MOLECULAR ECOLOGY

Molecular Ecology (2016) **25**, 6012–6023 doi: 10.1111/mec.13801

FROM THE COVER

Foraging environment determines the genetic architecture and evolutionary potential of trophic morphology in cichlid fishes

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Abstract

Phenotypic plasticity allows organisms to change their phenotype in response to shifts in the environment. While a central topic in current discussions of evolutionary potential, a comprehensive understanding of the genetic underpinnings of plasticity is lacking in systems undergoing adaptive diversification. Here, we investigate the genetic basis of phenotypic plasticity in a textbook adaptive radiation, Lake Malawi cichlid fishes. Specifically, we crossed two divergent species to generate an F_3 hybrid mapping population. At early juvenile stages, hybrid families were split and reared in alternate foraging environments that mimicked benthic/scraping or limnetic/sucking modes of feeding. These alternate treatments produced a variation in morphology that was broadly similar to the major axis of divergence among Malawi cichlids, providing support for the flexible stem theory of adaptive radiation. Next, we found that the genetic architecture of several morphological traits was highly sensitive to the environment. In particular, of 22 significant quantitative trait loci (QTL), only one was shared between the environments. In addition, we identified QTL acting across environments with alternate alleles being differentially sensitive to the environment. Thus, our data suggest that while plasticity is largely determined by loci specific to a given environment, it may also be influenced by loci operating across environments. Finally, our mapping data provide evidence for the evolution of plasticity via genetic assimilation at an important regulatory locus, *ptch1*. In all, our data address long-standing discussions about the genetic basis and evolution of plasticity. They also underscore the importance of the environment in affecting developmental outcomes, genetic architectures, morphological diversity and evolutionary potential.

Keywords: cichlid, craniofacial, cryptic genetic variation, phenotypic plasticity, quantitative trait loci

Received 3 May 2016; revision received 13 July 2016; accepted 21 July 2016

Introduction

Over 150 years after the publication of *On the Origin of Species*, the phenotype is still recognized as the primary target of natural selection; however, the mechanisms through which adaptive phenotypic variations arise are not fully understood (Mayr 1997; Schlichting & Pigliucci 1998; Hendrikse *et al.* 2007; Pigliucci 2008). Whereas much of the last century has focused on revealing the genetic determinants of phenotypic variation, it is becoming increasingly apparent that the environment plays a major role in determining the phenotypic

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variation that is expressed in populations (Hendrikse *et al.* 2007; Pfennig *et al.* 2010; Laland *et al.* 2014). In this regard, phenotypic plasticity has become a principal topic of interest and is now recognized as a key progenitor of variation that enables populations to develop adaptive phenotypes under alternate environmental conditions, potentially leading to new ecological opportunities and facilitating broader patterns of evolution (Pfennig *et al.* 2010; Moczek *et al.* 2015).

Emerging from a relatively neglected topic to a mainstream interest in evolutionary theory, plasticity research has matured over the past two decades (Schlichting & Pigliucci 1998). It now focuses on a range of hypotheses, many of which were first developed over a century ago and refined during the 1990s and early 2000s, with a keen interest in determining how plasticity influences evolution and how plasticity itself evolves (West-Eberhard 2003). For example, theories such as the Baldwin effect, genetic assimilation and the 'flexible stem' hypothesis all suggest that plasticity can initiate adaptive divergence. The Baldwin effect suggests that plasticity allows populations to persist in novel environments enabling further evolution to occur (Baldwin 1896). Baldwin's ideas were foundational in that they established the environment as an important inducer of phenotypic change in a population, on which natural selection could subsequently act (Crispo 2007). Genetic assimilation predicts that phenotypes initially induced by environmental cues can become canalized into 'normal' development (West-Eberhard 2003). This idea, originally put forth by Waddington (1953), provided a mechanistic framework for studying the evolutionary origins of novel traits. Finally, the flexible stem hypothesis suggests that the trajectory of ancestral reaction norms should mirror larger patterns of phenotypic divergence (i.e. plasticity initiates the direction of evolution) (West-Eberhard 2003; Wund *et al.* 2008). This theory brings together ideas and concepts from phenotypic plasticity and adaptive diversification to better understand the factors that promote and shape the evolutionary radiations. Understanding the genetic basis of plasticity could advance these theories by allowing connections at the molecular level to be made between plastic responses and larger patterns of divergence.

Questions about the genetic nature of plasticity have been around for decades and were the source of a major debate during the emergence of current plasticity research. On one side, researchers proposed that 'plasticity genes' did not exist per se, but rather plasticity evolved as a secondary outcome of selection on phenotypes in different environments (Via 1993). The counterargument was that plasticity could indeed evolve independently of the phenotype through the expression of distinct sets of loci in different environments, or via loci expressed across habitats that possess environmentally sensitive alleles (Scheiner 1993; Pigliucci 2001). Over the past two decades, the emerging consensus is that plasticity loci do exist (Gibson & Dworkin 2004); however, specific details with respect to how such loci may promote adaptive divergence remain unclear (Moczek *et al.* 2011; Ehrenreich & Pfennig 2015). Empirical progress on this topic has been limited in part because studies on the genetic determinants of plasticity have mainly occurred using laboratory (e.g. *Caenorhabditis elegans*, Gutteling *et al.* 2007; *Drosophila melanogaster,* Bergland *et al.* 2008; *Arabidopsis thaliana,* Bloomer *et al.* 2014; *Saccharomyces cerevisiae,* Bhatia *et al.* 2014) or agricultural organisms (e.g. maize, Zhu *et al.* 2006; winter wheat, Zhai *et al.*, 2014; *canola,* Fletcher *et al.* 2015), leaving uncertainty about the genetic control of plasticity in evolutionary systems (Ledon-Rettig *et al.* 2014). Therefore, our ability to connect the molecular mechanisms that underlie an environmental response directly to broader patterns of evolution has been limited. Making such direct connections would lend invaluable weight to the theories mentioned above and firmly cement plasticity into modern evolutionary biology (Ehrenreich & Pfennig 2015).

The cichlid fishes from the African Rift Valley provide an exemplary system for evolutionary biologists to examine the genetic basis of plasticity within the context of adaptive diversification. Our previous research has demonstrated that cichlid lineages within Lakes Malawi, Tanganyika and Victoria vary along a common ecomorphological axis whereby species have diverged in oral jaw length and craniofacial profile (Cooper *et al.* 2010). Variation along this axis is broadly correlated with divergence in foraging mode with short-jawed species foraging with a primarily benthic/biting mode, and long-jawed species feeding with a pelagic/suction mode. The genetic basis for foraging-related traits has also been extensively explored in the cichlid system (e.g. Albertson *et al.*, 2003; Albertson *et al.*, 2005; Cooper *et al.*, 2011; Parnell *et al.*, 2012; Parsons *et al.* 2015). Plasticity has also been demonstrated in cichlid oral jaw morphology (Bouten *et al.* 2002; Stauffer & Gray 2004; Parsons *et al.* 2014). Notably, the variation induced by diet exhibits a striking similarity to that observed among cichlid species (Parsons *et al.*, 2014), which is consistent with the hypothesis that the cichlid jaw represents a morphological flexible stem (West-Eberhard 2003). In addition, we have recently shown that two closely related Malawi cichlid species (*Labeotropheus fuelleborni* and *Tropheops* 'red cheek') differ in the amount of craniofacial plasticity they exhibit when presented with alternate foraging environments (Parsons *et al.*, 2014). This observation suggests that plasticity is actively evolving in this group, and provides an ideal scenario

for examining the genetic basis for this trait. Therefore, using an F3 hybrid cross between these two species, we explored the genetic architecture of craniofacial shape in distinct foraging environments. This approach allowed us to quantitatively test whether the patterns of phenotypic plasticity mirror the larger patterns of divergence among Malawi cichlids (i.e. to address the flexible stem hypothesis) and to determine the genetic basis of plasticity and extent to which the genotype–phenotype map is influenced by the environment.

Materials and methods

Species and pedigree

A single wild-caught *Labeotropheus fuelleborni* (LF) female from Makanjila Point was crossed to a single wild-caught *Tropheops* 'red cheek' (TRC) male from Chizumulu Island. LF is an obligate biting species that forages almost exclusively on firmly attached algae in the near-shore rocky habitat (Ribbink *et al.* 1983; Konings 2001). It possesses extremely short, wide jaws and a steeply descending craniofacial profile to accommodate this task. LF defines the outer edge of craniofacial morphospace among East African cichlids (Cooper *et al.* 2010). TRC also feeds on attached algae, but it possesses very narrow jaws with which it feeds with plucks strands of algae from the substrate (Ribbink *et al.* 1983; Konings 2001). While this species forages in the wild with a benthic mode, it is part of a more ecologically diverse species complex that exhibits biting, shifting and sucking modes (Ribbink *et al.* 1983; Konings 2001; Albertson, 2008). Consistent with this, TRC possesses a more generalized craniofacial phenotype with quantitatively longer jaws and more shallow profiles relative to LF (Cooper *et al.* 2010; Parsons *et al.* 2015). Also, TRC was found to exhibit greater plasticity in craniofacial morphology in response to different diet treatments (Parsons *et al.*, 2014). A full-sibling F_1 family was interbred to produce F_2 individuals for genotyping and the subsequent creation of a genetic map (see details below and 28); 265 F_3 individuals were then derived from 25 separate F_2 families.

Diet treatments

We hypothesized that morphology in our F_3 population would exhibit phenotypic plasticity in response to the variation in biomechanical demands. We therefore equally divided each family into one of two diet treatments consisting of food that mimicked 'benthic' and 'limnetic' conditions. For the benthic treatment, we ground a mixture of high-quality algae flake food, algae wafers and freeze-dried daphnia. We then embedded this mixture in 1.5% food-grade agar (Carolina Biological Supply Co., Burlington, NC, USA) and spread it over store-bought lava rocks. These 'algae rocks' were allowed to air-dry overnight and then sunk to the bottom of aquaria. This treatment was intended to induce a 'scraping' mode of feeding that is typical of many rock-dwelling cichlid species in the wild, including LF and TRC. For this mode of feeding, the animals were required to dislodge food from the substrate using a combination of biting, scraping and twisting actions (see Movie S1, Supporting information). For the alternate 'limnetic' treatment, the same ground food mixture was sprinkled into aquaria. In addition, two to three times a week, limnetic animals were given live daphnia. This mode of feeding required animals to actively 'hunt' for their food, and use a combination of suction and ram-feeding to gather prey items (see Movie S2, Supporting information). Foraging treatments began at 2 months of age when animals were large enough to accept both diets and were housed in 40-gallon aquaria. Up until this point, all animals were fed a typical larval diet of ground flake food. Following the 5-month feeding treatments, the animals were euthanized following a protocol approved by the Animal Care and Use Committee, fixed in 4% PFA and stored in 75% ethanol. Prior to fixation, flank musculature was taken for DNA extraction. The animals were dissected to reveal functionally salient landmarks, photographed in the left lateral view and ventral view using a Canon EOS digital camera (Canon EOS Rebel, Lake Success, NY, USA), and digital landmarks were placed on their heads using TPSdig2 according to the previous research (Cooper *et al.* 2010; Parsons *et al.* 2011).

Phenotypic analysis

Variation in the lateral and ventral view of the head in F_3 hybrids was quantified using a geometric morphometric (GM) approach. For the lateral view, a total of 16 landmarks were collected, while for the ventral view, 10 landmarks were collected. A generalized Procrustes analysis (GPA) was performed to minimize the variation due to orientation and size (Zelditch *et al.* 2012). Also, to minimize the potential effects of allometry, we performed a multiple regression analysis of shape on geometric centroid size for lateral landmarks to generate landmark data sets based on residuals for further analysis. Similarly, ventral landmark data were regressed upon a measure of standard length to generate residual landmark data. GPA was performed using Coordgen6h, and multiple regression analysis was carried out using Standard6 (all available at: http://www.life.bio.sunysb.edu/morph/). For statistical analysis, we performed a thin-plate spline (TPS) procedure to

generate the partial warp scores. TPS models the form of an infinitely thin metal plate that is constrained at some combination of points (i.e. landmarks), but is otherwise free to adopt a target form in a way that minimizes bending energy. In morphometrics, this interpolation is applied to a Cartesian coordinate system in which deformation grids are constructed from two landmark configurations (Bookstein 1991). The total deformation of the thin-plate spline can be decomposed into geometrically orthogonal components (partial warps) based on scale and their scores used in multivariate statistics.

To test whether our diet treatments had a significant influence on craniofacial shape, we performed a discriminant function analysis (DFA) using diet as the grouping variable. While this analysis allowed us to assess how diet affected shape it also provided a quantitative variable (canonical root scores) that described the differences in shape due to benthic and limnetic diet treatments. This provided a quantitative trait for examining the genetic basis of plasticity via quantitative trait loci (QTL) mapping. We also used our landmark data to extract additional variables related to specific anatomical regions of interest in our QTL analysis. These traits were eye diameter, jaw width and two measures of mechanical advantage (MA) in the lower jaw – opening and closing MA. On average, LF possess relatively smaller eyes, wider jaws and higher MA compared to TRC. In addition, we measured the overall body depth as the ratio between standard length and depth at the anterior edge of the dorsal fin. In the wild, LF has a deeper body than TRC. We also included a unique soft tissue trait in our analysis – the fleshy snout that extends rostrally from the upper jaw apparatus and wraps in on itself to form a flexible flap of tissue that runs along the rostral edge of the premaxilla. This trait is comprised predominantly of hypertrophied intermaxillary ligament and associated connective tissue, is pronounced in LF and is thought to facilitate foraging efficiency (Konings 2001) in the benthic environment. The trait is lacking in TRC, but is segregating in the cross (Concannon & Albertson 2015). The size of the flap was measured directly in cross-section. Because flap size scales allometrically with body size, residuals from a linear regression analysis were used for QTL mapping. Finally, we measured the ratio between the two superficial subdivisions of the adductor mandibulae muscle. This is the major muscle involved in the action of jaw closing, and parental species show discrete differences in the ratio of the superficial surface area of the A1 and A2 subdivisions. The A1 component inserts onto the maxilla and is relatively larger than the A2 division in LF, while the A2 division inserts primarily on the ascending arm of the articular process of the mandible and is relatively larger than the A1 in TRC. Because muscle ratios changed with body size, residuals were likewise used for mapping.

Test of the flexible stem hypothesis

We tested for support of the flexible stem hypothesis by comparing the trajectory of plastic responses in the craniofacial region of our F_3 hybrids to the primary trajectory of craniofacial divergence in the Malawi cichlid radiation as a whole. Quantitatively, this involved using the canonical root scores from our diet-based DFA on F_3 craniofacial shape, and the PC1 values derived from the same landmarks across a large sample of species (80% of extant genera) from Lake Malawi (Cooper *et al.* 2010). These variables represented the plastic responses in our F_3 and the primary trajectory of divergence in Malawi cichlids, respectively, and were used as the independent variable in regressions on the Procrustes superimposed landmark data from their respective data sets. These regressions identified a vector for each data set, which was normalized to unit length. The angle between both vectors was then calculated as the arc cosine. We then ran 2500 bootstraps with replacement for each group (i.e. F_3 plastic response and Malawi divergence) independently to produce a 95% confidence interval. The observed angle was then compared against the confidence intervals for both groups to determine whether it differed from random processes. These procedures were performed using the software Vecompare6 (http://www3.canisius.edu/~sheets/morphsoft.html).

Genotyping

Our genetic mapping experiments were performed within an F_3 hybrid cross to allow for a relatively higher number of recombination events compared to a typical F_2 design and thus increased resolution of mapping intervals. Because F_3 do not provide a tractable pattern of Mendelian segregation, we used a genetic map derived from the F_2 generation of the same pedigree and RAD genotyping (Albertson *et al.* 2014). Specifically, a subset ($n = 364$) of genetic markers spread evenly across the genome was genotyped in the F_3 via the same RAD-seq methodology used in the F_2. Genomic DNA was extracted from flank muscle tissue using DNeasy blood and tissue kits (Qiagen Inc., CA, USA), digested with the restriction enzyme SbfI and processed into RAD libraries following Chutimanitsakun *et al.* (2011). Barcoded, processed and purified DNA for each fish was sequenced using an Illumina HiSeq 2000 (Illumina, San Diego, CA) and single-read (1×100 bp) sequencing chemistry. Sequencing and

bioinformatics also followed Chutimanitsakun *et al.* (2011) and is described in greater detail in Albertson *et al.* (2014). QTL mapping was then performed using F_3 genotypes and phenotypes (benthic $n = 132$, limnetic $n = 133$).

QTL analysis and fine mapping

The genetic architecture of morphological plasticity was characterized via two main approaches: (i) QTL investigations of canonical root scores of lateral and ventral shape (derived from the DFAs described above with diet as a grouping variable) and (ii) through separate QTL analyses of the seven traits described above in each diet treatment group. The first approach could be viewed as a direct test of the hypothesis that plasticity is controlled by 'master control' genes that are active under both environments, while the second approach could be viewed as a test of the hypothesis that plasticity is the result of different loci being expressed in different environments. The second approach could be particularly useful for revealing cryptic genetic variation (CGV), whereby genotype–phenotype relationships may only be present under one environmental condition.

For both approaches, QTL analyses were divided into two steps: (i) a statistically liberal initial series of scans to identify putative loci and interactions and (ii) a more rigorous multiple QTL mapping (MQM) approach. For step 1, tests were conducted using the scanone and scantwo function within *r*/qtl (Broman & Sen 2009). From these tests, putative QTL loci were identified as having a logarithm of odds (LOD) score >3 or a LOD score greater than the 95% threshold (created by 1000 permutations for a given model). For step 2, this collection of putative loci were then tested for verification by maximum-likelihood-based backward elimination (to specify cofactors) and permutation tests (i.e. 95% threshold as determined by 1000 permutations) during the subsequent rounds of MQM scans (Arends *et al.* 2010).

For QTL identified from MQM mapping tests, we took advantage of the MZ genome to anchor QTL intervals to specific stretches of physical sequence in order to fine map select QTL. Specifically, the 95% LOD confidence interval was calculated for each QTL using the bayesint function in *r*/qtl, which allowed us to determine what mapped markers fell within each QTL. Because marker names were based on scaffold position (e.g. marker scaffold_1.6031297 corresponds to a SNP on scaffold #1 at 6 031 297 bp), we next identified contiguous stretches of physical sequence that corresponded to QTL intervals. We then identified additional, unmapped, RAD-seq SNPs within these intervals that were genotyped in the F_3. These were selected to span the region of interest with a spacing up to 1 marker every ~200 kb. This provided many additional genotypes that were used to assess genotype–phenotype associations across candidate intervals. In addition, we resequenced the *ptch1* locus in a panel of LF and TRC from the populations used to generate this cross. The specific SNP used was based on Roberts *et al.* (2011) (i.e. 'Ptch1Loc10_SNP1'). Primer, polymorphism and flanking sequence can be found in Table S1 (Supporting information) of this publication.

Results and discussion

Alternate foraging environments induce quantitative shifts in morphology in a hybrid mapping population

To examine the genetic basis of plasticity, we generated an F3 hybrid mapping population by crossing two closely related Lake Malawi cichlid species, LF and TRC, that differ in trophic and body shape. At 2 months of age (i.e. early juvenile stages), F_3 families were equally divided and reared on diets that mimicked natural 'benthic/hard' and 'limnetic/soft' prey (biting and suction feeding, respectively, Movies S1–S2, Supporting information). These diets require the functional tactics that define the major axis of evolution in African cichlid adaptive radiations (Cooper *et al.* 2010; Parsons *et al.* 2011) and were administered for 5 months, at which point the animals were euthanized, the tissue was taken for DNA extraction and the samples were prepared for phenotypic analysis.

We investigated both the lateral and ventral views of the head in F_3 hybrids using a standard GM approach. To test whether the diet treatments had a significant influence on the lateral and ventral shape, we performed a DFA using diet as a grouping variable. This analysis extracted the quantitative differences in shape induced by diet treatments, and scores along this 'plasticity axis' were subsequently used for QTL mapping across all F_3. In addition, we measured seven putatively adaptive traits related to feeding efficiency, namely eye diameter, jaw width, body depth, muscle architecture, ligament hypertrophy and two functional aspects of lower jaw shape (i.e. MA of jaw opening and closing). These traits were used for separate QTL analyses for each treatment.

Alternate foraging treatments led to a plastic response in some, but not all, traits measured (Table 1). In terms of the overall skull geometry in the lateral view, we found a support for the flexible stem hypothesis of adaptive radiation, which states that ancestral patterns of phenotypic plasticity will shape the direction of adaptive evolution (West-Eberhard 2003). We noted that alternate diets induced plastic changes in head

morphology that closely mimicked the variation across Malawi cichlids (Fig. 1). Specifically, when comparing the trajectory of these plastic responses in the craniofacial skeleton of our F_3 hybrids to the primary trajectory of craniofacial divergence in the Malawi radiation, we found that the shape variation was statistically indistinguishable between the two groups (observed angle of 58° between diet-based DFA in F_3 and PC1 of Malawi was within 95% bootstrapped CIs) (Fig. 1). While our F_3 hybrids may not meet the strict definition of an ancestor (and therefore ancestral plasticity), they are consistent with the predicted scenario for Lake Malawi cichlids whereby their explosive evolutionary diversification was facilitated by mass hybridization events (Seehausen 2004; Joyce *et al.* 2011). Thus, our feeding experiment induced a pattern of plasticity that is similar to the evolutionary divergence observed among cichlid species, providing a context for our genetic analysis.

The genetic basis of phenotypic plasticity

To characterize the genetic architecture of morphological plasticity, we used two main approaches: (i) QTL analyses of seven foraging-related traits in each diet treatment and (ii) QTL investigations of an induced plasticity axis (represented by DFA scores of lateral and ventral shape) across diet treatment. The first approach provided a test of the hypothesis that plasticity is the result of CGV. CGV builds upon earlier debates (Scheiner 1993; Via 1993) by recognizing that some genetic variation does not normally contribute to the range of phenotypes present in a population, but requires an environmental perturbation (or mutation) to be expressed (Gibson & Dworkin 2004; Schlichting 2008; Palmer 2012; Paaby & Rockman 2014). The release of such CGV will change the genotype–phenotype (G-P) map and is predicted to provide a rich source of evolutionary potential (Gibson & Dworkin 2004). While CGV has been well documented in several laboratory models, there is a conspicuous lack of empirical data with respect to its genetic basis in natural populations undergoing adaptive divergence (Ledon-Rettig *et al.* 2014).

We found a robust support for CGV-mediated plasticity. Across the seven traits, we detected a total of 22 QTL (Fig. 2, Table S1, Supporting information), nine of which were detected in animals reared on a benthic diet, while 13 were detected in limnetic animals. All QTL exhibited a modest effect on the phenotype, explaining between 10% and 17% of the phenotypic variation. Allele effects generally ranged from additive to dominance, with only two QTL exhibiting an overdominant mode of inheritance, both of which were detected in the limnetic population. Notably, of these 22 QTL, only a single locus was detected in both foraging

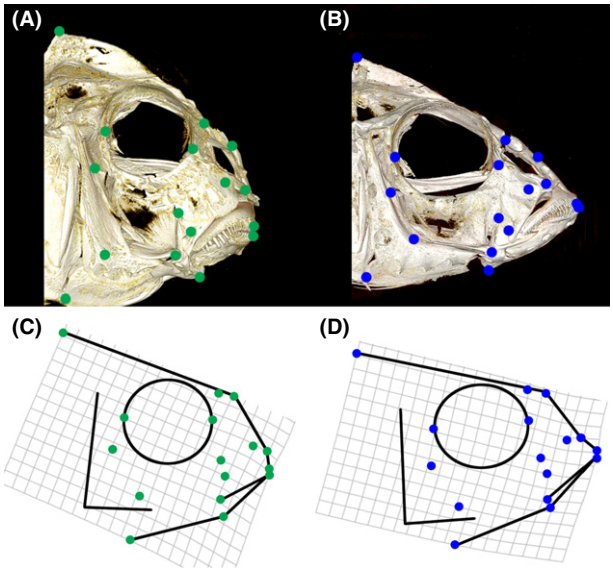

Fig. 1 Cichlids as a flexible stem. Microcomputed tomography scans of the craniofacial skeleton of two Lake Malawi cichlid species representing benthic (A) and limnetic (B) ecomorphologies. Shape differences among benthic and limnetic cichlids represent the major axis of divergence in Lakes Victoria, Malawi and Tanganyika (Cooper *et al.* 2010). These include coordinated variation in craniofacial profile, head depth, jaw rotation and jaw length. Notably, diet-induced plasticity in the F_3 mapping population resulted in a very similar pattern of variation (C, D). Animals reared on a benthic diet possessed, on average, deeper heads, more rounded craniofacial profiles and shorter more ventrally directed jaws (C). Alternatively, the animals reared on a limnetic diet developed more shallow heads, gradually sloping craniofacial profiles and longer more horizontally directly jaws (D). Landmarks used in morphometric shape analyses are shown and depicted in green on benthic fish and blue on limnetic fish.

Table 1 Mean differences in phenotype induced by alternate foraging environment. Significant differences in mean phenotypes are indicated in boldfaced lettering. Note, in particular, the pronounced plastic response in MA_O

	Averages				
Trait	Benthic	Limnetic	*t*	d.f.	*P*
Eye Size	0.5472654	0.5314805	2.515	235	**0.013**
Body depth	−1.140046	1.113116	0.200	240	0.842
Muscle ratio	0.025797	−0.027277	3.006	249	**0.003**
Flap	0.007957	−0.007546	0.508	188	0.612
Jaw width	0.208795	0.207584	0.5562	254	0.579
MA_0	0.029611	−0.029198	10.192	254	**<0.0001**
MA_C	0.009653	−0.008197	2.042	254	**0.042**

MA_C, mechanical advantage of lower jaw closing; MA_O, mechanical advantage of lower jaw opening.

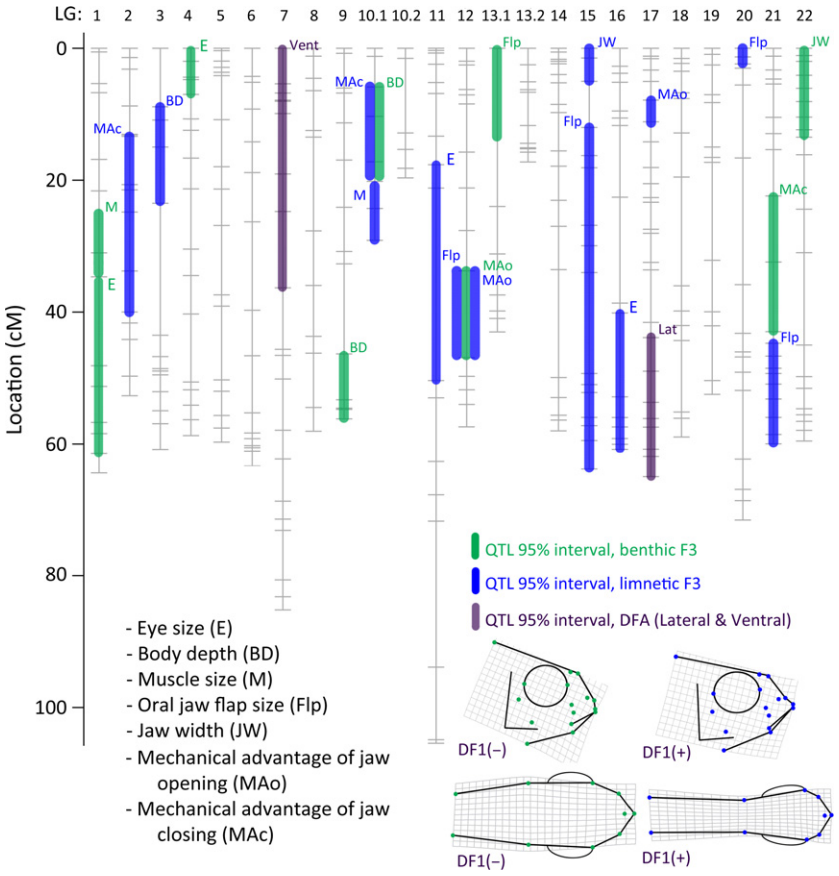

Fig. 2 The G-P map for foraging-related traits is distinct between alternate feeding environments. The 95% confidence intervals for quantitative trait loci (QTL) are shown. QTL from the benthic population are shown in green, whereas those in blue are from the limnetic population. Purple QTL intervals are for DF1 scores derived from the lateral (Lat) and ventral (Vent) views of F_3 fish. The list of traits and abbreviations are provided on the figure. Shape variation along DF1 is also depicted via deformation grids for both lateral and ventral views. In each analysis, benthic animals possessed, on average, more negative DF1 scores, whereas limnetic animals exhibited positive DF1 scores. Shape variation along these axes included the differences in head depth, craniofacial profile, jaw length and rotation and head width.

environments. It is unlikely that this trend is the result of a lack of statistical power as LOD association profiles from each environment are dissimilar from one another (i.e. QTL for one treatment that exceeded the significance threshold were not even marginally significant for the alternate treatment, see Fig 3). Further, we tested for the correlations between the LOD scores of fish reared under different diets (using all loci exhibiting a LOD > 1). Using 10 000 bootstraps, we found that LOD scores showed a strongly negative relationship (all r values >0.36) for all traits except MAo. These data suggest that CGV is widespread for these traits, with negative correlations between LOD scores, suggesting exclusive environment-specific G-P relationships. Moreover, we found that the degree of plastic response for a given trait did not predict the degree of CGV. Three of seven traits did not exhibit a plastic response (Table 1), but still exhibited nonoverlapping G-P maps (Fig. 2, Table S1, Supporting information). These data suggest that canalization/buffering has an environmentally dynamic genetic basis (Gibson & Dworkin 2004). Alternatively, the single QTL detected in both foraging environments was for mechanical advantage of jaw opening (MAo), which was by far the most plastic of those examined. Specifically, when mean phenotypic values from each environment were compared, the t-value for MAo was an order of magnitude greater than that for other traits (Table 1). Thus, not only is the G-P map unique with respect to foraging environment, but the degree of overlap in G-P relationships cannot be predicted by the degree of plasticity.

While CGV may inform us about the mechanisms of plasticity by revealing loci acting in distinct environments, it cannot account for G × E interactions underlain by loci operating across environments. Because allelic variation at such loci could also be evolutionarily relevant, our second approach was designed to determine whether some portion of plasticity is controlled by loci with allele sensitivity (Via 1993; Via *et al.* 1995). Under this scenario, plasticity loci are predicted to act across environments, but with alternate alleles playing more (or less) prominent roles in different environments. Such loci could represent 'master control' switches for plasticity whereby alternate alleles activate different downstream pathways in distinct environments. Our data provide evidence for this type of plasticity locus as well. When reared on alternate diets, F_3 animals exhibited significant differences in craniofacial morphology in both the ventral and lateral views (Figs 1 and 2). When mapping the variation along the axes that distinguished foraging-specific

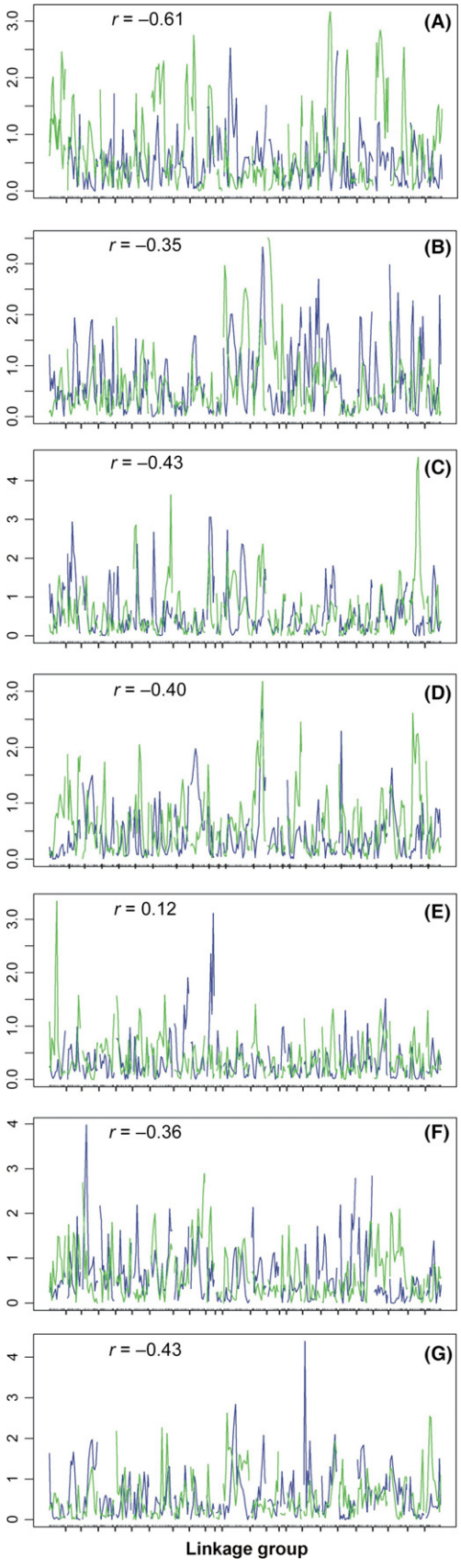

Fig. 3 Cryptic genetic variation is highly prevalent for determining cichlid morphology. Each panel represents line plots of LOD scores for a given trait under benthic (green) and limnetic (blue) foraging conditions. Traits examined include muscle architecture (A), body depth (B), eye diameter (C), mouth flap (D), mechanical advantage opening (E), mechanical advantage of the jaw opening (F) and jaw width (G). Notably, the peaks of LOD scores rarely overlap between foraging environments, indicating that the genetic basis of these traits is plastic.

shapes, we detected a robust support for QTL acting across environments (Fig. 2, Table S1, Supporting information). Notably, the QTL for the ventral view overlapped with the sex-determining locus on LG7 and is similar to other sex-linked craniofacial QTL detected for this cross (Parsons *et al*. 2015). This observation raises the interesting possibility of a sex-by-environment effect on ventral jaw shape. Allelic sensitivity at a single locus was also evidenced by a QTL for lateral shape. This QTL mapped to a region on LG17 that is distinct from any of the previously identified 20+ craniofacial QTL in this cross (Parsons *et al*. 2015), suggesting a distinct genetic basis for plasticity. Given that this trait encompasses the plastic response of the entire craniofacial complex, we hypothesize that this QTL on LG17 may contain genetic variation that regulates the plastic responses via 'master control switches' that initiate a cascade of effects across a number of downstream genes or signal transduction pathways. Collectively, our mapping study demonstrates that phenotypic plasticity has a robust genetic signature in Malawi cichlids, providing a critical foundation upon which the proximate molecular mechanisms that regulate its manifestation and evolution may be studied.

The evolution of plasticity via genetic assimilation at ptch1

Genetic assimilation was first put forward by Waddington (1953) as a process by which phenotypes originally induced by environmental cues become genetically determined (i.e. canalized) through selection. In spite of its importance to evolutionary theory, evidence for genetic assimilation in natural systems has been elusive (e.g. Aubret & Shine 2009), with some suggesting it has little importance to evolution (Gibson & Dworkin 2004; Pigliucci *et al*. 2006). Notably, our data, combined with prior knowledge of genetic evolution in this group, support genetic assimilation at a QTL for MAo on LG12 that is common to both foraging environments (Fig. 2, Table S1, Supporting information). In both treatments, the LF allele increases the trait value, while the TRC allele decreases the trait value. This is expected based on parental phenotypes (Roberts *et al*. 2011). Thus, the trend of allele effects is robust to the foraging environment. However, the

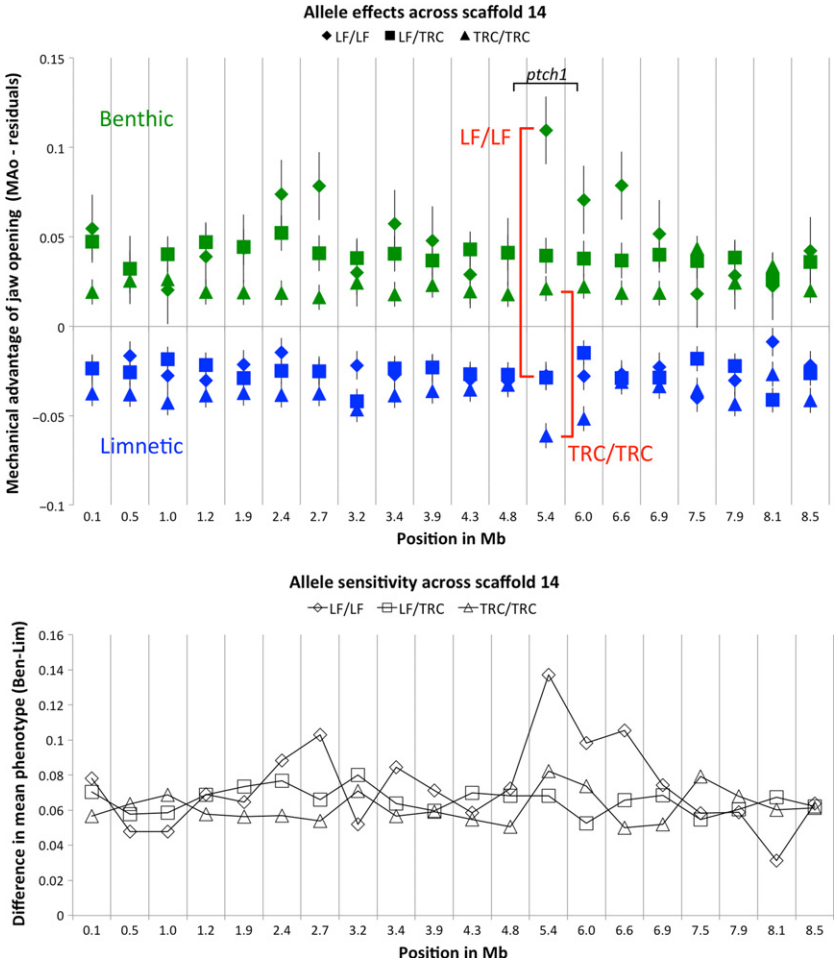

Fig. 4 The evolution of plasticity via genetic assimilation at the *ptch1* locus. The quantitative trait loci (QTL) for MA_O mapped to scaffold 14 in both benthic and limnetic treatments and corresponds to a QTL previously found to be due to the variation at *ptch1* (Roberts *et al.* 2011). Additional RAD-seq markers were used to fine map this region and peak genotype–phenotype association was observed in markers adjacent to *ptch1*, which is at ~5.2 Mb on scaffold 14. Regardless of genotype, means phenotypes did not overlap between the diet treatments, which means that foraging environment is a better predictor of shape than is genotype at this locus. Furthermore, we find that haplotypes are differentially sensitive to the environment at this locus. The difference in mean phenotype for the LF/LF genotype at 5.4 Mb is nearly twice as large as that for the TRC/TRC genotype (red brackets, top panel; bottom panel). Given that the LF allele is ancestral, this represents an unambiguous example of genetic assimilation.

range and sensitivity of genotypic effects is markedly different between the environments (Fig. 4, Table S1, Supporting information). For example, the mean phenotype for F_3 animals homozygous for the LF allele is lower in the limnetic environment than that for animals homozygous for the TRC allele in the benthic environment. In other words, at this locus, the environment is a better predictor of mean trait value than is genotype. Moreover, our data show that the LF allele is more sensitive to foraging environment than the TRC allele. The difference in mean trait value between treatments is ~50% greater for animals with the LF/LF genotype (mean = 0.087) compared to animals with the TRC/TRC genotype (mean = 0.059). This is consistent with the LF allele increasing plasticity through genetic accommodation, or the TRC allele decreasing plasticity through genetic assimilation. Given the evolutionary history of this locus, genetic assimilation is more likely.

This locus corresponds to a previously identified QTL for MAo that was determined to be caused by the variation at *ptch1* (Roberts *et al.* 2011). LF was used in both crosses, and TRC segregates the same allele as the species used in the previous cross, *Maylandia zebra* (MZ). It is therefore likely that *ptch1* underlies this QTL peak as well, and fine mapping using additional markers placed every ~500 kb confirmed that peak association between the variation in MAo and genotype was at a marker adjacent to *ptch1* (Fig. 4). Further, the difference in haplotype

sensitivity to foraging environment became even more pronounced when additional markers were added to span this interval, with the difference in mean phenotype being almost twice as large in LF/LF animals compared to TRC/TRC animals. While the 5′ region of *ptch1* implicated in the evolutionary divergence of jaw morphology in cichlids was missing from our SNP data set, we genotyped a panel of 20 wild LF and 20 wild TRC at the SNP previously shown to exhibit the highest levels of divergence between LF and MZ, and confirmed that TRC carry a high frequency of the MZ allele (F_{ST} = 0.85). The action of this gene is to determine jaw shape early in development by mediating bone deposition around the cartilaginous precursor of the retroarticular process, with higher levels associated with more robust bone deposition and lower levels associated with less bone deposition (Roberts *et al.* 2011; Hu & Albertson 2014). MZ and TRC both exhibit reduced levels of bone deposition relative to LF (Powder *et al.* 2015) and harbour the evolutionarily derived *ptch1* allele (Roberts *et al.* 2011). Thus, recent selection at the *ptch1* locus appears to favour the development of more gracile jaw morphologies that are advantageous for a more limnetic mode of feeding (Roberts *et al.* 2011). A reduction in the sensitivity of the evolutionarily derived TRC/MZ allele to foraging environment is therefore consistent with genetic assimilation acting to decrease plasticity in the limnetic ecomorphology of MAo.

Conclusions: towards an eco-devo approach

An ongoing challenge in evolutionary genetics is to identify the 'salient' molecular changes that underlie evolutionary divergence (Hendrikse *et al.* 2007), which refers to characterizing the genetic variation that natural selection acts upon. While pedigree mapping has been a useful and productive methodology in this pursuit, especially with recent technological advances in high-throughput genotyping (Nadeau & Jiggins 2010), there is an emerging view that additive genetic variation accounts for a relatively small percentage of phenotypic variation and rather it is the context in which traits develop that determine their final form (Hendrikse *et al.* 2007; Jamniczky *et al.* 2010; Pfennig *et al.* 2010; Hallgrimsson *et al.* 2014). Our work supports this idea and suggests that the genetic basis of a trait can be a 'moving target' for selection, with some regions of the genome being consistently involved across environments (i.e. loci with allele sensitivity), while many others are specific to the current conditions. Therefore, we argue for a shift towards an eco-devo (or eco-evo-devo) approach (Abouheif *et al.* 2014; Gilbert & Epel 2015), wherein the salient environment is considered and whenever possible incorporated into evo-devo studies. This will be especially important for complex traits that are (by definition) heavily influenced by genetic background and the environment. In all, such an integrative approach should provide a much richer (and perhaps more realistic) picture of the genetic basis of adaptive morphological variation.

Acknowledgements

We thank past and present members of the Albertson lab for helpful comments on various aspects of this project. This work was supported by start up funds from the University of Glasgow to K. J. P. and a grant to R. C. A. from the NSF (IOS-1054909).

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K.J.P. and R.C.A. conceived the project, designed all experiments, performed QTL mapping, and wrote the manuscript. M.C., D.N., J.W., I.E., and K.G., phenotyped the F3 for discrete traits and helped with QTL mapping C.C. collected morphometric data of the ventral view of the F3 and performed statistical analyses.

Data accessibility

Data are accessible in the online supporting information files and at Dryad: doi:10.5061/dryad.m2j85.

Supporting information

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

Table S1. Results of QTL analyses for seven foraging-associated traits in each environment.

Movie S1. Benthic foraging behaviour (separate attachment).

Movie S2. Limnetic foraging behaviour (separate attachment).

[Correction added on 07 December, 2016, after first online publication: Dryad doi no. was added]