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Parsing parallel evolution: ecological divergence and differential gene expression in the adaptive radiations of thick-lipped Midas cichlid fishes from Nicaragua

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Abstract

The study of parallel evolution facilitates the discovery of common rules of diversification. Here, we examine the repeated evolution of thick lips in Midas cichlid fishes (the *Amphilophus citrinellus* species complex)—from two Great Lakes and two crater lakes in Nicaragua—to assess whether similar changes in ecology, phenotypic trophic traits and gene expression accompany parallel trait evolution. Using next-generation sequencing technology, we characterize transcriptome-wide differential gene expression in the lips of wild-caught sympatric thick- and thin-lipped cichlids from all four instances of repeated thick-lip evolution. Six genes (apolipoprotein D, myelin-associated glycoprotein precursor, four-and-a-half LIM domain protein 2, calpain-9, GTPase IMAP family member 8-like and one hypothetical protein) are significantly underexpressed in the thick-lipped morph across all four lakes. However, other aspects of lips' gene expression in sympatric morphs differ in a lake-specific pattern, including the magnitude of differentially expressed genes (97-510). Generally, fewer genes are differentially expressed among morphs in the younger crater lakes than in those from the older Great Lakes. Body shape, lower pharyngeal jaw size and shape, and stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) differ between all sympatric morphs, with the greatest differentiation in the Great Lake Nicaragua. Some ecological traits evolve in parallel (those related to foraging ecology; e.g. lip size, body and head shape) but others, somewhat surprisingly, do not (those related to diet and food processing; e.g. jaw size and shape, stable isotopes). Taken together, this case of parallelism among thick- and thin-lipped cichlids shows a mosaic pattern of parallel and nonparallel evolution.

Keywords: crater lakes, divergent selection, geometric morphometrics, next-generation sequencing, RNA-Seq, sympatric speciation

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Introduction

'Parallel phenotypic evolution' describes the repeated, independent evolution of similar phenotypes in closely related lineages (reviewed in Elmer & Meyer 2011). Many classic cases of ecological speciation are,

in fact, examples of parallel evolution (Schluter 2000; Salzburger & Meyer 2004), including the Holarctic marine to freshwater invasions of stickleback fishes (Bell & Foster 1994), adaptive radiations such as Darwin's finches on the Galapagos Islands (Grant & Grant 2008) or cichlid fishes in African and Central American lakes (Meyer 1993; Barlow 2000). Instances of parallel evolution raise the question, *how* and *why* does evolution repeat itself in similar environments? Specifically, we can ask: does the repeated evolution of a similar trait coincide with parallel ecological and transcriptomic diversification, or are similar phenotypes evolving by different ecological and genomic mechanisms? Recent advances on this fundamental topic in evolutionary biology (Meyer 1999; West-Eberhard 2002; Sanetra *et al.* 2005; Losos 2011) have been aided by comparative developmental analyses and new genomic approaches –including the transcriptome-wide expression analyses as we use here (Johnson *et al.* 2010; Mahler *et al.* 2010; Kolbe *et al.* 2011; Kusumi *et al.* 2011; Losos & Pringle 2011; Eckalbar *et al.* 2012; Sanger *et al.* 2012).

Differences in gene expression or gene sequence can directly underpin ecologically relevant phenotypic variation in populations (e.g. Oleksiak *et al.* 2002; Whitehead & Crawford 2006; Hoekstra & Coyne 2007), thus providing the raw material upon which selection acts (Ellegren & Sheldon 2008). Gene expression and function link the genotype to the phenotype and, in this role, are central to understanding adaptation, phenotypic diversification, repeated evolution and ecological speciation (e.g. Sanetra *et al.* 2005; Manceau *et al.* 2010; Pavey *et al.* 2010). For instance, in Darwin's finches and African cichlids, it is variation in the expression of *bmp*4 that is linked to phenotypic variation in beak and jaw morphology, ecologically relevant morphological traits essential for each groups' adaptive radiation (Abzhanov *et al.* 2004; Albertson *et al.* 2005). In three-spined sticklebacks, pelvic reduction repeatedly evolved via a reduction of *Pitx*1 expression, with fixation in populations dependent on factors such as the presence of gape-limited predators and calcium limitation, among others (Shapiro *et al.* 2004; Chan *et al.* 2010).

However, parallel phenotypic evolution does not always arise from parallel genomic and transcriptomic changes (reviewed in Elmer & Meyer 2011). At the genomic level, either standing genetic variation or new mutations can be the target of selection (reviewed in Barrett & Schluter 2008). For example, the parallel evolution of cryptic coat coloration in *Peromyscus* mice is driven by an assortment of changes in gene function and expression, and the fixation of ancestral or, at times, unique, derived genetic mutations (reviewed in Manceau *et al.* 2010). In freshwater sticklebacks, selection repeatedly favours alleles present in the ancestral marine populations in generating repeated, similar phenotypes (Schluter & Conte 2009). How, when and to what extent parallel phenotypes evolve under a nonparallel genetic basis may prove as interesting as the parallel examples, because it suggests restricted phenotypic solutions to ecological problems (Losos 2011; Wake *et al.* 2011).

Recent advances in DNA sequencing technology allow the rapid acquisition of vast, genomic-scale resources and permit the collection of transcriptomic data for ecological as well as evolutionary model species (Stapley *et al.* 2010). Before sequence-based transcriptomics, it was microarrays and qPCR techniques based on a priori sequence information that revealed the importance of gene expression differentiation in the diversification of natural populations (Oleksiak *et al.* 2002; Storey *et al.* 2007; Pavey *et al.* 2010), particularly in the phenotypic divergence of teleost populations (e.g. Derome *et al.* 2006; Kobayashi *et al.* 2006; St-Cyr *et al.* 2008). Comparative transcriptomic approaches permit an expanded purview into gene expression by detecting differential expression in novel genes and the interaction of many genes of small effect (Mackay *et al.* 2009; Stapley *et al.* 2010), both of which are likely to be important in the evolution of novel phenotypes. In short, both the novel genomic tools—and the morphological and ecological tools—now exist to parse evolutionary events into their repeated vs. unique components in cases of repeated evolution of particular phenotypic traits (e.g. Manceau *et al.* 2010; Elmer & Meyer 2011). Here, we leverage these new tools to investigate the similarity of ecomorphological and transcriptomic changes accompanying the repeated evolution of hypertrophied lips in Central American Midas cichlid fishes (*Amphilophus* species complex, Gunther 1864) from the Great Lakes and crater lakes of Nicaragua (Fig. 1).

Midas cichlids provide repeated natural experiments in ecological speciation (Barlow & Munsey 1976; Meyer 1990a; Elmer *et al.* 2010a). In Nicaragua, tectonic and volcanic activity led to the formation of numerous, small crater lakes. At least eight crater lakes were colonized by Midas cichlids originating from the Great Lakes–Lake (L.) Managua and L. Nicaragua (Wilson *et al.* 2000; Barluenga & Meyer 2004, 2010; Elmer *et al.* 2010b, 2012). In several isolated crater lakes, similar morphological types are repeatedly observed, including a deep-body benthic morph, an elongate, limnetic morph and a thick-lipped morph (reviewed in Elmer *et al.* 2010a). Despite the similarity of these repeated phenotypes, phylogenetic analyses have shown that individuals within any given crater lake are more closely related to one another than they are to individuals in any other lake (Wilson *et al.* 2000; Barluenga &

Fig. 1 Map of western Nicaragua and relevant lakes. Representative thick- and thin-lipped Midas cichlid morphs from the Great Lakes Nicaragua and Managua (left) and crater lakes Apoyeque and Masaya (right).

Meyer 2010). Thus, the occurrence of common morphological types across crater lakes reflects parallel phenotypic evolution.

Thick lips ('hypertrophied lips') are thought to be an adaptation for foraging between rock crevices and a key trait allowing for ecomorphological differentiation of sympatric populations (Barlow & Munsey 1976; Arnegard & Snoeks 2001; Elmer *et al.* 2010b). Thick-lipped Midas cichlids from the Great Lakes, L. Nicaragua and L. Managua, are called *Amphilophus labiatus* (Barlow & Munsey 1976). *Amphilophus labiatus* is only weakly genetically differentiated at neutral loci (Barluenga & Meyer 2010) and in body morphometrics (Meek 1907; Barlow & Munsey 1976) from their more abundant sympatric sister species, the thin-lipped *A. citrinellus*—but are clearly different in body shape (Elmer *et al.* 2010a). Similarly, while considerable ecomorphological differentiation separates the thin- and thick-lipped Midas morphs in crater lake Apoyeque, this is not accompanied by significant genetic differentiation (Elmer *et al.* 2010b). Thick-lipped Midas cichlids are found in sympatry with thin-lipped Midas cichlids in three crater lakes: Apoyeque, occasionally in Masaya and extremely rarely in Xiloá (Barlow 1976; Waid *et al.* 1999; McKaye *et al.* 2002; Fig. 1). It is not clear yet in all crate lake instances whether thick-lipped Midas cichlids represent a different species than the sympatric thin-lipped morphs; all are currently considered part of the polyphyletic species *A. citrinellus* (Elmer *et al.* 2010b). However, a growing body of evidence suggests that the thick-lipped morphs from the crater lakes may actually represent biological species (McKaye *et al.* 2002; Meyer *et al.*, unpublished data, including observations of assortative mating). Ecological differentiation between thin- and thick-lipped morphs within crater lakes, and the similarity of ecomorphological differentiation across lakes, has to date not been assessed.

The repeated evolution of thick lips in crater lake Midas cichlids is well suited to address questions regarding the ecological and transcriptomic aspects of parallel phenotypic evolution, given the environmental homogeneity within (and heterogeneity across) lakes and the recent diversification of sympatric morphs. From a genomic perspective, the short time period since crater lake colonization [~100 years for Apoyeque (Elmer *et al.* 2010b) to maximally 6000 years for Masaya (Kutterolf *et al.* 2007; Elmer *et al.* 2012)] from the Great Lakes would be expected to limit the extent of novel mutations and hence constrain the evolution of phenotypic variation. In the present study, we aim to test for parallel ecomorphological and transcriptomic changes accompanying the repeated evolution of the thick-lipped phenotypes in Midas cichlids in the two Great Lakes that were the source populations and in two crater lakes of Nicaragua that were colonized from them during the last few thousand years. Specifically, we quantify trophic and morphological differentiation using correlates of foraging ecology, diet and habitat, namely head and body shape, jaw morphology, lip size and stable isotopes. We test for differences in the expression levels of genes between the lip tissue of thin- and thick-lipped morphs using RNA-Seq (Wang *et al.* 2009). Considered together, we assess the degree to which parallel ecomorphological and transcriptomic differentiation is associated with the repeated evolution of the thick-lipped phenotype.

Methods

Field collections

We collected adult specimens of *A. citrinellus* and *A. labiatus* from Great Lakes Nicaragua and Managua and thin- and thick-lipped *A.* cf. *citrinellus* from crater

lakes Apoyeque and Masaya in 2001, 2003, 2005, 2007 and 2010 (Fig. 1; Table S1, Supporting information). Some individuals from L. Masaya were smaller than those collected in other lakes and may be subadult (see Results ii). Individuals were captured using gill nets or speargun, or purchased live from local fishermen. In 2007 and 2010, 0.5–1 cm^3 of the upper lip was dissected in a subset of individuals and preserved in RNAlater (Qiagen). Standardized photographs from lateral view were taken for all specimens. Specimens were preserved in ethanol for later extraction of pharyngeal jaws and muscle tissue. Crater lake *A.* cf. *citrinellus* were categorized as thick-lipped if the upper lip protruded markedly beyond the margin of the snout and thin-lipped if it did not. Table 1 describes the number of individuals used in each analysis.

Ecology and morphology

Lip state and size. We quantified lip size as the area of the upper lip relative to standard body length in standardized photographs of living or recently dead fish, both measured in ImageJ (Table 1, P.M.H., ver 1.43u, Rasband 1997). Variation in lip size within and among lakes and morphs was tested with an analysis of covariance on top lip area with standard length as a covariate (ANCOVA, ln-transformed variables).

Stable isotope analysis. Stable carbon ($\partial^{13}C$) and nitrogen ($\partial^{15}N$) isotope ratios were analysed using gas chromatography/combustion isotope ratio mass spectrometry (GC-C-IRMS) (Table 1). Briefly, approximately 1 g of white muscle tissue was extracted from the back of the head of ethanol-preserved specimens, dried for 24–48 h at 50–60 °C and pulverized, and a 0.9–1.4 mg subsample was analysed for isotopes (see Fig. S1, Supporting information, for additional details). $\partial^{13}C$ values were corrected for lipid content using C:N ratios (Kiljunen *et al.* 2006); all values in this text refer to the defatted results.

Isotopic differences between sympatric morphs were assessed jointly using a nonparametric multivariate permutation test (nonparametric MANOVA, analysed in PAST, Hammer *et al.* 2001) and for each stable isotope independently using a Wilcoxon two-sample test. Niche variation between morphs was examined with two measures of within-morph $\delta^{13}C$-$\delta^{15}N$ isotopic variability: (i) the total area of the convex-hull (TA) following Layman *et al.* (2007) and (ii) the adjusted standard ellipse area (SEAc) as developed by Jackson *et al.* (2011) with reduced sensitivity to differences in sample size. In both methods, larger values indicate increased isotopic niche size. Cross-lake differences in stable isotope values and niche width are not considered, as a number of environmental factors affect baseline carbon and nitrogen values and variation therein (Post 2002; Casey & Post 2011; interlake offsets shown and discussed in Fig. S2, Supporting information).

Head and body shape. We examined head and body shape differentiation between thick- and thin-lipped fishes using geometric morphometrics. Eighteen homologous body landmarks were digitized in TPSDIG2.16 (Rohlf 2001) from standardized images (Table 1; Fig. S3, Supporting information). Shape analyses were performed in MorphoJ 1.03d (Klingenberg 2011) and included landmark alignment by Procrustes superimposition (Zelditch *et al.* 2004) and a correction for allometry using centroid size (8.3% of shape variance explained by centroid size, $P < 0.0001$; methods detailed in Elmer *et al.* 2010a). Multivariate variation in individual body shapes within and across lakes was visualized

Table 1 Specimen count summary. Specimen counts are presented by lake, species, morph and analysis

			Morphology (*n*)					
Lake	Species	Morph	Body shape	Jaw shape	Jaw measurements	Lip size	Isotopes (*n*)	Expression analysis (*n*)
Great Lakes								
Nicaragua	*A. citrinellus*	Thin	224	62	62	62	36	5
	A. labiatus	Thick	145	43	44	44	22	5
Managua	*A. citrinellus*	Thin	177	45	46	46	40	6
	A. labiatus	Thick	37	20	21	21	33	6
Crater Lakes								
Apoyeque	*A.* c.f. *citrinellus*	Thin	83	122	125	125	31	4
	A. c.f. *citrinellus*	Thick	39	45	46	46	29	5
Masaya	*A.* c.f. *citrinellus*	Thin	54	54	53	53	32	5
	A. c.f. citrinellus	Thick	18	17	18	18	21	6
Total			777	408	415	415	244	42

using principal component analysis (PCA) on the regression residuals from the allometric size correction (Fig. S4, Supporting information). Shape differences between groups were visualized using thin-plate splines (Dryden & Mardia 1998) (Fig. S5, Supporting information), and differentiation of thin- and thick-lipped fish within lakes was tested with Hotelling's T^2 test.

Lower pharyngeal jaw shape. Lower pharyngeal jaws (LPJ) were extracted, cleaned, dried and photographed. Seventeen landmarks were digitized (P.M.H.) from the images for geometric morphometrics (Table 1; Fig. S3, Supporting information). Procrustes coordinates were corrected for allometry using body standard length (8.3% of shape variance explained, $P < 0.0001$) and analysed and visualized using the same methods as described in the body shape analyses (section 'Head and body shape'.).

LPJ measurements. Lower pharyngeal jaws were weighed and measured with calipers for depth, width and length (Table 1). These measurements were collapsed to a single variable ($PC1_{jaw}$) using PCA. An analysis of covariance (ANCOVA, $PC1_{jaw}$ with ln-transformed standard length as a covariate) was used to test for differentiation between morphs within and among lakes.

Gene expression

RNA extraction. Total RNA was extracted from preserved lip samples for Illumina library preparation (Table S1, Supporting information). Samples were removed from RNAlater and homogenized in 1 ml of Trizol (Invitrogen) in an MP Biosciences homogenizer (medium–high intensity, 45-s duration). RNA extraction was performed following the manufacturer's protocol and re-precipitated for 1 h with 0.1 volume of 3M NaOAc and two volumes of 100% EtOH. Quantity and quality of total RNA were assessed by spectrophotometry, agarose gel electrophoresis and microfluidic electrophoresis (Bioanalyzer 2100, Agilent Technologies). Only high-quality RNA samples (RNA integrity number values > 7) with more than 2 μg of total RNA were sequenced.

Illumina sequencing. Libraries were generated for 42 individuals using the Illumina TruSeq RNA sample preparation kit (low-throughput protocol) according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Library quantification and quality assessment was performed on a Bioanalyzer 2100 (Agilent Technologies). Each of the 42 samples was barcoded with one of the 12 available unique sequence indexes. Equimolar volumes of 12 different barcoded samples (i.e. 12 individuals) were pooled for each lane. For technical replication, each indexed sample was loaded in two different lanes of the flowcell. Single-end sequencing of clustered template DNA on the Genome Analyzer II was performed using four-color DNA Sequencing-By-Synthesis (SBS) technology with 79 cycles (including seven cycles for the barcode sequences). A total of seven lanes were sequenced simultaneously on a single flowcell.

Quality filtering and transcriptome assembly. Raw reads were quality controlled before *de novo* assembly, read mapping and further analysis. Read trimming was performed in CLC Genomics Workbench v4.9 (CLC bio, Aarhus, Denmark) and quality control in FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). Low-quality reads were identified (CLC parameter 'limit' set to 0.05) and excluded. Reads were then trimmed of adapters and ambiguous bases at both ends. After processing, the data consisted of a total of 224 438 049 reads (2 159 364–9 848 493 reads per individual) with a mean length of 43 nucleotides.

A *de novo* assembly was conducted with Oases v0.1.22 (Schulz *et al.* 2012) by combining the output of the Illumina sequencing run described above with in-house transcriptomic resources for Midas cichlids to maximize assembly accuracy (additional reads: 233 420 425; Henning *et al.* in prep; paired-end with 72 cycles per read). We selected an assembly kmer length (kmer = 23) on the basis of N50 after a kmer search (kmer 19-41). Assembled transcripts had a minimum coverage of 10 (i.e. >10 reads contributing to a contig) and length of 200 bp. The final assembly included 78 106 contigs or alternative transcripts that were assigned by Oases to 32 033 loci with N50 size of 2740 bp. We conducted a BLASTX (Altschul *et al.* 1997) search of the assembled contigs against the vertebrate sequences of GenBank protein database (*e*-value < e−10) to exclude sequences resulting from contamination or misassembly. Contamination may have arisen, for example, from the accidental inclusion of fish parasites in lip samples or misassembly from the inaccurate assignment of reads to contigs. In total, we obtained 15 069 genes (comprised of 39 922 contigs). These genes were used in all further analyses.

Read mapping was performed in CLC Genomics Workbench with voting for conflict matches, ungapped alignment with mismatch cost for the nucleotides 2, limit 8, and random assignment of reads with multiple matches to only one of the matched contigs. Technical replicates were assessed by projecting the expression profiles (in read per kilo base model or RPKM values) of each sample (two samples per individual) in a PCA

conducted in CLC Genomics Workbench v4.9; results indicated almost identical expression patterns between replicates.

Differential expression analysis, SNP identification and functional annotation. Identification of differentially expressed (DE) genes was performed in DESeq (Anders & Huber 2010), using read count data for each individual (after pooling the reads of the two technical replicates as suggested in DESeq manual). Differential expression was tested among individuals grouped according to lip morphology (i.e. thick- or thin-lipped) for each lake separately. Individuals within groups represented the 'biological replicates' of the group. The relative size of each individual's sequencing output was estimated with the function 'estimateSizeFactors'. The dispersion of read counts for each contig was estimated by accounting for depth of sequencing (using normalized values) and the variation of biological replicates with the function 'estimateVarianceFunctions'. Finally, we compared the magnitude of expression in each contig between the two groups of each lake with the function 'nbinomTest' applying a false discovery rate (FDR) cut-off of 0.01 and extracted the DE contigs. The probability of sharing DE genes among lake pairs by chance alone was calculated using a binomial probability density function (probability of 'success' is equal to the ratio of the total number of DE genes in the paired lake with the highest number of DE genes to the total number of the assembled genes, the number of trials were defined by the total number of DE genes in the paired lake with the least number of DE genes, and the number of shared genes across the lake pair comprised the number of 'success' cases).

Single nucleotide polymorphism (SNP) discovery and genotype calling were performed with SAMtools (Li *et al.* 2009), using the mpileup and var_filter functions. SNPs with quality lower than 20 (Phred-scaled probability of all the samples being homozygous to the reference) or with fewer than 10 reads of coverage were excluded. SNP calling was performed in two levels: within lake by including all the individuals and within morph per lake by doing separate SNP calling for the morphs with each lake. Note that given the lower number of individuals used for the within-morph analysis, the two analyses are not comparable. Population-level SNP allele frequencies were not estimated due to the low statistical power given our sample size of individuals per morph within lakes.

Functional annotation was performed with BLAST2GO (Conesa *et al.* 2005). The contigs were annotated after a BLASTX search against SWISSPROT database (*e*-value $< e-10$, annotation cut-off > 55 and a GO weight > 5) and assignment of the corresponding gene ontology (GO) terms. We used Fisher's exact test to compare GO terms for the DE contigs and the SNP-containing contigs per lake (FDR < 0.01).

Analysis of parallelism

In order to visualize the direction and magnitude of parallelism across ecomorphological and transcriptomic analyses, for each analysis we calculated the median distance between morphs relative to the upper 95th-percentile pairwise distance among all individuals in a given lake. For stable isotope and jaw measurements ($PC1_{jaw}$), median group and pairwise individual distances were calculated for the residuals after a lake-wide regression on body size (ln (standard length)). Body and jaw shape median and pairwise distances were calculated using Procrustes distances on the residuals after a cross-lake regression on size (as described previously in Ecology and morphology sections 'Head and body shape' and 'Lower pharyngeal jaw shape'). This parallelism metric could not be calculated for the transcriptomic data, which is instead visualized as the median DE $\log_2$-fold change within lake normalized by the cross-lake maximum for the DE genes shared by all lakes.

Results

Ecology and morphology

Lip state and size. Lip size as a function of body length is significantly different among thick- and thin-lipped fish across and within all lakes (ANCOVA on ln-transformed top lip area and morph with ln-transformed standard length as a covariate, $P < 0.001$, see Table S2, Supporting information, for details). At the cross-lake scale, body size (ln-transformed standard length) does not have a significant effect on lip size ($F_{1,408} = 2.21$, $P = 0.08$), indicating that there is a common relationship between lip size and body length across lakes.

Stable isotope analysis. $\delta^{13}C$ and $\delta^{15}N$ values differ between morphs within Lakes Nicaragua, Managua and Apoyeque ($P < 0.05$ for the univariate Wilcoxon and multivariate permutation tests, Fig. 2). There is mixed evidence for isotopic differentiation between morphs in L. Masaya: $\delta^{13}C$ values were significant ($P < 0.05$ for Wilcoxon's test), but $\delta^{15}N$ values and the multivariate permutation test are not. Body size (ln-transformed standard length) explains nearly half the variance in $\delta^{15}N$ values in L. Masaya ($r^2 = 0.47$, $P < 0.001$), although notably body size is uncorrelated with $\delta^{15}N$ values in Lakes Nicaragua, Managua and Apoyeque. L. Masaya fish in our study are generally small (<10 cm

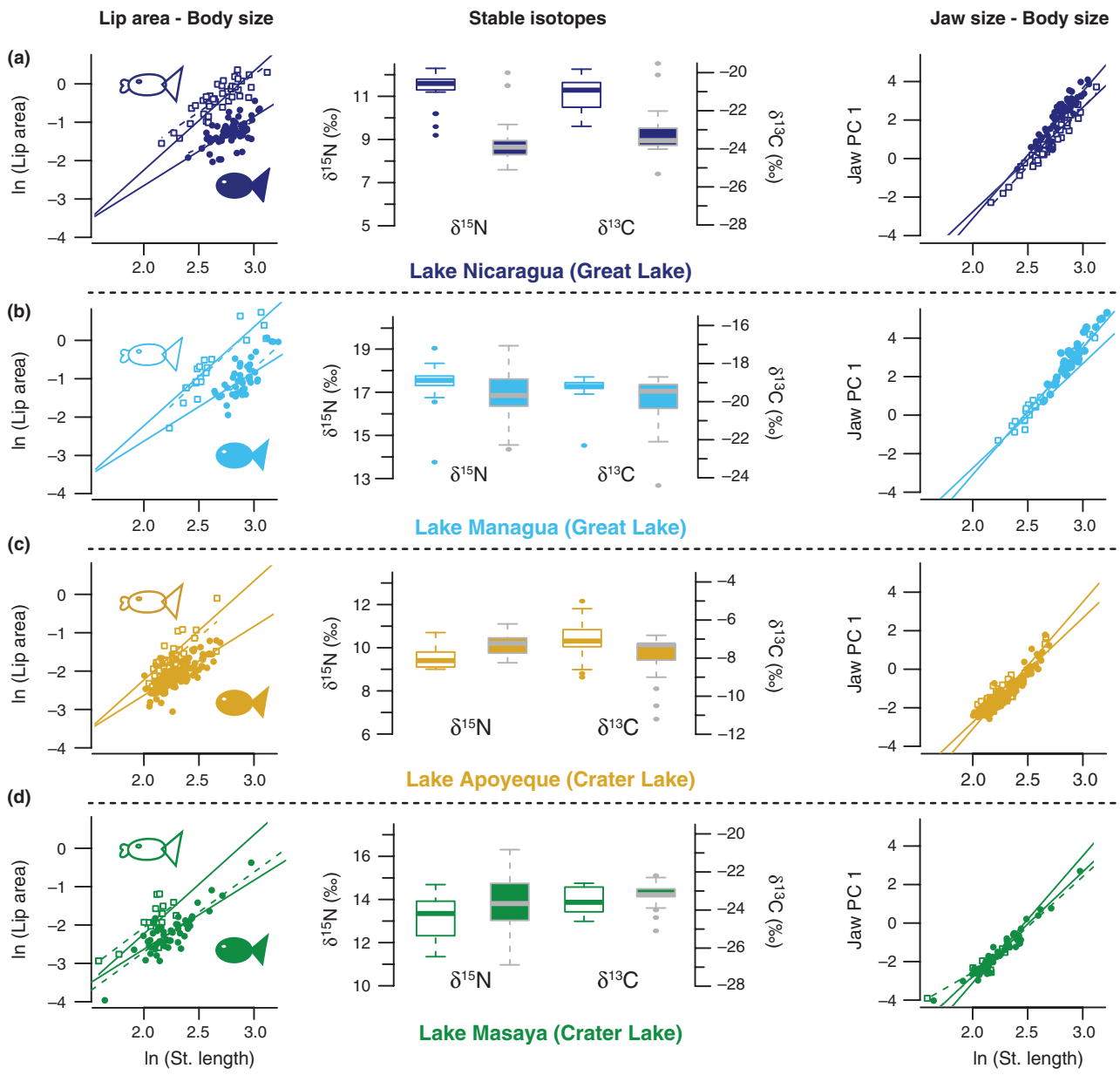

Fig. 2 Ecomorphological differentiation of thick- and thin-lipped Midas cichlids. Column 1 – Lip area as a function of body size (ln (top lip area) vs. ln(standard length)), Column 2 – stable nitrogen ($\delta^{15}N$, two leftmost box plots) and carbon ($\delta^{13}C$, two rightmost box plots) by lip group (thick-lipped in open backgrounds; thick-lipped in grey backgrounds) and Column 3 – jaw size as a function of body size (PC 1 of jaw measurement data vs. ln(standard length) for each of the Great Lakes (A) L. Nicaragua and (B) L. Managua and crater lakes (C) L. Apoyeque and (D) L. Masaya. Thick-lipped fish shown in open squares and thin-lipped fish in solid circles; colour reflects lake (Nicaragua = dark blue, Managua = light blue, Masaya = green, Apoyeque = yellow). Solid regression lines indicate cross-lake regressions by lip type; dotted regression lines indicate within-lake regressions by lip type (within-lake regressions typically cannot be seen behind cross-lake regression lines).

standard length on average compared to > 10 cm in other lakes, Fig. S2, Supporting information). While most fish from L. Masaya were adults, our sample includes some subadults as well; thus, the observed significant size–$\delta^{15}N$ relationship could reflect ontogenetic change in diet. In each of the four within-lake niche width comparisons, values for both TA and SEAc are larger in the thin-lipped morph, although significantly different only for L. Managua (Table S4, Supporting information).

Body and head shape. Within both Great Lakes, *A. citrinellus* and *A. labiatus* have significantly different body shapes ($P < 0.0001$, Table 2), with similar changes in

Table 2 Morphological differentiation of body and jaw shape by morph and pairwise stable isotope differentiation

	Body shape (thin–thick lip comparison)			Jaw shape (thin–thick lip comparison)			Stable isotope (thin–thick lip comparison)			
							$\delta^{15}N$ (‰)		$\delta^{13}C$ (‰)	
Lake	Procrust. Dist.	Hotel. T^2	*P*-value	Procrust. Dist.	Hotel. T^2	*P*-value	Wilcox. W	*P*-value	Wilcox. W	*P*-value
L. Nicaragua	0.029	1655.01	<0.0001	0.125	757.69	<0.0001	750.5	<0.0001	737	<0.0001
L. Managua	0.025	500.33	<0.0001	0.05	191.63	0.004	908.5	0.005	909.5	0.005
L. Apoyeque	0.010	161.16	<0.0001	0.021	64.11	0.053	165	<0.0001	614.5	0.014
L. Masaya	0.020	192.11	<0.0001	0.028	87.79	0.19	241	0.0857	225.5	0.045

Hotel. T^2 = Hotelling's T^2 statistic, Procrust. Dist. = mean Procrustes distance between group means, and Wilcox. W = Wilcoxon's rank sum test statistic.

both head and body shape between sympatric morphs. *Amphilophus labiatus* has a shallower body [landmark (LM) 6, 9-10] and an elongate head and snout (LM 1-5, 16-18) relative to *A. citrinellus* (Figs S3 and S4, Supporting information). We similarly find significant body shape differentiation between morphs within crater lakes Apoyeque and Masaya ($P < 0.001$, Table 2), with thick-lipped individuals also having shallower bodies and more elongate heads. Generally, sympatric crater lake morphs are less differentiated than sympatric *A. citrinellus* and *A. labiatus*, as described by Procrustes distance (Table 2). An overall assessment of body shape variation reveals a lake-independent morphological parallelism in body and head shape: within lakes, pairs of thin- and thick-lipped Midas cichlids differ in the same directions in the multidimensional shape space, that is, along PC1 and PC2 (Fig. 3). These major axes of differentiation account for 21.54% and 17.59% of the total shape variation and represent major changes in body depth (LM 6 and 9), mid-body width (LM 7-10), relative bulkiness of the snout and head region (LM 1-5, 16-18) and relative elongation of the whole body (LM 6-15) (Fig. S5, Supporting information).

LPJ size and shape. The four measurement traits analysed—jaw depth, width, length and ln (weight)—are all highly correlated (pairwise r^2 ranging from 0.90 to 0.95) and are subsequently collapsed into a single metric, $PC1_{jaw}$, using a PCA on the correlation matrix ($PC1_{jaw}$ contained 97.6% of jaw measurements variance). $PC1_{jaw}$ as a function of standard length differs significantly among morphs within and across lakes (ANCOVA on $PC1_{jaw}$; $P < 0.001$, Table S3, Supporting information) and supports the observation that jaw size differs between thick- and thin-lipped morphs but that the direction and manner of differentiation is lake specific (see Table S3, Supporting information for lake-specific patterns of differentiation). Generally speaking, morph differentiation in $PC1_{jaw}$ is concordant with patterns of

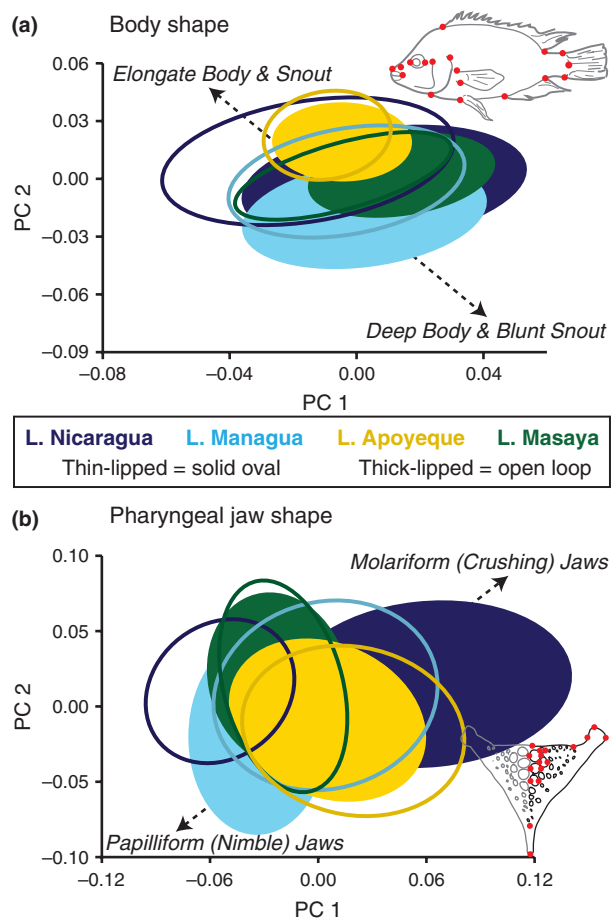

Fig. 3 Body and jaw shape differentiation by lip group and lake. Principal component analyses of body shape (18 landmarks) and jaw shape (17 landmarks) in (A) and (B), respectively (see landmark details Fig. S3, Supporting information). Results shown as 90% confidence ellipses for clarity (see all data Fig. S6, Supporting information). Thin-lipped morphs indicated with solid ovals by lake (Nicaragua = dark blue, Managua = light blue, Masaya = green, Apoyeque = yellow), and thick-lipped morphs indicated with open loops. Major morphological trends along PCs indicated in each plot by italicized descriptors and dotted arrows.

LPJ shape change as revealed by geometric morphometric analysis.

Across all lakes, PC1 and PC2 capture 36.2% and 17.9% of total jaw shape variation, respectively. Positive PC1 scores coincide with jaws with a posterior jaw margin (defined by LM 1 to LM 2) that is flat to posteriorly arched and contains large teeth (particularly teeth A, B, E in Figs S3 and S4, Supporting information) shifted anteriorly (teeth B, C) and posteriorly (teeth A, D). Positive PC2 scores coincide with jaws with short, stout posterior horns (LM 2-5), an elongate anterior horn (LM 6, 7) and slightly enlarged, posteriorly shifted teeth (teeth A–E). More positive PC1 and PC2 values (together describing relatively large-toothed and short posterior horned jaws) coincide with larger (more robust) jaws at a given body size ($PC1_{jaw}$). This robust, large-toothed LPJ morphology is known as 'molariform' in contrast to relatively gracile, small-toothed 'papilliform' jaws (e.g. Meyer 1990a,b).

Within both Great Lakes, *A. citrinellus* and *A. labiatus* have significantly different LPJ shapes ($P < 0.01$, Table 2), but the relative jaw shapes are in opposite directions in both morphs in these two large lakes (Fig. 3). In L. Nicaragua, *A. citrinellus* are positively loaded on PC1 (i.e. relatively molariform) and *A. labiatus* is negatively loaded on PC1 (i.e. relatively papilliform). The opposite pattern is observed in L. Managua, where the thin-lipped *A. citrinellus* are loaded more negatively along PC 1 and PC 2 and *A. labiatus* more positively.

Crater lake Apoyeque follows the general patterns observed in L. Managua—thick-lipped morphs load relatively positively along PC 1 (although slightly more negatively along PC 2) and are borderline significantly different ($P = 0.05$) (see also Elmer *et al.* 2010b). The pattern is further supported by jaw measurement results.

In L. Masaya, LPJ shape does not differ significantly between morphs (Table 2). As with body shape, morphs within crater lakes are separated by smaller Procrustes differences than those in Great Lakes (Table 2), but differ in that across lakes LPJs exhibit clearly nonparallel patterns of morph differentiation (nonparallelism confirmed across PCs accounting for 95% of shape variance: PC1-PC15, see thin-plate spline representation of jaw shape change in Fig. S5, Supporting information).

Transcriptome analysis

Differential gene expression. A large number of genes are significantly differentially expressed between morphs in each lake (FDR < 0.01): 512 (1833 contigs) in L. Managua, 490 (2125 contigs) in L. Nicaragua, 149 (481 contigs) in L. Apoyeque and 97 (397 contigs) in L. Masaya (Table 3; Fig. 4; Fig. S7, Supporting information; Table S5, Supporting information). Each lake contains a partially unique profile of DE genes, while many DE genes are shared across a subset of lakes (Table 3). The probability of finding so many shared DE genes across pairs of lakes by chance alone is very low (Table 3). The most DE genes are shared between the species pairs in L. Nicaragua and L. Managua, and the least between morphs in L. Apoyeque and L. Masaya (Table 3).

Six genes are differentially expressed between the morphs in all four lakes in the same direction (downregulated in thick-lipped morphs) and annotated as apolipoprotein D, myelin-associated glycoprotein precursor, four-and-a-half LIM domain protein 2, calpain-9, GTPase IMAP family member 8-like and one hypothetical protein (Table 4). A BLASTN search against the genome sequences of five African cichlids, *Oreochromis niloticus*, *Astatotilapia burtoni*, *Malawi (Maylandia) zebra*, *Pundamila nyererei* and *Neolamprologus brichardi* (BROAD Institute http://www.broadinstitute.org/ – unpublished data), indicates that all six genes are distributed in different linkage groups, at least in African cichlid genomes.

The over- and under-represented GO terms in the three gene ontology domains (biological process, molecular function and cellular component) are listed for DE

Lakes	L. Nicaragua	L. Managua	L. Apoyeque	L. Masaya
Total DE	490	512	149	97
Shared DE				
L. Managua	84 (5.24E−34)	–	–	–
L. Apoyeque	44 (2.55 E−29)	65 (2.68 E−54)	–	–
L. Masaya	29 (4.88 E−20)	36 (8.39 E−28)	26 (1.37 E−29)	–
Total SNPs	6330	4818	3525	5225
Shared SNPs				
L. Managua	2583	–	–	–
L. Apoyeque	1985	1827	–	–
L. Masaya	2705	2199	1750	–
Total SNPs/individual	633	402	392	475

Table 3 Differentially expressed genes between thick- and thin-lipped Midas cichlids and SNPs comparison across lakes. Total and pairwise comparison of differentially expressed genes for each lake. In brackets is the probability density function of observing such a high number of shared genes in each lake pair comparison. In the same context, we show the amount of SNPs observed within the total lake populations and SNP comparisons between lakes

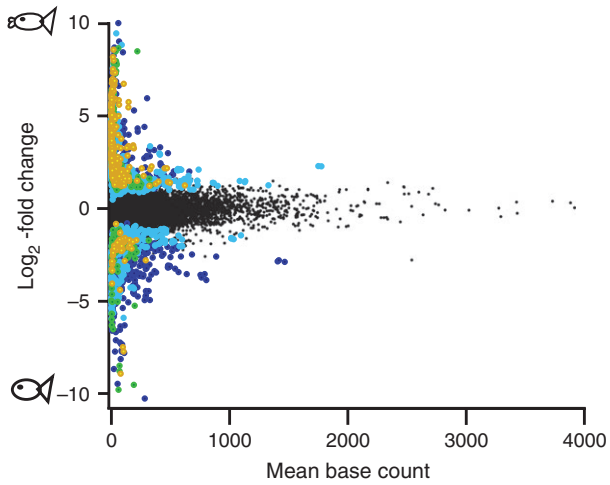

Fig. 4 Differential expression (mean base count to the $\log_2$-fold change) by morph across all four lakes. Differentially expressed contigs between the two morphs (mean of the base counts from all the individuals of each morph to the $\log_2$-fold change; FDR < 0.01) (Nicaragua = dark blue, Managua = light blue, Masaya = green, Apoyeque = yellow). Positive $\log_2$-fold values indicate relative overexpression in thin-lipped morph; negative $\log_2$-fold values indicate overexpression in thick-lipped morph.

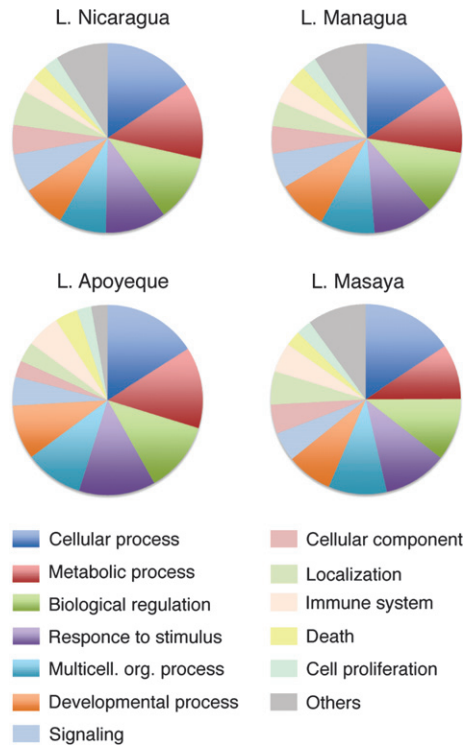

Fig. 5 GO terms summary for the differentially expressed contigs by lake. Representation of Gene Ontology terms of 'Biological Process' for the differentially expressed contigs per lake. Categories are ranked based on their size respective to that of L. Managua.

contigs for each lake relative to the whole contigs set (Table S6, Supporting information). Notably, while GO terms of DE genes are highly similar among lakes (Fig. 5), there are no specific (high)-level GO terms over- or under-represented in the DE genes and shared among all lakes (Table S6, Supporting information).

SNP diversity. We identify between 3525 and 6330 polymorphic sites within each lake, many of which are shared among lakes (Table 3). L. Apoyeque has the fewest SNPs and L. Nicaragua the most, even after controlling for sample size in each group by dividing the total number of SNPs with the sample size (Table 3). The number of SNPs shared between lake populations ranges from 2705 (L. Masaya and L. Nicaragua) to 1750 (L. Masaya and

Table 4 Shared differentially expressed genes between morphs across all four lakes. Annotation by BLASTX search against vertebrate sequences of GenBank protein database

Candidates	BLASTX best hit			Direction of expression in thick-lipped fish
	GI number	Description	*e*-value	
1	348533281	Calpain-9 [*Oreochromis niloticus*]	4.00E−99	Under
2	348522161	Apolipoprotein D-like [*Oreochromis niloticus*]	8.00E−48	Under
3	348542207	Hypothetical protein LOC100692391 [*Oreochromis niloticus*]	1.00E−16	Under
4	100305242	Myelin-oligodendrocyte glycoprotein precursor [*Oncorhynchus mykiss*]	3.00E−22	Under
5	348531006	PREDICTED: four-and-a-half LIM domain protein 2-like [*Oreochromis niloticus*]	4.00E−175	Under
6	348545456	PREDICTED: GTPase IMAP family member 8-like [*Oreochromis niloticus*]	3.00E−85	Under

L. Apoyeque). A total of 4994 contigs in 4362 loci (~13% and ~29% of total contigs and loci, respectively) contain SNPs in at least one lake. In GO comparison of those 4994 contigs to the total assembly, the cellular component 'mitochondrion' is over-represented (GO term analysis, Table S6, Supporting information). Finally, in the lakewise analysis, no SNPs are found to be fixed between the two morphs in any of the lakes.

When focusing within morphs in each lake (SNP calling separately for thick-lipped and thin-lipped individuals per lake), there are a number of polymorphisms observed in only one of the two morphs for each lake (Table 5), indicating either the fixation of an allele in one of the two morphs, the emergence of new alleles to the alternative morph or sampling bias due to the small number of individuals used. Thick-lipped morphs consistently have more polymorphic sites than their sympatric thin-lipped morphs. Normalization of the number of polymorphisms with sample size reveals that the difference between thick- and thin-lipped fish is greatest in L. Apoyeque and smallest in L. Managua (Fig. S8, Supporting information). L. Masaya was the only lake where the thin-lipped fish had more SNPs per individual than the thick-lipped.

Three of six parallel DE genes contain SNPs (Table S7, Supporting information). In total, these three genes contain 26 SNPs distributed throughout their transcripts. Five SNPs are found in synonymous sites, 15 in nonsynonymous and five in UTRs with one generating a stop codon (open-reading frame of each contig was defined based on sequence alignment with the most similar publicly available sequence). The majority of these SNPs are found in L. Nicaragua (18), with nine, ten and five SNPs in L. Managua, L. Masaya and L. Apoyeque populations, respectively. Only two SNPs are shared across all lakes and are not morph specific. Finally, we find unique SNPs in each lake population (L. Nicaragua: 12, L. Masaya: 5, L. Apoyeque: 2) except L. Managua.

Parallelism

Parallelism, here defined as significant differentiation between species/morphs with the same direction of change, is evident in the morphological traits of lip size to body length and body shape (Fig. 6). Nonparallel ecomorphological change is found, however, in jaw shape, jaw measurements and stable isotopes. The six DE genes shared across lakes share a similar median $\log_2$-fold change across genes, with the greatest average $\log_2$-fold change in L. Apoyeque and the least in L. Masaya. Significant differentiation (Fig. 6) is tested within each analysis (see preceding Results sections).

Table 5 SNPs observed within morphs. In each lake, the SNPs found within each morph were grouped to morph specific or shared

	L. Nicaragua	L. Managua	L. Apoyeque	L. Masaya
Thick-lipped	1896	1419	1349	1252
Thin-lipped	1577	1205	555	1232
Shared	1176	1129	667	1261

Discussion

Thin- and thick-lipped Midas cichlids in all lakes differ significantly from each other in a whole range of ecologically relevant traits, including body shape, pharyngeal jaw size and shape, and dietary niche. Some ecomorphological traits exhibit striking parallelisms, whereas others do not, suggesting that the evolution of

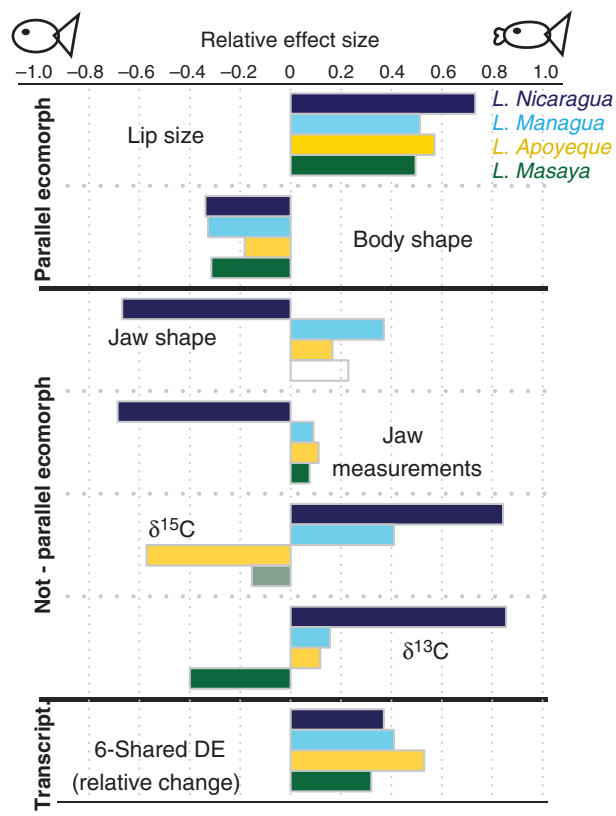

Fig. 6 Direction and magnitude of parallelism across lakes and analyses. Ecomorphological parallelism shown as the median distance between morphs relative to the upper 95th-percentile pairwise distance among all individuals in a given lake (Nicaragua = dark blue, Managua = light blue, Masaya = green, Apoyeque = yellow). Transcriptomic parallelism shown as the median DE $\log_2$-fold value within lake normalized by the cross-lake maximum for the DE genes shared by all lakes. Significant morph differentiation is shown with solid bars, nonsignificant with white bars and mixed significance with grey bars.

thick- and thin-lipped morphs—including their general ecomorphological differentiation—occurs by a combination of shared and unique evolutionary changes across lakes. This combination of parallel and nonparallel evolution (in other words, partial parallelism) also characterizes the gene expression differentiation of the otherwise to date genetically indistinguishable (Barluenga & Meyer 2010), sympatric, thick- and thin-lipped Midas morphs. Ecologically, L. Managua and crater lake fishes exhibit less extreme intermorph differentiation than fishes from L. Nicaragua, possibly due to weaker dietary niche separation in those lake environments. In contrast, the smaller number of DE genes between morphs in both of the younger crater lakes as compared to the older, larger Great Lakes could reflect more recent (or less complete) ecological and reproductive isolation between morphs or greater similarity in environmental conditions, possibly including a greater similarity of their trophic niches.

Morphological, ecological and transcriptomic diversification: parallel paths

Across lakes, we find evidence for parallel ecomorphological differentiation of lip size, head and body shape, isotopic niche width and a subset of DE genes. Thick-lipped Midas cichlids share, of course, a significant increase in lip size (Fig. 2; Table 3) compared to their sympatric thin-lipped morphs, but also a set of six parallel DE genes (Table 4).

Field observations suggest that thick-lipped cichlids use their lips as an aid for feeding: large lips are hypothesized to increase crevice-feeding efficacy either by creating a more effective seal or gasket for suction feeding (Barlow & Munsey 1976), a 'bumper' that protects the fish against injury when it bumps into the sharp-edged volcanic rock during feeding (A. Meyer, pers. obs.), or by providing an enlarged olfactory surface for detecting out-of-sight prey (Arnegard & Snoeks 2001; Oliver & Arnegard 2010).

Three lines of evidence suggest that the thick-lipped phenotype likely develops in response to changes in gene expression in both early ontogeny and development and maintenance during adulthood. First, thick-lipped Midas cichlids from L. Masaya raised in the laboratory develop less distinctly protruding lips than those caught in the field (Barlow & Munsey 1976). This observation suggests that early developmental signalling alone is unlikely to account for the full adult thick-lipped phenotype. Moreover, lips of adult *A. labiatus* seem to shrink in captivity if they are not allowed to feed in their natural way (A. Meyer, pers. obs.). However, in the laboratory, thick- to thin-lipped hybrids exhibit intermediate-lip phenotypes, demonstrating that lip size is at least partially heritable (G. Machado-Schiaffino, unpublished data). Second, there is general evidence for diet-related trophic apparatus remodelling in juvenile-to-adult teleosts (e.g. Meyer 1987; Huysseune 1995; Park *et al.* 2012) and phenotypic plasticity in trophic traits of Nicaraguan cichlids (e.g. Meyer 1987, 1990a,b; Muschick *et al.* 2011). Third, in this study, we show that thick-lipped fish in all Nicaraguan lakes have steeper allometric growth curves of lip size with body length (Fig. 2), also potentially due to ongoing differential gene expression in thick-lipped morphs. However, it remains possible that environmental conditions during juvenile stages determine the long-term growth trajectory of adult lips, and would not be captured by differential gene expression analysis on adult fish alone.

Six common genes with differential expression in cichlids with and without thick lips. Across the four lakes, we identify six parallel DE genes in common, five of which could be annotated. These are candidate genes for maintenance, growth, tissue composition and plasticity of thick lips in adult Midas cichlids, which we consider in the light of the proposed functional hypotheses—olfaction and mechanical advantage—and thick-lip composition (typically a partly keratinized epidermis with greatly thickened dermis containing collagen and nerve fibres, capillaries and cartilage; Benjamin 1990; Arnegard & Snoeks 2001). DE genes can act as activators or inhibitors in the expression of the thick- or thin-lip trait; thus, functional assays are needed to evaluate their specific role in the growth of Midas cichlid lips. The olfaction hypothesis garners support from the differential expression of apolipoprotein D (*ApoD*) and myelin-associated glycoprotein precursor (*Magp*), which both have important nervous system functions. *ApoD* is involved in nervous system development, maintenance and regeneration (Rassart *et al.* 2000; Sánchez *et al.* 2001) and has been shown in cancer research to inhibit cell growth (Sugimoto *et al.* 1994; Jin *et al.* 2006). *Magp* is likewise involved in neural regeneration (Filbin 2006). The downregulation of both genes in thick-lipped individuals could, for instance, reflect the innervation of the increased density of taste buds observed in thick-lipped cichlids (Arnegard & Snoeks 2001; Oliver & Arnegard 2010).

Two additionally downregulated genes, four-and-a-half LIM domain protein 2 (*Fhl2*) and calpain-9 (*Capn9*), are involved in the formation of muscle and cartilage (Yajima & Kawashima 2002; Higuchi *et al.* 2012; Rafael *et al.* 2012). *Fhl2* has recently been implicated in the development of craniofacial musculature, mineralized tissue and skin in the gilt-head bream, *Sparus aurata*, during late fish development (Rafael *et al.* 2012). *Fhl2* has the greatest change in expression of the six DE

genes, suggesting that this gene candidate may be particularly promising.

GTPase IMAP family member 8-like (*Gimap8*), the fifth annotated DE gene, is intriguingly the only gene candidate to contain SNPs shared across all lakes. Although not morph specific, they are nonsynonymous SNPs. At present, little is known about this candidate gene [e.g. no teleost homolog in Ensembl (Ensembl 66, Hubbard *et al.* 2005)] excepting predicted genes for cod and tilapia, with a known T-lymphocyte role in mammals, Nitta *et al.* 2006). Together, the five annotated gene candidates span roles including the development of the muscle, nervous and cartilage tissues and generally support both proposed lip function hypotheses.

Same genes for hypertrophied lips in cichlids from the New and Old World?. When compared the total DE genes found in this analysis with a previous study by Kobayashi *et al.* (2006), we found that four DE genes in the New World Midas cichlids are shared with thick-lipped African cichlid species. These are microfibril-associated glycoprotein 4 (*Mfap4*) differentially expressed in L. Masaya, anterior gradient protein 2 homolog (*Agr2*) differentially expressed in L. Apoyeque, CUB and zona pellucida-like domain-containing protein 1 (*Cuzd1*) differentially expressed in L. Apoyeque and a nonannotated cichlid-specific gene differentially expressed in L. Managua. All but *Cuzd1* are differentially expressed in the same direction (i.e. under-expressed in thick-lipped fish). As the probability of finding these four shared DE genes in both of those studies by chance alone is very low (probability density function of binomial distribution: 7.1197e-08), they are strong candidates for further analysis on convergent evolution of the thick-lipped phenotype across the family Cichlidae and possibly other fishes, more generally, because thick lips are found in several other lineages of teleosts.

Concomitant changes in lips and other phenotypic traits: Jack of all trades, master of none?. In addition to possessing larger lips, thick-lipped fish have more elongate bodies and snouts than thin-lipped fish in each of the four lakes examined (Fig. 3A; Table 2; see also: Klingenberg *et al.* 2003; Elmer *et al.* 2010a,b). We hypothesize that the parallel diversification of these three traits (lip size, head shape and body shape) may be all driven by the unique foraging challenge of foraging from rock crevices, including the capture of difficult to handle prey such as crabs. Broadly speaking, trophic ecology (including foraging mode and dietary composition) is one of the possible key drivers of cichlid body shape (Liem & Osse 1975; Meyer 1993; Clabaut *et al.* 2007) and skull morphology (e.g. Albertson 2008; Cooper *et al.* 2010). The functional trade-offs accompanying such changes in body and skull shape are well quantified (e.g. Liem 1993; Svanback & Eklov 2003; Andersson *et al.* 2006).

Four lines of evidence support the crevice foraging ecology as the tie between enlarged lips and relative snout and body elongation in Midas cichlids. First, there are the coincident observations that Neotropical and African species of thick-lipped fish feed by placing their lips in and among boulders and appear to have relatively elongate, conical heads, regardless of interspecific differences in depth habitat, phylogeny or prey hardness (morphological and ecological descriptions from Fryer & Iles 1972; Barlow & Munsey 1976; Greenwood 1981; Eccles & Trewavas 1989; Arnegard & Snoeks 2001; Oliver & Arnegard 2010). Second, the more conical and slender head shape of thick-lipped fish has significant functional advantages for suction mode feeding (Liem 1993; Klingenberg *et al.* 2003) and for reaching into narrow crevices (Konings 1998; Kohda *et al.* 2008; Spreitzer *et al.* 2012), feeding advantages also thought to accompany the development of enlarged lips (Barlow & Munsey 1976). Third, lip size and body and snout elongation may be phenotypically plastic in Midas cichlids (A. Meyer, pers. obs., Muschick *et al.* 2011); when raised in aquaria, *A. labiatus* from L. Nicaragua and thick-lipped morphs from L. Masaya exhibit a reduction in lip size with a concomitant shortening of snout length and deepening of body aspect (Barlow & Munsey 1976; A. Meyer, pers. obs.). Fourth, our stable isotope niche width analyses suggest that on average, thick-lipped morphs in all lakes feed on a more specialized, less isotopically variable diet than thin-lipped morphs (Table S4, Supporting information). While the absolute difference in isotopic niche width is only significant for sympatric morphs in L. Managua, the shared direction of niche width change across four lakes provides some additional support for our inference of a relatively specialized trophic niche exploited by fish with large lips and relatively elongate snouts and bodies.

Morphological and ecological diversification: nonparallel paths

We find evidence of nonparallel ecomorphological and transcriptomic evolution in the divergence of thick- and thin-lipped morphs. To start, while LPJ morphology and stable isotope ratios differ significantly among thick- and thin-lipped morphs (Table 2), the direction of differentiation is decidedly not parallel (Fig. 3; Fig. 6). The admixture of parallel and nonparallel ecomorphological diversification suggests that while thick- and thin-lipped fish are foraging in a similar manner across lakes, their diet varies considerably between lakes.

Pharyngeal jaw morphology reflects some combination of the prey type exploited during times of food limitation and the dominant dietary composition (e.g. Meyer 1989, 1990a,b; Wainwright 1996; Hulsey *et al.* 2005). Two jaw-type extremes are known in Midas cichlids: (i) robust, large-toothed molariform jaws adept at snail crushing and (ii) gracile, fine-toothed papilliform jaws adept at rapidly processing soft-bodied prey (Meyer 1989, 1990a,b; Muschick *et al.* 2011). Midas cichlids—as feeding generalists—consume a mixture of algae, invertebrates and small fish (Barlow 1976; Meyer 1990a; Vivas & McKaye 2001; Barluenga *et al.* 2006). However, there are significant differences in proportional diet composition and, subsequently, pharyngeal jaw type among species and morphs (Meyer 1990a; Barluenga *et al.* 2006; Elmer *et al.* 2010b).

Here, we find that in L. Nicaragua the thin-lipped *A. citrinellus* possesses strongly molariform LPJs (most positive PC 1 scores), while the thick-lipped *A. labiatus* possesses distinctly papilliform ones (most negative PC1 scores) (Fig. 3B; Table 3). The opposite trend is found in L. Managua and L. Apoyeque, where the thin-lipped morph has relatively papilliform LPJs in comparison with the thick-lipped morph. This pattern is independently confirmed by jaw measurements (Fig. 2; Table 3), which quantify the relative robustness of LPJs. In L. Masaya, evidence for LPJ differentiation among morphs is restricted to significant allometric differentiation of jaw measurements but matches L. Managua and L. Apoyeque in direction. In short, both the LPJ measurements and shape data strongly indicate that the relative prey field exploited by thick- and thin-lipped Midas cichlids is, at least to some degree, lake specific and possibly dependent on the absolute and relative abundance of particular prey types and their seasonal fluctuations.

Further, we find significant differences in $\delta^{13}C$ and $\delta^{15}N$ between morphs (Fig. 2), a signal also consistent with a change in source dietary carbon and nitrogen (e.g. Post 2002; Casey & Post 2011). As with jaw morphology, the direction of isotopic differentiation differs among lakes. The thick-lipped *A. labiatus* are significantly enriched in both ^{15}N and ^{13}C relative to the thin-lipped *A. citrinellus* in the Great Lakes Nicaragua and Managua. The opposite pattern (thin-lipped enriched in ^{15}N and ^{13}C relative to thick) occurs in L. Masaya and a mix of both (thick-lipped morphs relatively depleted in ^{15}N and enriched in ^{13}C) in L. Apoyeque. One likely way for the morphs to be isotopically distinct is whether each morph feeds on prey with a different basal food chain isotope composition, for example, by differing in feeding habitat, prey or water depths.

Cichlid fishes are remarkable for their trophic and morphological diversity, as well as their species-level richness (reviewed in Kuraku & Meyer 2008). Behind their expansive trophic diversity lies a high degree of craniofacial modularity (Albertson *et al.* 2005; Parsons *et al.* 2011, 2012) that allows for opposing selective pressures to act independently on, for instance, the opening vs. the closing of the jaw (Albertson *et al.* 2005). Here, we find evidence for parallelism in traits related to Midas cichlid foraging ecology, including lip size, and body and head shape (on foraging ecology and lip size: Barlow & Munsey 1976; Arnegard & Snoeks 2001; Elmer *et al.* 2010b; on foraging ecology and head shape: Liem 1993; Klingenberg *et al.* 2003; and on foraging ecology and body shape: e.g. Liem 1993; Clabaut *et al.* 2007). In contrast, we find two lines of evidence for nonparallel diversification of dietary composition by lip morph: muscle stable isotope ratios and jaw size and shape (on dietary composition and jaw size and shape: e.g. Meyer 1989, 1990a,b; Muschick *et al.* 2011; on dietary composition and stable isotopes: e.g. Harrod *et al.* 2010; Casey & Post 2011). Thus, we hypothesize that the combination of shared and unique ecomorphological differentiation reflects two separate trophic niche axes: foraging ecology (rock crevices vs. open sand; parallel) and diet (hard vs. soft food; nonparallel). Parsing ecomorphology and stable isotope variation in Midas cichlids by two aspects of trophic ecology—foraging ecology and dietary composition—is warranted both by the established functional ecology of the traits and proxies used and by the extent of craniofacial modularity in cichlid fishes.

Much of the gene expression differences between sympatric thick- and thin-lipped morphs are lake specific: 93–99% of DE genes are not shared among all lakes. The high number of lake-specific DE genes is not unexpected and could—as with diet—reflect both environmental differences, as well as effects of genetic drift because the founding populations of each of these crater lake radiations were likely to be very small. The founder effects (Kolbe *et al.* 2012) due to the combination of sampling only a subset of the genetic variation contained in the large and old ancestral populations from the two large lakes in Nicaragua as well as the somewhat different ecological conditions of each crater lake could likely explain those differences. Alternatively, the majority of DE genes could reflect neutral transcriptomic divergence accounting for large differences among lakes. Neutral divergence of Great and crater lakes is expected to occur (e.g. Oleksiak *et al.* 2002; Harrison *et al.* 2012) due to a combination of founding effects and drift as well. Distinguishing neutral from selective transcriptomic evolution is nontrivial, with many studies pointing towards the importance of selection in transcriptome evolution (reviewed in Gilad *et al.* 2006). A third possibility is that

nonparallel gene expression underlies parallel morphs. The shared DE genes are therefore the strongest genetic candidates for commonality of divergence underlying thin- and thick-lipped phenotypes. This is a direction we are planning to extend this work into, but these RNA-Seq data obviously do not permit to address this important issue as of yet.

Magnitude and implication of ecomorphological and transcriptomic differentiation

Across the four lakes, we examine the relative magnitude of differentiation among morphs in regard to standing population variation for (i) parallel ecomorphological differentiation, (ii) nonparallel ecomorphological differentiation; and (iii) total within-lake DE. First, the evolution of thick-lipped Midas cichlids with elongated head and body shape is generally parallel in magnitude as well as in direction (Fig. 4), suggesting similar selective pressure on foraging ecology, and foraging-related traits, in all lakes.

Likewise, the relative magnitude of dietary niche separation (e.g. pharyngeal jaw shape and size, and stable isotopes) is—as with the relative direction—decidedly nonparallel (Fig. 6). Across lakes, dietary niche separation is greatest in L. Nicaragua, with L. Managua and particularly the crater lake radiations showing less extreme intermorph differentiation. A parsimonious explanation for variation in the magnitude of differentiation is that it directly relates to the strength of diversifying selection with regard to diet. Thus, we hypothesize a weaker dietary niche separation in L. Managua and the crater lakes than in L. Nicaragua.

We find a approximately fivefold difference in the number of DE genes between morphs in the Great Lakes as compared to crater lakes (Fig. 7; Table 3). This difference could reflect either the more recent (or less complete) isolation between morphs or the greater similarity in environmental conditions experienced in the small crater lakes. The large, environmentally diverse Great Lakes Nicaragua and Managua allow ample opportunity for the habitat segregation that occurs on fine scales between morphs (boulders vs. sand for thick- and thin-lipped, respectively, Barlow 1976) to play out over greater spatial and environmental scales. The crater lakes are significantly smaller (three orders of magnitude by surface area), providing less opportunity for spatial segregation of thick- and thin-lipped morphs and less environmental heterogeneity across space. Thus, the fivefold difference in DE between morphs in Great Lakes and crater lakes could simply reflect greater environmental segregation—with commensurate physiological adaptations—in the larger lakes (Fig. 7). Alternatively, the accumulation of neutral divergence of

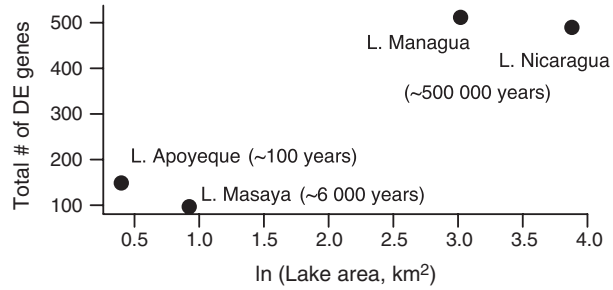

Fig. 7 Lake size and age vs. number of DE genes. Total number of DE genes between morph within lake as a function of lake area, a rough proxy for environmental heterogeneity and opportunity for spatial segregation by morphs. Lake age is indicated for Great Lakes and crater lakes as a metric of relative time since morph divergence, although lake colonization by Midas cichlids is typically more recent [e.g. ~100 years for Apoyeque (Elmer *et al.* 2010b), 6000 years for Masaya (Elmer *et al.* 2012)].

transcriptomes over time could account for the difference in DE in Great Lakes and crater lakes (e.g. Oleksiak *et al.* 2002; Harrison *et al.* 2012). The Great Lakes are much older and support larger populations of Midas cichlids than the younger and smaller crater lakes (Barluenga & Meyer 2010; Elmer *et al.* 2010a,b, 2012). Currently, the Great Lake thick- and thin-lipped morphs are recognized as species, while the crater lake morphs may be in the midst of incipient speciation (previously argued for L. Apoyeque morphs in Elmer *et al.* 2010b). Together, population size, time since (and effectiveness of) genetic isolation, and founder effects could drive the fivefold difference in DE between Great Lakes and crater lakes.

In contrast to the excess in DE genes between Great Lakes and crater lake Midas cichlids, a similar magnitude of SNPs differences was identified in each of the four lakes (Table 3) and across morphs within each lake (Table 5). The lack of alternatively fixed alleles in the transcriptome of the lips between the two morphs is readily explained by incomplete (or weak) assortment of common polymorphisms and possible ongoing gene flow—especially for crater lakes (as supported by other analyses of neutral markers, e.g. Elmer *et al.* 2010b; Barluenga & Meyer 2010). While variation in population allele frequencies between morphs is expected as a result of divergent selection, small sample size limits our inference regarding the amount of within-morph SNPs per individual (Fig. S8, Supporting information). Finally, SNP accumulation is greater in genes related to 'mitochondrion' (GO analysis, Table S6, Supporting information), possibly supporting the importance of energy metabolism in speciation (reviewed in Bernatchez *et al.* 2010).

However, additional genomewide sequencing with greater intrapopulation sample sizes is required to discern population genetic patterns. Within the scope of our study, we assess the transcriptomic differences in the lips of the two morphs to understand the functional and ecological implications of the tissue. As an important next step, transcriptomic, genomic and ecological differentiation also in earlier developmental stages should be studied to obtain a fuller picture of diversification of thin- and thick-lipped Midas cichlid fishes.

Conclusions

The relative roles of selection vs. genomic constraints are often debated as to their influence in driving parallel phenotypic evolution. Here, we parse the problem of parallel evolution into two components: ecomorphology and gene expression.

Concerning the first, this study provides clear evidence for the importance of selection in both ecomorphological parallelism and nonparallelism in thick- and thin-lipped Midas cichlids. It thus expands the limited number of studies that investigated parallelism beyond the trait of interest to find an admixture of modes (e.g. Kaeuffer *et al.* 2012). We find evidence that all traits related to foraging ecology exhibit parallel evolution (lip size, head shape, body shape and isotopic niche width). In contrast, in the separate—but related—dietary ecological niche, we also find evidence for nonparallel, lake-specific evolutionary trajectories (jaw size and shape, and stable isotopes). These morphological traits are linked by ecology and suggest a strong role for selection in driving parallel lip evolution in Midas cichlids.

Second, we use transcriptomics to identify genes that are differentially expressed (downregulated in thick-lipped morphs) in the lips of thick- and thin-lipped cichlids across all lakes. Six genes accompany the repeated and parallel evolution of the thick-lipped phenotype, while most DE genes are specific to a given lake–morph pair. We find that the number of DE genes between sympatric morphs varies across lakes, possibly due to some combination of demographics, population age and environmental heterogeneity. Such a pattern suggests the role for either lake-specific transcriptomic evolution between morphs or local physiological adaptations in total DE. Combined this provides evidence for the importance of selection during sympatric speciation, along both parallel and nonparallel evolutionary trajectories.

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T.M. conducted the transcriptomic analyses and drafted the manuscript. P.M.H. conducted the lip size, jaw shape, stable isotope, ecomorphological and parallelism analyses and drafted the manuscript. H.K. conducted body shape analyses and provided the raw data on pharyngeal jaw morphology. G.M.S. performed the RNA extraction. P.F. performed the Illumina sequencing. C.H. produced isotope data and conducted isotopic niche width analysis. K.R.E. and A.M. designed research. H.K., G.M.S., K.R.E. and A.M. collected samples in the field. All authors contributed to writing and approved the final manuscript.

Data accessibility

The raw sequence data are available at NCBI's Short Read Archive, accession SRA057285. The transcriptomic resources, ecological data and morphometric data have been deposited on DRYAD entry doi:10.5061/dryad.3gq12.

Supporting information

Additional Supporting Information may be found in the online version of this article.

Fig. S1 Year (and laboratory) effects on Lake Managua stable isotope values.

Fig. S2 Cross-lake comparison of stable isotopes in thick- and thin-lipped fish.

Fig. S3 Body and Jaw landmarks and descriptions.

Fig. S4 PC 1 and PC 2 of body and jaw shape change.

Fig. S5 Thick- to thin-lipped body and jaw shape change.

Fig. S6 Body and jaw shape differentiation by morph and lake.

Fig. S7 Volcano plot of the DE genes for the four lakes.

Fig. S8 SNPs within morphs per lake.

Table S1 Sample details for all of individuals used in all analyses.

Table S2 ANCOVAS of ln (top lip area) as a function of morph and lake.

Table S3 ANCOVAS of $PC1_{jaw}$ as a function of morph & lake.

Table S4 Niche variation between morphs for each lake.

Table S5 Differential expression between the two morphs in the four lakes.

Table S6 GO term analyses.

Table S7 Description of the SNPs found within the contigs of the differentially expressed genes that are downregulated in thick-lipped fish in every lake.

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