

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

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21

22 **Abstract**

23 Phenotypic plasticity allows organisms to change their phenotype in response to shifts in
24 the environment. While a central topic in current discussions of evolutionary potential, a
25 comprehensive understanding of the genetic underpinnings of plasticity is lacking in
26 systems undergoing adaptive diversification. Here we investigate the genetic basis of
27 phenotypic plasticity in a textbook adaptive radiation, Lake Malawi cichlid fishes.

28 Specifically, we crossed two divergent species to generate an F₃ hybrid mapping
29 population. At early juvenile stages, hybrid families were split and reared in alternate
30 foraging environments that mimicked benthic/scraping or limnetic/sucking modes of
31 feeding. These alternate treatments produced variation in morphology that was broadly

32 similar to the major axis of divergence among Malawi cichlids, providing support for the
33 flexible stem theory of adaptive radiation. Next we found that the genetic architecture of
34 several morphological traits was highly sensitive to the environment. In particular, of 22
35 significant quantitative trait loci (QTL), only one was shared between environments. In
36 addition, we identified QTL acting across environments with alternate alleles being
37 differentially sensitive to the environment. Thus, our data suggest that while plasticity is
38 largely determined by loci specific to a given environment, it may also be influenced by
39 loci operating across environments. Finally, our mapping data provide evidence for the
40 evolution of plasticity via genetic assimilation at an important regulatory locus, *ptch1*. In
41 all, our data address longstanding discussions about the genetic basis and evolution of
42 plasticity. They also underscore the importance of the environment in affecting
43 developmental outcomes, genetic architectures, morphological diversity, and
44 evolutionary potential.

45

46

47 **Introduction**

48 Over 150 years after the publication of *On the Origin of Species*, the phenotype is still
49 recognized as the primary target of natural selection, however the mechanisms through
50 which adaptive phenotypic variation arise are not fully understood (Mayr 1997,
51 Schlichting and Pigliucci 1998, Hendrikse et al. 2007, Pigliucci 2008). Whereas much of
52 the last century has focused on revealing the genetic determinants of phenotypic
53 variation, it is becoming increasingly apparent that the environment plays a major role in
54 determining the phenotypic variation that is expressed in populations (Hendrikse, Parsons
55 et al. 2007, Pfennig, Wund et al. 2010, Laland, Uller et al. 2014). In this regard
56 phenotypic plasticity has become a principal topic of interest, and is now recognized as a
57 key progenitor of variation that enables populations to develop adaptive phenotypes
58 under alternate environmental conditions, potentially leading to new ecological
59 opportunities, and facilitating broader patterns of evolution (Pfennig et al. 2010, Moczek
60 et al. 2015).

61

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

84

85 Questions about the genetic nature of plasticity have been around for decades, and were
86 the source of a major debate during the emergence of current plasticity research. On one
87 side, researchers proposed that ‘plasticity genes’ did not exist *per se*, but rather plasticity
88 evolved as a secondary outcome of selection on phenotypes in different environments
89 (Via 1993). The counter argument was that plasticity could indeed evolve independently
90 of the phenotype through the expression of distinct sets of loci in different environments,
91 or via loci expressed across habitats that possess environmentally-sensitive alleles
92 (Scheiner 1993, Pigliucci 2001). Over the past two decades the emerging consensus is

93 that plasticity loci do exist (Gibson and Dworkin 2004), however specific details with
94 respect to how such loci may promote adaptive divergence remains unclear (Moczek,
95 Sultan et al. 2011, Ehrenreich and Pfennig 2015). Empirical progress on this topic has
96 been limited in part because studies on the genetic determinants of plasticity have mainly
97 occurred using laboratory (e.g., *C. elegans*, Gutteling et al., 2007; *D. melanogaster*,
98 Bergland et al., 2008; *A. thaliana*, Bloomer et al., 2014; *S. cerevisiae*, Bhatia et al., 2014)
99 or agricultural organisms (e.g., maize, Zhu et al., 2005; winter wheat, Zhai et al., 2014;
100 *canola*, Fletcher et al., 2015), leaving uncertainty about the genetic control of plasticity in
101 evolutionary systems (Ledon-Rettig, Pfennig et al. 2014). Therefore our ability to connect
102 the molecular mechanisms that underlie an environmental response directly to broader
103 patterns of evolution has been limited. Making such direct connections would lend
104 invaluable weight to the theories mentioned above and firmly cement plasticity into
105 modern evolutionary biology (Ehrenreich and Pfennig 2015).

106

107 The cichlid fishes from the African Rift Valley provide an exemplary system for
108 evolutionary biologists to examine the genetic basis of plasticity within the context of
109 adaptive diversification. Our previous research has demonstrated that cichlid lineages
110 within lakes Malawi, Tanganyika, and Victoria vary along a common eco-morphological
111 axis whereby species have diverged in oral jaw length and craniofacial profile (Cooper et
112 al. 2010). Variation along this axis is broadly correlated with divergence in foraging
113 mode with short-jawed species foraging with a primarily benthic/biting mode, and long-
114 jawed species feeding with a pelagic/suction mode. The genetic basis for foraging-related
115 traits has also been extensively explored in the cichlid system (e.g., Albertson et al.,
116 2003; Albertson et al., 2005; Cooper et al., 2011; Parnell et al., 2012; Parsons et al.,
117 2015). Plasticity has also been demonstrated in cichlid oral jaw morphology (Bouton et
118 al. 2002; Stauffer and van Snik Gray 2004; Parsons et al. 2014). Notably, variation
119 induced by diet exhibits striking similarity to that observed among cichlid species
120 (Parsons et al., 2014), which is consistent with the hypothesis that the cichlid jaw
121 represents a morphological flexible stem (West-Eberhard, 2003). In addition, we have
122 recently shown that two closely related Malawi cichlid species (*Labeotropheus*
123 *fuelleborni*, *Tropheops* “red cheek”) differ in the amount of craniofacial plasticity they

124 exhibit when presented with alternate foraging environments (Parsons et al., 2014). This
125 observation suggests that plasticity is actively evolving in this group, and provides an
126 ideal scenario for examining the genetic basis for this trait. Therefore, using an F3 hybrid
127 cross between these two species, we explored the genetic architecture of craniofacial
128 shape in distinct foraging environments. This approach allowed us to quantitatively test
129 whether patterns of phenotypic plasticity mirror larger patterns of divergence among
130 Malawi cichlids (i.e., to address the flexible stem hypothesis), and to determine the
131 genetic basis of plasticity and extent to which the genotype-phenotype map is influenced
132 by the environment.

133

134 **Materials and methods.**

135 *Species and Pedigree*

136 A single wild-caught *Labeotropheus fuelleborni* (LF) female from Makanjila Point was
137 crossed to a single wild-caught *Tropheops* ‘red cheek’ (TRC) male from Chizumulu
138 Island. LF is an obligate biting species that forages almost exclusively on firmly attached
139 algae in the near-shore rocky habitat (Ribbink et al., 1983; Konings 2001). It possesses
140 extremely short, wide jaws and a steeply descending craniofacial profile to accommodate
141 this task. LF defines the outer edge of craniofacial morphospace among East African
142 cichlids (Cooper et al., 2010). TRC also feeds on attached algae, but it possesses very
143 narrow jaws with which it feeds with plucks strands of algae from the substrate (Ribbink
144 et al., 1983; Konings 2001). While this species forages in the wild with a benthic mode, it
145 is part of a more ecologically diverse species complex that exhibits biting, shifting and
146 sucking modes (Ribbink et al., 1983; Konings 2001; Albertson, 2008). Consistent with
147 this, TRC possesses a more generalized craniofacial phenotype with quantitatively longer
148 jaws and more shallow profiles relative to LF (Cooper et al., 2010; Parsons, Wang et al.
149 2015). Also, TRC was found to exhibit greater plasticity in craniofacial morphology in
150 response to different diet treatments (Parsons et al., 2014). A full sibling F₁ family was
151 interbred to produce F₂ individuals for genotyping and the subsequent creation of a
152 genetic map (see details below, and 28). 265 F₃ individuals were then derived from 25
153 separate F₂ families.

154

155 *Diet Treatments*

156 We hypothesized that morphology in our F₃ population would exhibit phenotypic
157 plasticity in response to variation in biomechanical demands. We therefore equally
158 divided each family into one of two diet treatments consisting of food that mimicked
159 ‘benthic’ and ‘limnetic’ conditions. For the benthic treatment, we ground a mixture of
160 high-quality algae flake food, algae wafers and freeze-dried daphnia. We then embedded
161 this mixture in 1.5% food-grade agar (Carolina Biological Supply Co., Burlington, NC,
162 USA) and spread it over store-bought lava rocks. These ‘algae rocks’ were allowed to air-
163 dry overnight and then sunk to the bottom of aquaria. This treatment was intended to
164 induce a ‘scraping’ mode of feeding that is typical of many rock-dwelling cichlid species
165 in the wild, including LF and TRC. For this mode of feeding animals were required to
166 dislodge food from the substrate using a combination of biting, scraping and twisting
167 actions (see Movie S1). For the alternate ‘limnetic’ treatment, the same ground food
168 mixture was sprinkled into aquaria. In addition, two to three times a week, limnetic
169 animals were given live daphnia. This mode of feeding required animals to actively
170 “hunt” for their food, and use a combination of suction and ram-feeding to gather prey
171 items (see Movie S2). Foraging treatments began at 2 months of age when animals were
172 large enough to accept both diets and were housed in 40-gallon aquaria. Up until this
173 point all animals were fed a typical larval diet of ground flake food. Following the 5
174 month feeding treatments animals were euthanized following a protocol approved by the
175 animal care and use committee, fixed in 4% PFA and stored in 75% ethanol. Prior to
176 fixation, flank musculature was taken for DNA extraction. Animals were dissected to
177 reveal functionally salient landmarks, photographed in the left lateral view, and ventral
178 view using a Canon EOS digital camera (Canon EOS Rebel, Lake Success, NY, USA)
179 and digital landmarks were placed on their heads using TPSdig2 according to previous
180 research (Cooper, Parsons et al. 2010, Parsons 2011).

181

182 *Phenotypic Analysis*

183 Variation in the lateral and ventral view of the head in F₃ hybrids was quantified using a
184 geometric morphometric approach. For the lateral view a total of 16 landmarks were
185 collected, while for the ventral view 10 landmarks were collected. A generalized

186 Procrustes analysis (GPA) was performed to minimize variation due to orientation, and
187 size (Zelditch, Swiderski et al. 2012). Also, to minimize the potential effects of allometry
188 we performed a multiple regression of shape on geometric centroid size for lateral
189 landmarks to generate landmark data sets based on residuals for further analysis.
190 Similarly, ventral landmark data was regressed upon a measure of standard length to
191 generate residual landmark data. GPA was performed using Coordgen6h, and multiple
192 regression was done using Standard6 (all available at:
193 <http://www.life.bio.sunysb.edu/morph/>). For statistical analysis we performed a thin-plate
194 spline (TPS) procedure to generate partial warp scores. TPS models the form of an
195 infinitely thin metal plate that is constrained at some combination of points (i.e.,
196 landmarks) but is otherwise free to adopt a target form in a way that minimizes bending
197 energy. In morphometrics, this interpolation is applied to a Cartesian coordinate system
198 in which deformation grids are constructed from two landmark configurations (Bookstein
199 1991). The total deformation of the thin-plate spline can be decomposed into
200 geometrically orthogonal components (partial warps) based on scale and their scores used
201 in multivariate statistics.

202

203 To test whether our diet treatments had a significant influence on craniofacial shape we
204 performed a discriminant function analysis using diet as the grouping variable. While this
205 analysis allowed us to assess how diet affected shape it also provided a quantitative
206 variable (canonical root scores) that described differences in shape due to benthic and
207 limnetic diet treatments. This provided a quantitative trait for examining the genetic basis
208 of plasticity via QTL mapping. We also used our landmark data to extract additional
209 variables related to specific anatomical regions of interest in our QTL analysis. These
210 traits were eye diameter, jaw width, and two measures of mechanical advantage (MA) in
211 the lower jaw – i.e., opening and closing MA. On average, LF possess relatively smaller
212 eyes, wider jaws and higher MA compared to TRC. In addition, we measured overall
213 body depth as the ratio between standard length and depth at anterior edge of the dorsal
214 fin. In the wild, LF has a deeper body than TRC. We also included a unique soft-tissue
215 trait in our analysis – the fleshy snout that extends rostrally from the upper jaw apparatus
216 and wraps in on itself to form a flexible flap of tissue that runs along the rostral edge of

217 the premaxilla. This trait is comprised predominantly of hypertrophied intermaxillary
218 ligament and associated connective tissue, is pronounced in LF, and is thought to
219 facilitate foraging efficiency (Konings 2001) in the benthic environment. The trait is
220 lacking in TRC, but is segregating in the cross (Concannon and Albertson 2015). The size
221 of the flap was measured directly in cross section. Because flap size scales allometrically
222 with body size, residuals from a linear regression were used for QTL mapping. Finally,
223 we measured the ratio between the two superficial subdivisions of the adductor
224 mandibulae muscle. This is the major muscle involved in the action of jaw closing, and
225 parental species show discrete differences in the ratio of the superficial surface area of the
226 A1 and A2 subdivisions. The A1 component inserts onto the maxilla and is relatively
227 larger than the A2 division in LF, while the A2 division inserts primarily on the
228 ascending arm of the articular process of the mandible and is relatively larger than the A1
229 in TRC. Because muscle ratios changed with body size, residuals were likewise used for
230 mapping.

231

232 *Test of the Flexible Stem Hypothesis*

233 We tested for support of the flexible stem hypothesis by comparing the trajectory of
234 plastic responses in the craniofacial region of our F₃ hybrids to the primary trajectory of
235 craniofacial divergence in the Malawi cichlid radiation as a whole. Quantitatively this
236 involved using the canonical root scores from our diet based DFA on F₃ craniofacial
237 shape, and the PC1 values derived from the same landmarks across a large sample of
238 species (80% of extant genera) from Lake Malawi (Cooper, Parsons et al. 2010). These
239 variables represented the plastic responses in our F₃ and the primary trajectory of
240 divergence in Malawi cichlids, respectively, and were used as the independent variable in
241 regressions on the Procrustes superimposed landmark data from their respective data sets.
242 These regressions identified a vector for each data set, which was normalized to unit
243 length. The angle between both vectors was then calculated as the arc cosine. We then
244 ran 2500 bootstraps with replacement for each group (i.e., F₃ plastic response, and
245 Malawi divergence) independently to produce a 95% confidence interval. The observed
246 angle was then compared against the confidence intervals for both groups to determine

247 whether it differed from random processes. These procedures were performed using the
248 software Vecompare6 (<http://www3.canisius.edu/~sheets/morphsoft.html>).

249

250 *Genotyping*

251 Our genetic mapping experiments were performed within an F₃ hybrid cross to allow for
252 a relatively higher number of recombination events compared to a typical F₂ design, and
253 thus increased resolution of mapping intervals. Because F₃ do not provide a tractable
254 pattern of Mendelian segregation, we used a genetic map derived from the F₂ generation
255 of the same pedigree and RAD genotyping (Albertson et al. 2014). Specifically, a subset
256 (n=364) of genetic markers spread evenly across the genome was genotyped in the F₃ via
257 the same RAD-seq methodology used in the F₂. Genomic DNA was extracted from flank
258 muscle tissue using DNeasy blood and tissue kits (Qiagen Inc. CA, USA), digested with
259 the restriction enzyme SbfI and processed into RAD libraries following Chutimanitsakun
260 et al. (2011). Barcoded, processed and purified DNA for each fish was sequenced using
261 an Illumina HiSeq 2000 (Illumina San Diego CA) and single-read (1 x 100 bp)
262 sequencing chemistry. Sequencing and bioinformatics also followed Chutimanitsakun
263 et al. (2011), and is described in greater detail in Albertson et al. (2014). QTL mapping was
264 then performed using F₃ genotypes and phenotypes (benthic n=132, limnetic n=133).

265

266 *QTL Analysis and Fine Mapping*

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

277

278 For both approaches QTL analyses were divided into two steps: (1) a statistically liberal
279 initial series of scans to identify putative loci and interactions; and (2) a more rigorous
280 multiple QTL mapping (MQM) approach. For step 1 tests were conducted using the
281 scanone and scantwo function within r/qtl (Broman 2009). From these tests putative QTL
282 loci were identified as having a LOD score greater than 3 or a LOD score greater than the
283 95% threshold (created by 1000 permutations for a given model). For step 2 this
284 collection of putative loci were then tested for verification by maximum likelihood-based
285 backward elimination (to specify cofactors) and permutation tests (i.e., 95% threshold as
286 determined by 1000 permutations) during subsequent rounds of MQM scans (Arends,
287 Prins et al. 2010).

288
289 For QTL identified from MQM mapping tests we took advantage of the MZ genome to
290 anchor QTL intervals to specific stretches of physical sequence in order to fine map
291 select QTL. Specifically, the 95% LOD confidence interval was calculated for each QTL
292 using the bayesint function in r/qtl, which allowed us to determine what mapped markers
293 fell within each QTL. Since marker names were based on scaffold position (e.g., marker
294 scaffold_1.6031297 corresponds to a SNP on scaffold #1 at 6031297bp) we next
295 identified contiguous stretches of physical sequence that corresponded to QTL intervals.
296 We then identified additional, unmapped, RAD-seq SNPs within these intervals that were
297 genotyped in the F₃. These were selected to span the region of interest with a spacing up
298 to 1 marker every ~200kb. This provided many additional genotypes that were used to
299 assess genotype-phenotype associations across candidate intervals. In addition we
300 resequenced the *ptch1* locus in a panel of LF and TRC from the populations used to
301 generate this cross. The specific SNP used was based on Roberts et al. (2011) (i.e.,
302 “Ptch1Loc10_SNP1”). Primer, polymorphism, and flanking sequence can be found in
303 Table S1 of this publication.

304

305

306 **Results and discussion**

307

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

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

319

320 We investigated both the lateral and ventral views of the head in F₃ hybrids using a
321 standard geometric morphometric (GM) approach. To test whether diet treatments had a
322 significant influence on lateral and ventral shape we performed a discriminant function
323 analysis (DFA) using diet as a grouping variable. This analysis extracted quantitative
324 differences in shape induced by diet treatments, and scores along this ‘plasticity axis’
325 were subsequently used for QTL mapping across all F₃. In addition, we measured seven
326 putatively adaptive traits related to feeding efficiency, including eye diameter, jaw width,
327 body depth, muscle architecture, ligament hypertrophy, and two functional aspects of
328 lower jaw shape (i.e., mechanical advantage of jaw opening and closing). These traits
329 were used for separate QTL analyses for each treatment.

330

331 Alternate foraging treatments led to a plastic response in some, but not all, traits
332 measured (Table 1). In terms of overall skull geometry in the lateral view, we found
333 support for the flexible stem hypothesis of adaptive radiation, which states that ancestral
334 patterns of phenotypic plasticity will shape the direction of adaptive evolution (West-
335 Eberhard 2003). We noted that alternate diets induced plastic changes in head
336 morphology that closely mimicked variation across Malawi cichlids (Fig. 1). Specifically,
337 when comparing the trajectory of these plastic responses in the craniofacial skeleton of
338 our F₃ hybrids to the primary trajectory of craniofacial divergence in the Malawi

339 radiation, we found that shape variation was statistically indistinguishable between the
340 two groups (observed angle of 58° between diet-based DFA in F₃ and PC1 of Malawi
341 was within 95% bootstrapped CIs) (Fig. 1). While our F₃ hybrids may not meet the strict
342 definition of an ancestor (and therefore ancestral plasticity), they are consistent with the
343 predicted scenario for Lake Malawi cichlids whereby their explosive evolutionary
344 diversification was facilitated by mass hybridization events (Seehausen 2004, Joyce, Lunt
345 et al. 2011). Thus, our feeding experiment induced a pattern of plasticity that is similar to
346 the evolutionary divergence observed among cichlid species, providing a context for our
347 genetic analysis.

348

349 *The genetic basis of phenotypic plasticity*

350 To characterize the genetic architecture of morphological plasticity we used two main
351 approaches: (1) QTL analyses of seven foraging-related traits in each diet treatment; and
352 (2) QTL investigations of an induced plasticity axis (represented by DFA scores of lateral
353 and ventral shape) across diet treatment. The first approach provided a test of the
354 hypothesis that plasticity is the result of cryptic genetic variation (CGV). CGV builds
355 upon earlier debates (Scheiner 1993, Via 1993) by recognizing that some genetic
356 variation does not normally contribute to the range of phenotypes present in a population,
357 but requires an environmental perturbation (or mutation) to be expressed (Gibson and
358 Dworkin 2004, Schlichting 2008, Palmer 2012, Paaby and Rockman 2014). The release
359 of such CGV will change the genotype-phenotype (G-P) map and is predicted to provide
360 a rich source of evolutionary potential (Gibson and Dworkin 2004). While CGV has been
361 well documented in several laboratory models, there is a conspicuous lack of empirical
362 data with respect to its genetic basis in natural populations undergoing adaptive
363 divergence (Ledon-Rettig, Pfennig et al. 2014).

364

365 We found robust support for CGV mediated plasticity. Across the 7 traits we detected a
366 total of 22 QTL (Fig. 2, Table S1), 9 of which were detected in animals reared on a
367 benthic diet, while 13 were detected in limnetic animals. All QTL exhibited a modest
368 effect on the phenotype, explaining between 10-17% of the phenotypic variation. Allele
369 effects generally ranged from additive to dominance, with only 2 QTL exhibiting an

370 overdominant mode of inheritance, both of which were detected in the limnetic
371 population. Notably, of these 22 QTL only a single locus was detected in both foraging
372 environments. It is unlikely that this trend is the result of a lack of statistical power as
373 LOD association profiles from each environment are dissimilar from one another (i.e.,
374 QTL for one treatment that exceeded the significance threshold were not even marginally
375 significant for the alternate treatment, see Fig 3). Further, we tested for correlations
376 between the LOD scores fish reared under different diets (using all loci exhibiting a
377 LOD>1). Using 10,000 bootstraps we found that LOD scores showed a strongly negative
378 relationship (all r values> 0.36) for all traits except MAo. These data suggest that CGV is
379 widespread for these traits, with negative correlations between LOD scores suggesting
380 exclusive environment specific G-P relationships. Moreover, we found that the degree of
381 plastic response for a given trait did not predict the degree of CGV. Three out of seven
382 traits did not exhibit a plastic response (Table 1), but still exhibited non-overlapping G-P
383 maps (Fig. 2, Table S1). These data suggest that canalization/buffering has an
384 environmentally dynamic genetic basis (Gibson and Dworkin 2004). Alternatively, the
385 single QTL detected in both foraging environments was for mechanical advantage of jaw
386 opening (MAo), which was by far the most plastic of those examined. Specifically, when
387 mean phenotypic values from each environment were compared, the t-value for MAo was
388 an order of magnitude greater than that for other traits (Table 1). Thus, not only is the G-
389 P map unique with respect to foraging environment, but the degree of overlap in G-P
390 relationships cannot be predicted by the degree of plasticity.

391

392 While CGV may inform us about the mechanisms of plasticity by revealing loci acting in
393 distinct environments, it can't account for G x E interactions underlain by loci operating
394 across environments. Since allelic variation at such loci could also be evolutionarily
395 relevant, our second approach was designed to determine whether some portion of
396 plasticity is controlled by loci with allele sensitivity (Via 1993, Via et al. 1995). Under
397 this scenario, plasticity loci are predicted to act across environments, but with alternate
398 alleles playing more (or less) prominent roles in different environments. Such loci could
399 represent "master control" switches for plasticity whereby alternate alleles activate
400 different downstream pathways in distinct environments. Our data provide evidence for

401 this type of plasticity locus as well. When reared on alternate diets, F₃ animals exhibited
402 significant differences in craniofacial morphology in both the ventral and lateral views
403 (Figs. 1 and 2). When mapping variation along the axes that distinguished foraging-
404 specific shapes, we detected robust support for QTL acting across environments (Fig. 2,
405 Table S1). Notably, the QTL for the ventral view overlapped with the sex-determining
406 locus on LG7, and is similar to other sex-linked craniofacial QTL detected for this cross
407 (Parsons, Wang et al. 2015). This observation raises the interesting possibility of a sex-
408 by-environment effect on ventral jaw shape. Allelic sensitivity at a single locus was also
409 evidenced by a QTL for lateral shape. This QTL mapped to a region on LG17 that is
410 distinct from any of the previously identified 20+ craniofacial QTL in this cross (Parsons,
411 Wang et al. 2015), suggesting a distinct genetic basis for plasticity. Given that this trait
412 encompasses the plastic response of the entire craniofacial complex we hypothesize that
413 this QTL on LG17 may contain genetic variation that regulates plastic responses via
414 ‘master control switches’ that initiate a cascade of effects across a number of downstream
415 genes or signal transduction pathways. Collectively our mapping study demonstrates that
416 phenotypic plasticity has a robust genetic signature in Malawi cichlids, providing a
417 critical foundation upon which the proximate molecular mechanisms that regulate its
418 manifestation and evolution may be studied.

419

420 *The evolution of plasticity via genetic assimilation at ptch1*

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

432 sensitivity of genotypic effects is markedly different between environments (Fig. 4, Table
433 S1). For example, the mean phenotype for F₃ animals homozygous for the LF allele is
434 lower in the limnetic environment than that for animals homozygous for the TRC allele in
435 the benthic environment. In other words, at this locus, the environment is a better
436 predictor of mean trait value than is genotype. Moreover, our data show that the LF allele
437 is more sensitive to foraging environment than the TRC allele. The difference in mean
438 trait value between treatments is ~50% greater for animals with the LF/LF genotype
439 (mean = 0.087) compared to animals with the TRC/TRC genotype (mean = 0.059). This
440 is consistent with the LF allele increasing plasticity through genetic accommodation, or
441 the TRC allele decreasing plasticity through genetic assimilation. Given the evolutionary
442 history of this locus, genetic assimilation is more likely.

443

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

450 Further, the difference in haplotype sensitivity to foraging environment became even
451 more pronounced when additional markers were added to span this interval, with the
452 difference in mean phenotype being almost twice as large in LF/LF animals compared to
453 TRC/TRC animals. While the 5' region of *ptch1* implicated in the evolutionary
454 divergence of jaw morphology in cichlids was missing from our SNP dataset, we
455 genotyped a panel of 20 wild LF and 20 wild TRC at the SNP previously shown to
456 exhibit the highest levels of divergence between LF and MZ, and confirmed that TRC
457 carry a high frequency of the MZ allele ($F_{ST} = 0.85$). The action of this gene is to
458 determine jaw shape early in development by mediating bone deposition around the
459 cartilaginous precursor of the retroarticular process, with higher levels associated with
460 more robust bone deposition and lower levels associated with less bone deposition
461 (Roberts, Hu et al. 2011, Hu and Albertson 2014). MZ and TRC both exhibit reduced
462 levels of bone deposition relative to LF (Powder, Milch et al. 2015), and harbour the

463 evolutionarily derived *ptch1* allele (Roberts, Hu et al. 2011). Thus, recent selection at the
464 *ptch1* locus appears to favour the development of more gracile jaw morphologies that are
465 advantageous for a more limnetic mode of feeding (Roberts, Hu et al. 2011). A reduction
466 in the sensitivity of the evolutionarily derived TRC/MZ allele to foraging environment is
467 therefore consistent with genetic assimilation acting to decrease plasticity in the limnetic
468 eco-morphology of MAo.

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470 *Conclusions: Toward an eco-devo approach*

471 An ongoing challenge in evolutionary genetics is to identify the “salient” molecular
472 changes that underlie evolutionary divergence (Hendrikse, Parsons et al. 2007), which
473 refers to characterizing the genetic variation that natural selection acts upon. While
474 pedigree mapping has been a useful and productive methodology in this pursuit,
475 especially with recent technological advances in high through-put genotyping (Nadeau
476 and Jiggins 2010), there is an emerging view that additive genetic variation accounts for a
477 relatively small percentage of phenotypic variation and rather it’s the context in which
478 traits develop that determines their final form (Hendrikse et al. 2007, Jamniczky et al.
479 2010, Pfennig et al. 2010, Hallgrímsson et al. 2014). Our work supports this idea, and
480 suggests that the genetic basis of a trait can be a ‘moving target’ for selection, with some
481 regions of the genome being consistently involved across environments (i.e., loci with
482 allele sensitivity), while many others are specific to the current conditions. Therefore, we
483 argue for a shift toward an eco-devo (or eco-evo-devo) approach (Abouheif, Fave et al.
484 2014, Gilbert and Epel 2015), wherein the salient environment is considered and
485 whenever possible incorporated into evo-devo studies. This will be especially important
486 for complex traits that are (by definition) heavily influenced by genetic background and
487 the environment. In all, such an integrative approach should provide a much richer (and
488 perhaps more realistic) picture of the genetic basis of adaptive morphological variation.

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493 **Figure legends:**

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495 **Figure 1. Cichlids as a flexible stem.** Micro computed tomography scans of the
496 craniofacial skeleton of two Lake Malawi cichlid species representing benthic (A) and
497 limnetic (B) eco-morphologies. Shape differences among benthic and limnetic cichlids
498 represent the major axis of divergence in Lakes Victoria, Malawi and Tanganyika
499 (Cooper et al., 2010). These include coordinated variation in craniofacial profile, head
500 depth, jaw rotation, and jaw length. Notably, diet induced plasticity in the F₃ mapping
501 population resulted in a very similar pattern of variation (C-D). Animals reared on a
502 benthic diet possessed, on average, deeper heads, more rounded craniofacial profiles, and
503 shorter more ventrally directed jaws (C). Alternatively, animals reared on a limnetic diet
504 developed more shallow heads, gradually sloping craniofacial profiles, and longer more
505 horizontally directed jaws (D). Landmarks used in morphometric shape analyses are
506 shown, and depicted in green on benthic fish and blue on limnetic fish.

507

508 **Fig. 2. The G-P map for foraging related traits is distinct between alternate feeding**
509 **environments.** The 95% confidence intervals for QTL are shown. QTL from the benthic
510 population are shown in green, whereas those in blue are from the limnetic population.
511 Purple QTL intervals are for DF1 scores derived from the lateral (Lat) and ventral (Vent)
512 views of F₃ fish. The list of traits and abbreviations are provided on the figure. Shape
513 variation along DF1 is also depicted via deformation grids for both lateral and ventral
514 views. In each analysis, benthic animals possessed, on average, more negative DF1
515 scores, whereas limnetic animals exhibited positive DF1 scores. Shape variation along
516 these axes included differences in head depth, craniofacial profile, jaw length and
517 rotation, and head width.

518

519 **Figure 3. Cryptic genetic variation is highly prevalent for determining cichlid**
520 **morphology.** Each panel represents line plots of LOD scores for a given trait under
521 benthic (green) and limnetic (blue) foraging conditions. Traits examined include muscle
522 architecture (A), body depth (B), eye diameter (C), mouth flap (D), mechanical advantage
523 opening (E), mechanical advantage closing, and jaw width (G). Notably, the peaks of

524 LOD scores rarely overlap between foraging environments indicating that the genetic
525 basis of these traits is plastic.

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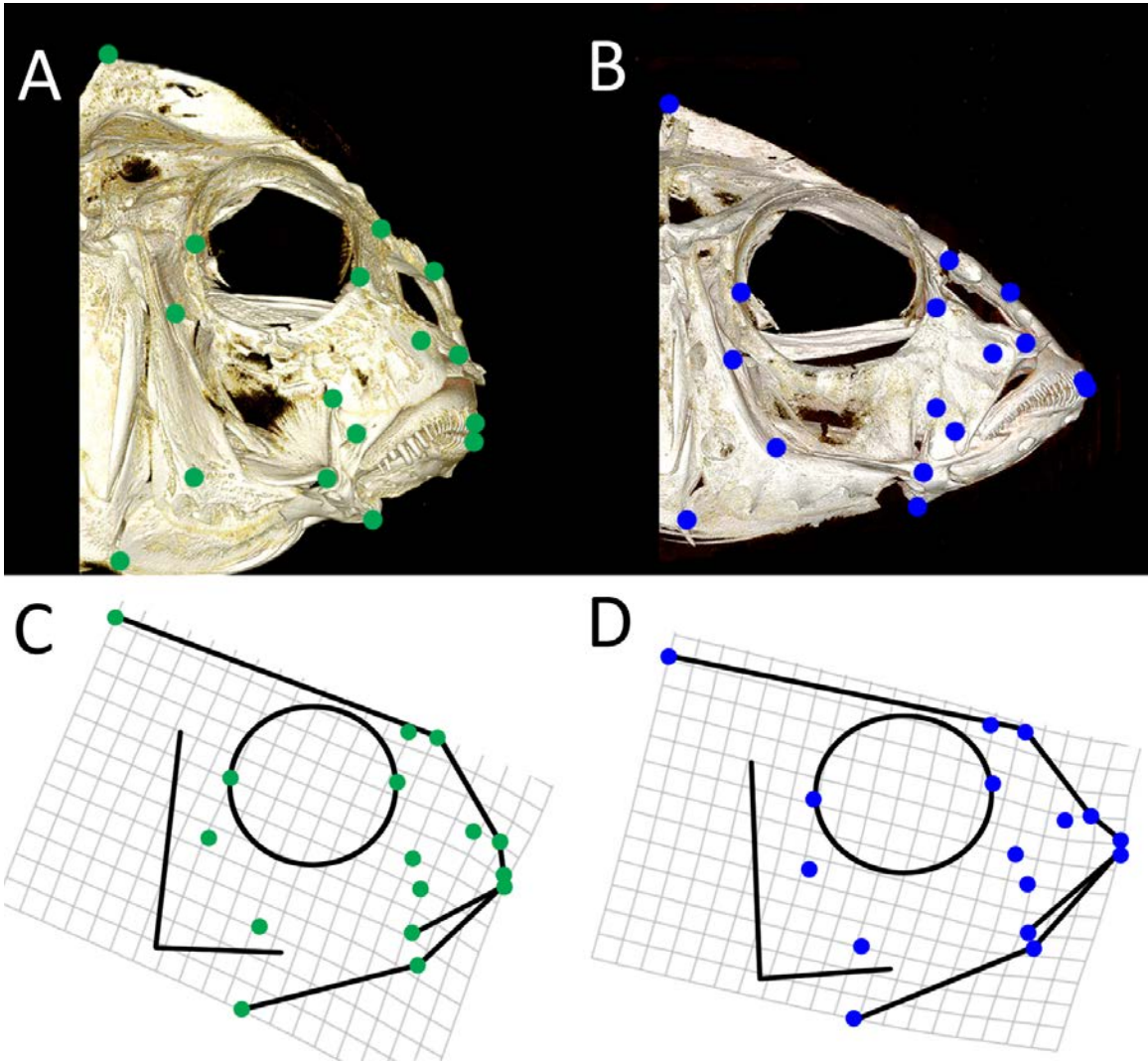
528 **Figure 4. The evolution of plasticity via genetic assimilation at the *ptch1* locus.** The
529 QTL for MA₀ mapped to scaffold 14 in both benthic and limnetic treatments, and
530 corresponds to a QTL previously found to be due to variation at *ptch1* (Roberts et al.,
531 2011). Additional RAD-seq markers were used to fine map this region and peak
532 genotype-phenotype association was observed in markers adjacent to *ptch1*, which is at
533 ~5.2Mb on scaffold 14. Regardless of genotype, means phenotypes did not overlap
534 between diet treatments, which means that foraging environment is a better predictor of
535 shape than is genotype at this locus. Furthermore, we find that haplotypes are
536 differentially sensitive to the environment at this locus. The difference in mean
537 phenotype for the LF/LF genotype at 5.4Mb is nearly twice as large as that for the
538 TRC/TRC genotype (red brackets, top panel; bottom panel). Given that the LF allele is
539 ancestral, this represents an unambiguous example of genetic assimilation.

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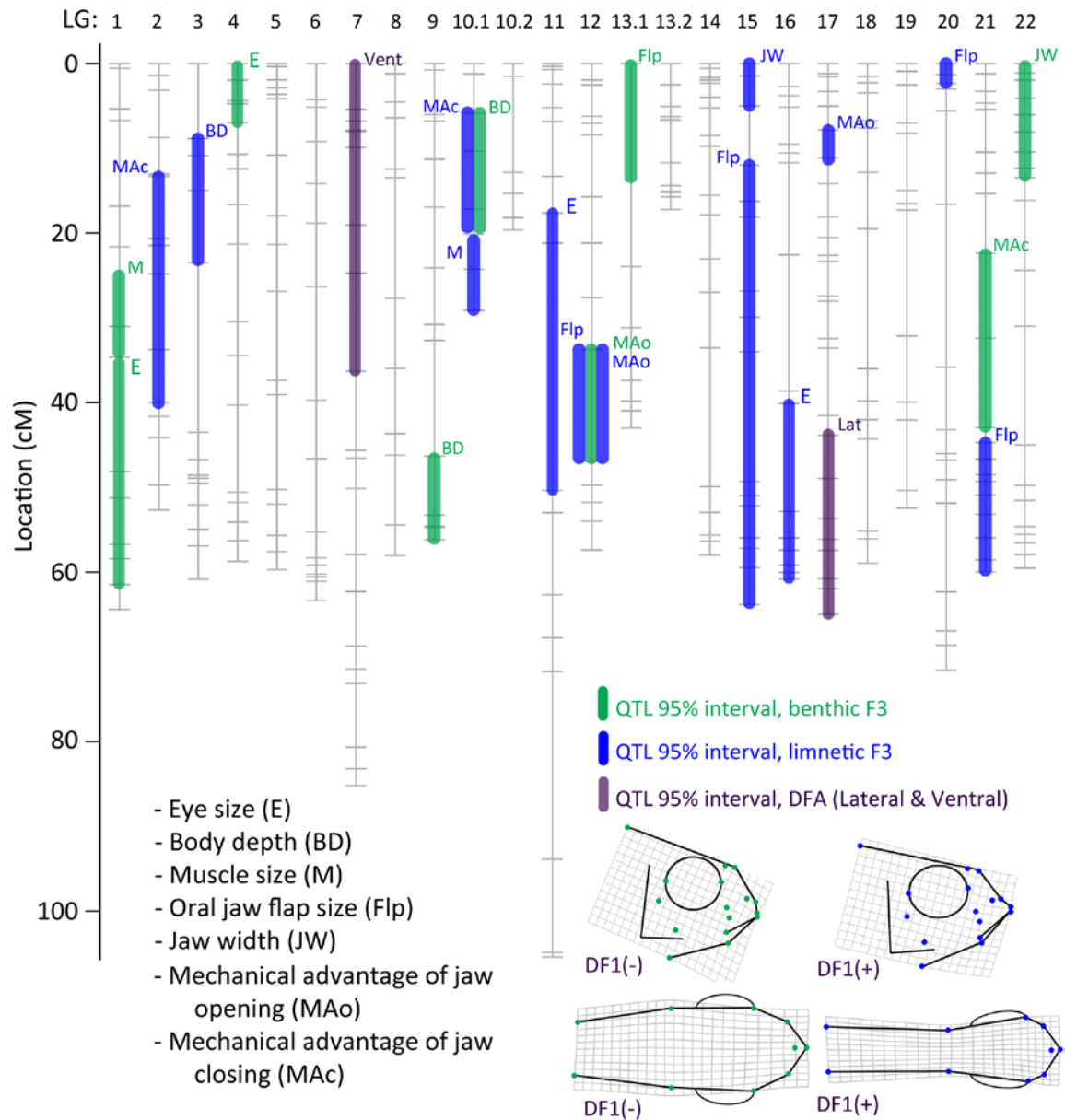
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543 **Figure 1**



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555 **Figure 2**

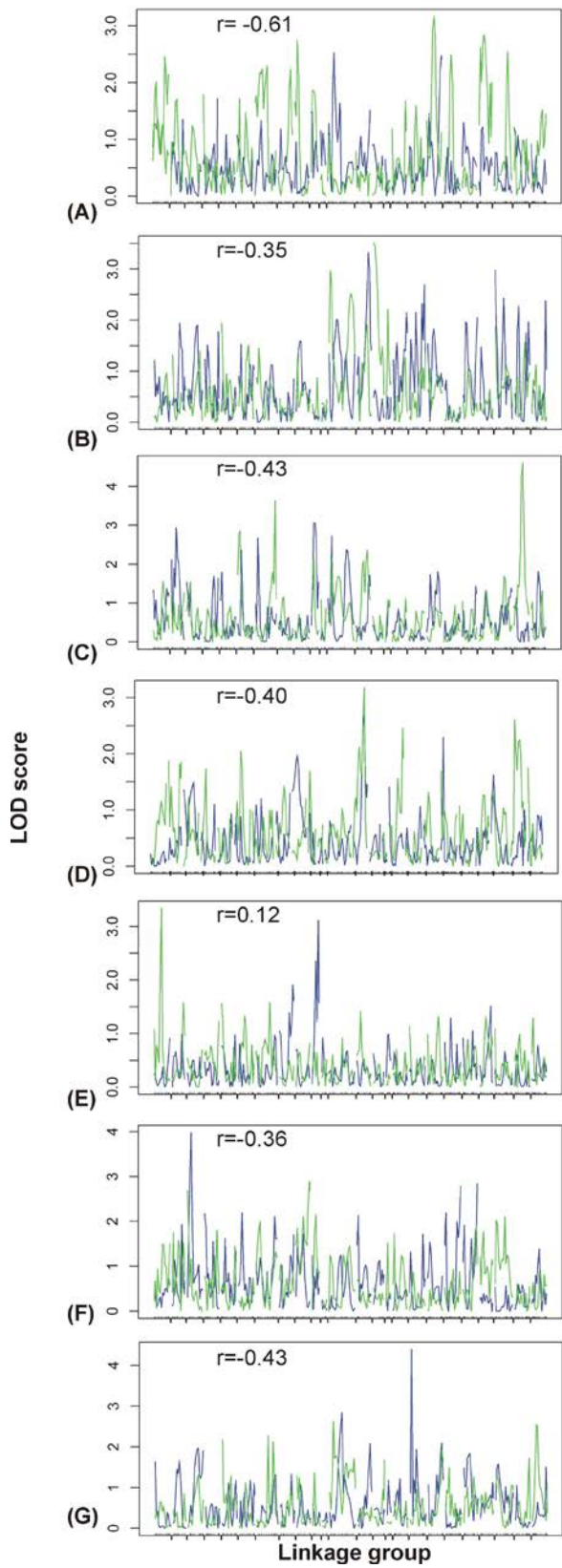


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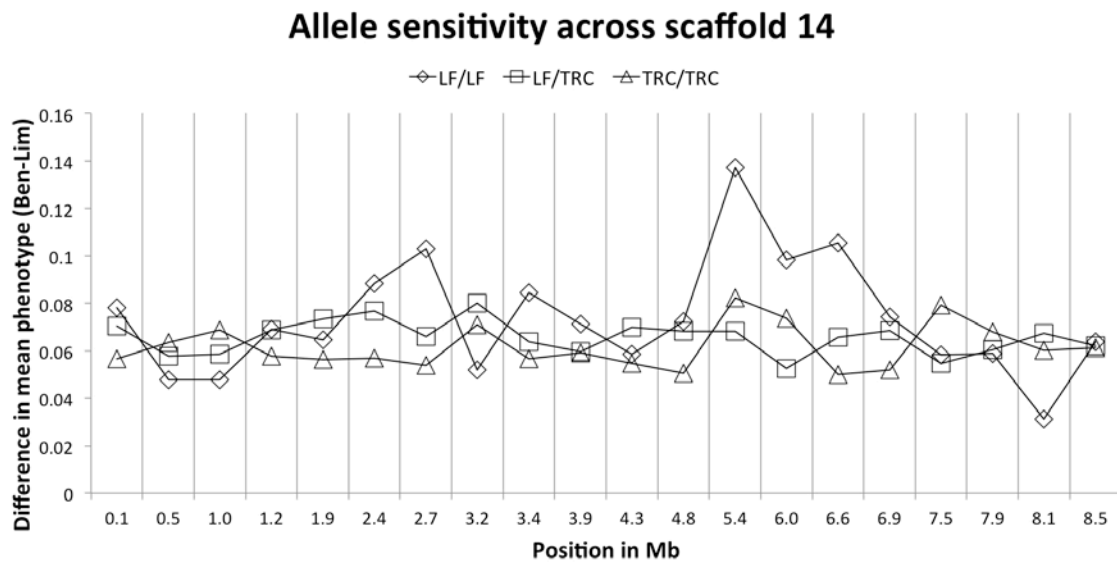
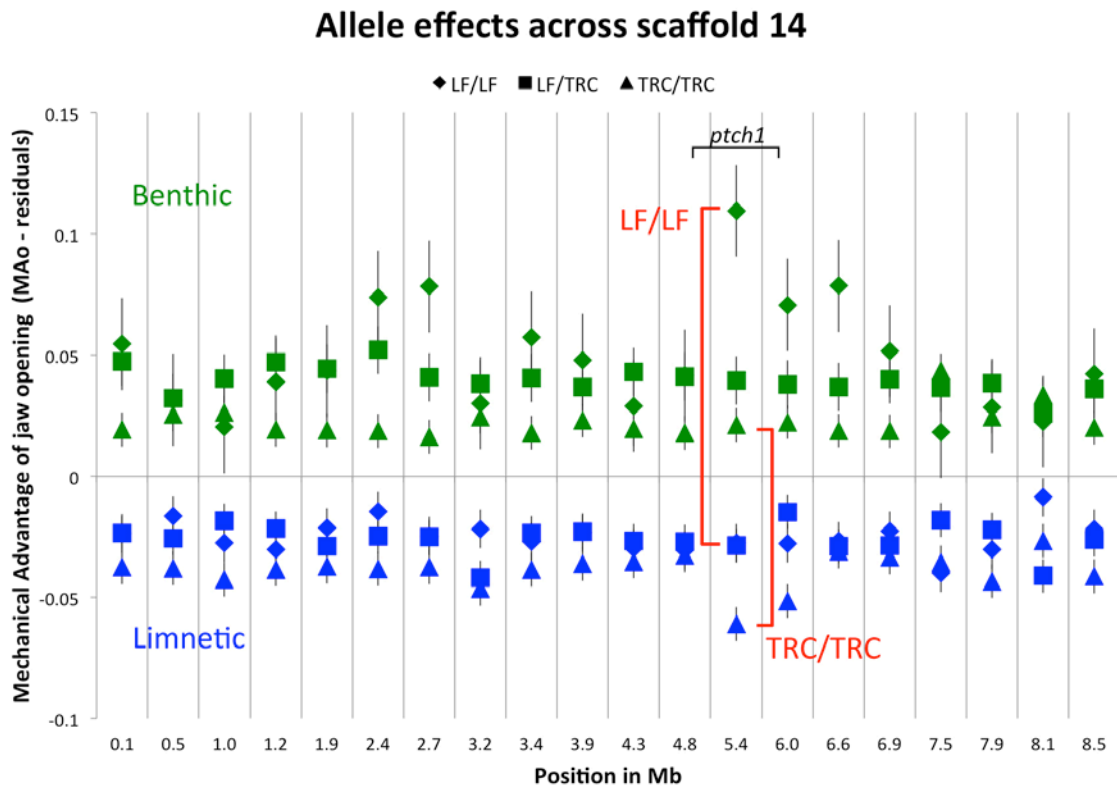
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559 **Figure 3**



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561 **Figure 4**



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Trait	Averages		t	df	p
	Benthic	Limnetic			
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 _o	0.029611	-0.029198	10.192	254	< 0.0001
MA _c	0.009653	-0.008197	2.042	254	0.042

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Table 1. Mean differences in phenotype induced by alternate foraging

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environment. Significant differences in mean phenotypes are indicated in boldfaced

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lettering. Note, in particular, the pronounced plastic response in MA_o.

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Abbreviations: MA_c, mechanical advantage of lower jaw closing; MA_o, mechanical

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advantage of lower jaw opening.

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Supplemental Information:

Movie S1: Benthic foraging behavior (separate attachment)

Movie S2: Limnetic foraging behavior (separate attachment)

Trait	Treatment	QTL	IG	cM	QTL interval	LOD	PVE (%)	Allele Effects					PVE sum (%)
								Lf/Lf	Lf/Trc	Trc/Trc	Add	Down	
Eye Size	Benthic	Eye.Ben ₁	1	50.0	34.65-61.47	4.372**	14.2	0.5579863	0.531293	0.4986151	0.0297	0.0030	24.5
		Eye.Ben ₂	4	0.0	0.00-7.00	3.126*	10.3	0.5240081	0.5356153	0.5636037	-0.0198	-0.0082	
	Limnetic	Eye.Lim ₁	11	25.0	17.64-50.37	3.326*	10.9	0.5549417	0.5199657	0.5306703	0.0121	-0.0228	21.3
		Eye.Lim ₂	16	60.0	40.13-60.82	3.151*	10.4	0.5302653	0.5376887	0.5225471	0.0039	0.0113	
Body Depth	Benthic	BD.Ben ₁	9	55.0	46.33-56.19	4.63**	14.9	31.13779	8.53832	-24.70940	27.9236	5.3241	26.5
		BD.Ben ₂	10.1	15.0	5.86-20.18	3.53**	11.6	-17.69934	-8.19794	114.40602	-66.0527	-56.5513	
	Limnetic	BD.Lim ₁	3	15.0	8.87-23.5	3.95**	12.8	-39.88877	-11.81503	49.69876	-44.7938	-16.7200	12.8
Muscle Ratio	Benthic	A1:A2.Ben ₁	1	30.0	25.00-34.65	4.82**	15.4	0.03693	-0.08005	0.11151	-0.0373	-0.1543	15.4
		A1:A2.Lim ₁	10.1	25.0	20.18-29.11	3.63**	11.9	-0.01619	-0.06672	0.05658	-0.0364	-0.0869	11.9
Flap	Benthic	Flap.Ben ₁	13.1	5.0	0.00-15.00	4.12**	13.3	0.19481	-0.00435	-0.20304	0.1989	-0.0002	13.3
		Flap.Lim ₁	12	45.0	33.74-51.78	3.90**	12.6	0.22450	-0.02541	-0.08808	0.1563	-0.0936	48.2
	Limnetic	Flap.Lim ₂	15	60.0	12.01-63.83	3.31*	10.8	-0.06651	0.06573	-0.08113	0.0073	0.1395	
		Flap.Lim ₃	20	0.0	0.00-3.01	4.39**	14.1	0.20994	0.17572	0.02899	0.0905	0.0563	
		Flap.Lim ₄	21	55.0	44.73-59.98	3.26*	10.7	0.09028	0.01384	0.21891	0.1546	0.0505	
Jaw Width	Benthic	Width.Ben ₁	22	20.0	13.47-24.39	4.09**	13.3	0.20713	0.21701	0.20539	0.0009	0.0107	13.3
	Limnetic	Width.Lim ₁	15	5.0	1.47-5.01	5.31**	16.8	0.21551	0.18732	0.20213	0.0067	-0.0215	16.8
MA ₀	Benthic	MA ₀ .Ben ₁	12	40.0†	33.74-51.78	3.86**	12.6	0.07500	0.03400	0.01900	0.0280	-0.0130	12.6
		MA ₀ .Ben ₂	12	40.0†	33.74-46.9	4.07**	13.2	-0.01200	0.02800	-0.04000	0.0140	-0.0020	23.4
	Limnetic	MA ₀ .Lim ₁	17	10.0	7.86-11.14	3.07*	10.2	-0.03400	-0.01800	-0.05300	0.0095	0.0255	
MA _c	Benthic	MA _c .Ben ₁	21	35.0	22.39-42.97	5.19**	16.5	0.06880	-0.00300	-0.01700	0.0429	-0.0289	16.5
		MA _c .Ben ₂	2	20.0	13.35-39.98	3.23*	10.7	0.03000	-0.01500	-0.02200	0.0260	-0.0190	24.2
	Limnetic	MA _c .Lim ₁	10.1	15.0	5.86-20.18	4.15**	13.5	0.02100	-0.02500	-0.01400	0.0175	-0.0285	
DF1 (Lateral)	All F ₃	DF1.Lat	17	55.0	43.87-57.44	3.60**	7.9	4.27377	3.35325	3.92451	0.1746	-0.2459	7.9
DF1 (Ventral)	All F ₃	DF1.Vent	7	25.0	0.00-35.00	4.45**	9.3	3.64362	4.13707	4.35429	-0.3553	0.1381	9.3

* genome wide significance at p < 0.1

** genome wide significance at p < 0.05

† This is the only shared QTL between foraging treatments in the dataset. The nearest marker is "scaffold_14:688397", which is ~1.5 Mb away from *ptchl* (5189942-5238428 Mb on scaffold 14).

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Table S1. Results of QTL analyses for seven foraging-associated traits in each environment. Boldfaced values indicate the haplotype that increases trait value in the F₃. Abbreviations are as follows: A1, the first subdivision of the adductor mandibulae muscle; A2, the second subdivision of the adductor mandibulae muscle; BD, body depth; DF1, canonical root scores from our diet based DFA on F₃ craniofacial shape; Flap, a measure of the hypertrophied intermaxillary ligament, which in LF forms a conspicuous flap that over hangs the upper jaw (see Concannon and Albertson, 2015 for more detail on this trait); MA_c, mechanical advantage of lower jaw closing; MA₀, mechanical advantage of lower jaw opening.

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