to contain binding sites for the mechanically responsive transcription factor AP1 in their promoter regions (Schneider et al., 2014). Thus, our observed changes in inducible plasticity may be underlain by promoter evolution, a notion that is supported by theoretical (Espinosa-Soto, Martin, & Wagner, 2011; Schlichting & Pigliucci, 1993) and empirical evidence ([Li et al., 2006; Suzuki & Nijhout, 2006; Ghalambor et al., 2015]; but see [Sikkink, Reynolds, Ituarte, Cresko, & Phillips, 2014]). Our study analysed a small portion of the transcriptome so we cannot infer the proportion of the genome that may have undergone genetic assimilation, or whether phenotypic plasticity is promoted through largely overlapping or unique mechanisms among these species.

Our putative identification of genetically assimilated loci within a cichlid adaptive radiation represents an important step in establishing a mechanism by which plasticity may have promoted their explosive speciation (Stauffer & van Snick Gray, 2004). This adds to other recent

studies that have demonstrated that selection on plastic loci may have contributed to adaptive diversification in other species (Ghalambor et al., 2015). Our work will enable future investigations that pinpoint the loci associated with reduced plasticity in *H. ishmaeli* and will open the door to comparative studies investigating other genetically assimilated loci in other Lake Victoria cichlid species. As these species display vast differences in LPJ shape, it is plausible that different loci would have been fixed through genetic assimilation. Comparisons between various species with different LPJ sizes and shapes will allow us to differentiate the loci associated with LPJ architecture, versus those that modulate phenotypic plasticity itself. It would also be very interesting to compare trophically equivalent species from different African lakes that are of different ages, as a means of studying various stages of genetic assimilation. For example, the Lake Victoria radiation is ~100,000 years old and the Tanganyika cichlid radiations are 9–12 M years old (Elmer et al., 2009; Friedman et al., 2013; Genner et al., 2007; Koblmüller, Sefc, & Sturmbauer, 2009; Verheyen, Salzburger, Snoeks, & Meyer, 2003). The highly specialized morphology of *T. moorii* hints that longer evolutionary time periods are associated with more specialized, canalized morphologies. However, solid evidence of this requires the comparison of trophically equivalent (snail crushing) species from Lake Tanganyika. This research will add to previous studies that have identified important loci that might be responsible for traits that are conducive for the formation of adaptive radiations in general (Abzhanov, Protas, Grant, Grant, & Tabin, 2004; Albertson & Kocher, 2006; Chan et al., 2010; Colosimo et al., 2005), with the added benefit of providing correlative evidence that phenotypic plasticity may have played a role in the initial accumulation and selection of these mutations.

One potential objection to the hypothesis that the formation of the cichlid adaptive radiations was facilitated by phenotypic plasticity is that it would be self-limiting, as genetic assimilation would restrict phenotypic plasticity, thus preventing further plasticity-mediated evolution. On the contrary, West-Eberhard (2003) argues that hypervariable flexible stems, such as those of cichlids have "the potential to rapidly evolve in any variety of new directions, should conditions change." Indeed, rapid evolution of increased plasticity has been observed in lineages under strong selection pressure (Nussey, Postma, Gienapp, & Visser, 2005). This scenario is backed up by the results of a range of experiments involving pond-based and natural populations, which indicate that adaptive traits fluctuate over extremely short timescales (year to year), with phenotypic plasticity providing the most plausible explanation (Kishe-Machumu, Witte, & Wanink, 2008; van Rijssel & Witte, 2013; van Rijssel et al., 2015). This initial flexibility might have assisted the evolution of cichlid diversification as they are clearly dominating the East African lakes in terms of biodiversity.

5 | CONCLUSION

Our investigation has provided support for the hypothesis that initial phenotypic plasticity has the potential to expand niche space and resist extinction, while later, during the formation of adaptive radiations, this plasticity can be lost and possibly become canalized into more stereotypical phenotypes of many derived species through the process of genetic assimilation. We find that derived, specialist lineages from cichlid adaptive radiations show reduced phenotypic plasticity compared to generalist lineages from outside of, and basal to, the adaptive radiations. Although it is not likely that all adaptive radiations are seeded by plastic generalist lineages, our result suggests that this scenario may be more common in adaptive radiations that were seeded by hyperflexible stem lineages that originally occupied fluctuating environments. Furthermore, through comparative gene expression studies, we have identified several putative candidates that may have undergone genetic assimilation, providing a potential genetic explanation for the reduced phenotypic plasticity displayed by the derived lineages. This research suggests that future studies should examine the selection of genetically assimilated loci in natural populations, a powerful next step in establishing a role for phenotypic plasticity in the formation of adaptive radiations—still a much debated hypothesis with only limited genetic evidence to date.

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DATA ACCESSIBILITY

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.84b09. Data files include raw relative gene expression data for qRT-PCR and linear and geometric morphometrics analyses.

AUTHOR CONTRIBUTION

H.M.G., A.M. and C.S. designed the study, and experimental work was carried out by I.K. and H.M.G. Data analysis was performed by R.F.S., I.K. and H.M.G. The initial draft of this manuscript was prepared by H.M.G. and R.F.S. and all authors assisted with additional drafts and reviewed the final version.

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