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Adaptive divergence between lake and stream populations of an East African cichlid fish

ANYA THEIS,[1] FABRIZIA RONCO,[1] ADRIAN INDERMAUR, WALTER SALZBURGER and BERND EGGER

Zoological Institute, University of Basel, Vesalgasse 1, 4051 Basel, Switzerland

Abstract

Divergent natural selection acting in different habitats may build up barriers to gene flow and initiate speciation. This speciation continuum can range from weak or no divergence to strong genetic differentiation between populations. Here, we focus on the early phases of adaptive divergence in the East African cichlid fish *Astatotilapia burtoni*, which occurs in both Lake Tanganyika (LT) and inflowing rivers. We first assessed the population structure and morphological differences in *A. burtoni* from southern LT. We then focused on four lake–stream systems and quantified body shape, ecologically relevant traits (gill raker and lower pharyngeal jaw) as well as stomach contents. Our study revealed the presence of several divergent lake–stream populations that rest at different stages of the speciation continuum, but show the same morphological and ecological trajectories along the lake–stream gradient. Lake fish have higher bodies, a more superior mouth position, longer gill rakers and more slender pharyngeal jaws, and they show a plant/algae and zooplankton-biased diet, whereas stream fish feed more on snails, insects and plant seeds. A test for reproductive isolation between closely related lake and stream populations did not detect population-assortative mating. Analyses of F1 offspring reared under common garden conditions indicate that the detected differences in body shape and gill raker length do not constitute pure plastic responses to different environmental conditions, but also have a genetic basis. Taken together, the *A. burtoni* lake–stream system constitutes a new model to study the factors that enhance and constrain progress towards speciation in cichlid fishes.

Keywords: adaptive divergence, *Astatotilapia burtoni*, East African cichlid fishes, Lake Tanganyika, lake–stream system, speciation continuum

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Introduction

Different environmental conditions constitute a major source of divergent natural selection between populations (reviewed in Schluter 2000; Nosil 2012). Adaptation to divergent habitats may ultimately lead to speciation, for example when reproductive isolation builds up as by-product of adaptive divergence ('ecological speciation'), or when different mutations become fixed in geographically separated populations adapting to similar environments ('mutation-order speciation') (Rundle & Nosil 2005; Schluter 2009). Both scenarios imply that speciation is a gradual process, which is evidenced by empirical data demonstrating substantial variation in the level of divergence between adjacent populations, even along environmental clines that are free of geographical barriers (Hendry *et al.* 2000; Schluter 2000; Rundle & Nosil 2005; Butlin *et al.* 2008; Mallet 2008; Berner *et al.* 2009; Nosil *et al.* 2009). This so-called speciation continuum can range from weak or no divergence between populations to strong genetic differentiation between what might then be novel pairs of sister species (Hendry *et al.* 2009; Nosil *et al.* 2009). What determines the strength of divergence between populations remains poorly understood, though.

Correspondence: Walter Salzburger and Bernd Egger, Fax: +41 61 267 0301; E-mails: walter.salzburger@unibas.ch and bernd.egger@unibas.ch

[1]These authors contributed equally to this work.

Adaptive divergence has mainly been studied in settings involving populations that differ in their degree of reproductive isolation, such as in stick insects (Nosil & Sandoval 2008), mosquitofish (Langerhans *et al.* 2007) or *Heliconius* butterflies (Mallet & Dasmahapatra 2012). Important model systems in fishes are three-spine sticklebacks and salmonids, which often occur along discrete environmental gradients such as marine–freshwater and/or lake–stream habitats (e.g. Hendry *et al.* 2000; Berner *et al.* 2008; Jones *et al.* 2012; Roesti *et al.* 2012). Stickleback lake–stream populations, for example, differ with regard to resource use and are morphologically distinct, with limnetic-foraging lake forms typically displaying shallower bodies and more and longer gill rakers than the benthic-foraging stream types (Schluter & McPhail 1992; Berner *et al.* 2008). The extent of divergence between lake and stream population pairs depends on the strength of divergent selection, on the level of gene flow and on the time since divergence (Hendry & Taylor 2004; Berner *et al.* 2010; Roesti *et al.* 2012; Hendry *et al.* 2013; Lucek *et al.* 2013). Studies in sticklebacks and salmonids also uncovered that diversification may proceed rapidly (see e.g. Hendry *et al.* 2007). In the sockeye salmon (*Oncorhynchus nerka*), for example, it took about a dozen of generations only until reproductive isolation occurred between two adjacent beach and stream populations that diverged after an introduction event (Hendry *et al.* 2000). However, ecological divergence might also fail to generate the evolution of reproductive isolation barriers (Raeymaekers *et al.* 2010).

In this study, we focus on the early phases of adaptive divergence in a prime model system for evolutionary biology, the East African cichlid fishes (see e.g. Kocher 2004; Salzburger 2009; Santos & Salzburger 2012). More specifically, we examine eco-morphological and genetic divergence in *Astatotilapia burtoni* (Günther 1894), which occurs both in East African Lake Tanganyika (LT) and inflowing rivers. Although *A. burtoni* is one of the most important cichlid model species in various fields of research including developmental biology, neurobiology, genetics and genomics, and behavioural biology (see e.g. Wickler 1962; Robison *et al.* 2001; Hofmann 2003; Lang *et al.* 2006; Salzburger *et al.* 2008; Baldo *et al.* 2011; Theis *et al.* 2012; Santos *et al.* 2014) and represents one of the five cichlid species whose genome has recently been sequenced (Brawand *et al.* 2014), surprisingly little is known about its ecology, phylogeographic distribution, population structure or genetic and phenotypic diversity in the wild.

Taxonomically, *A. burtoni* belongs to the Haplochromini, the most species-rich group of cichlids. Within the haplochromines, *A. burtoni* is nested in the derived 'modern' clade (as defined in Salzburger *et al.* 2005), the members of which are characterized by a pronounced sexual colour dimorphism with typically brightly coloured males and inconspicuous females, a polygynandrous mating system with maternal mouthbrooding, as well as egg-spots on the anal fin of males. The vast majority of haplochromines is endemic to a specific lake or river system, respectively, and specialized to certain habitat types therein. Only very few cichlid species exist that commonly occur in both truly riverine and lacustrine habitats. *Astatotilapia burtoni* is such a habitat generalist, inhabiting the shallow zones of LT as well as rivers and streams surrounding LT (Fernald & Hirata 1977; De Vos *et al.* 2001; Kullander & Roberts 2011), and thus represents an ideal species to study adaptive divergence across an environmental gradient in cichlid fishes.

So far, adaptive divergence in cichlids has mainly been investigated within lakes, for example along depth or habitat gradients (see e.g. Barluenga *et al.* 2006; Seehausen *et al.* 2008). In our study, we targeted divergence along a lake–stream environmental gradient to test whether similar mechanisms are involved in divergence along this habitat gradient as in other groups of fishes. To this end, we first established phylogeographic relationships and assessed the population structure in *A. burtoni* from the southern part of the LT drainage using mtDNA and microsatellite markers. Second, we examined morphological differences between these populations by analysing body shape, a complex quantitative trait encompassing morphological variation associated with multiple ecological factors (Webb 1984). We then focused on four lake–stream systems in detail. In addition to the body shape and population-genetic analyses, we quantified several ecologically relevant traits in these replicate lake–stream population groups, including the gill raker apparatus, which is known to respond to distinct feeding modes in fishes. The number and length of gill rakers have been identified as key elements influencing prey capture and handling in stickleback (Bentzen & McPhail 1984; Lavin & McPhail 1986; Schluter 1993, 1995; Robinson 2000). Furthermore, we examined the pharyngeal jaw apparatus, a highly diverse trait in cichlids linked to trophic diversification (Galis & Drucker 1996; Hulsey *et al.* 2006; Muschick *et al.* 2012), and used stomach content analysis as a proxy for divergent selection acting on foraging morphology. We then tested whether there were associations between shifts in resource use and trophic morphology along the lake–stream gradient that might reflect ecologically based adaptive divergence (Berner *et al.* 2009; Harrod *et al.* 2010). Finally, we conducted a mating experiment to test for reproductive isolation among a lake and stream populations. Additionally, offspring from this common garden setting was used to

evaluate levels of phenotypic plasticity in adaptive traits such as body shape and gill raker morphology.

Materials and methods

Study populations and sampling

Sampling of *A. burtoni* was carried out between February 2010 and July 2013 in the southern basin of LT and in inflowing rivers and streams, with a particular emphasis on four river systems, the Kalambo River, the Chitili Creek, the Lunzua River and the Lufubu River (Figs 1A and 2A) (see Appendix S1, Supporting information for a detailed description of these river systems). Specimens were collected using hook and line fishing, minnow traps and gill nets under the permission of the LT Research Unit, Department of Fisheries, Republic of Zambia. In total, we sampled 22 populations (several of these multiple times), resulting in a data set comprising 1425 individuals (see Tables S1 and S2A, Supporting information for details). Specimens were anaesthetized using clove oil (2–3 drops clove oil per litre water) and photographed in a standardized manner for morphometric analyses; a fin clip was taken and stored in ethanol (96%) for a DNA sample; specimens for gill raker measurements, pharyngeal jaw and stomach content analyses were preserved in ethanol (96%).

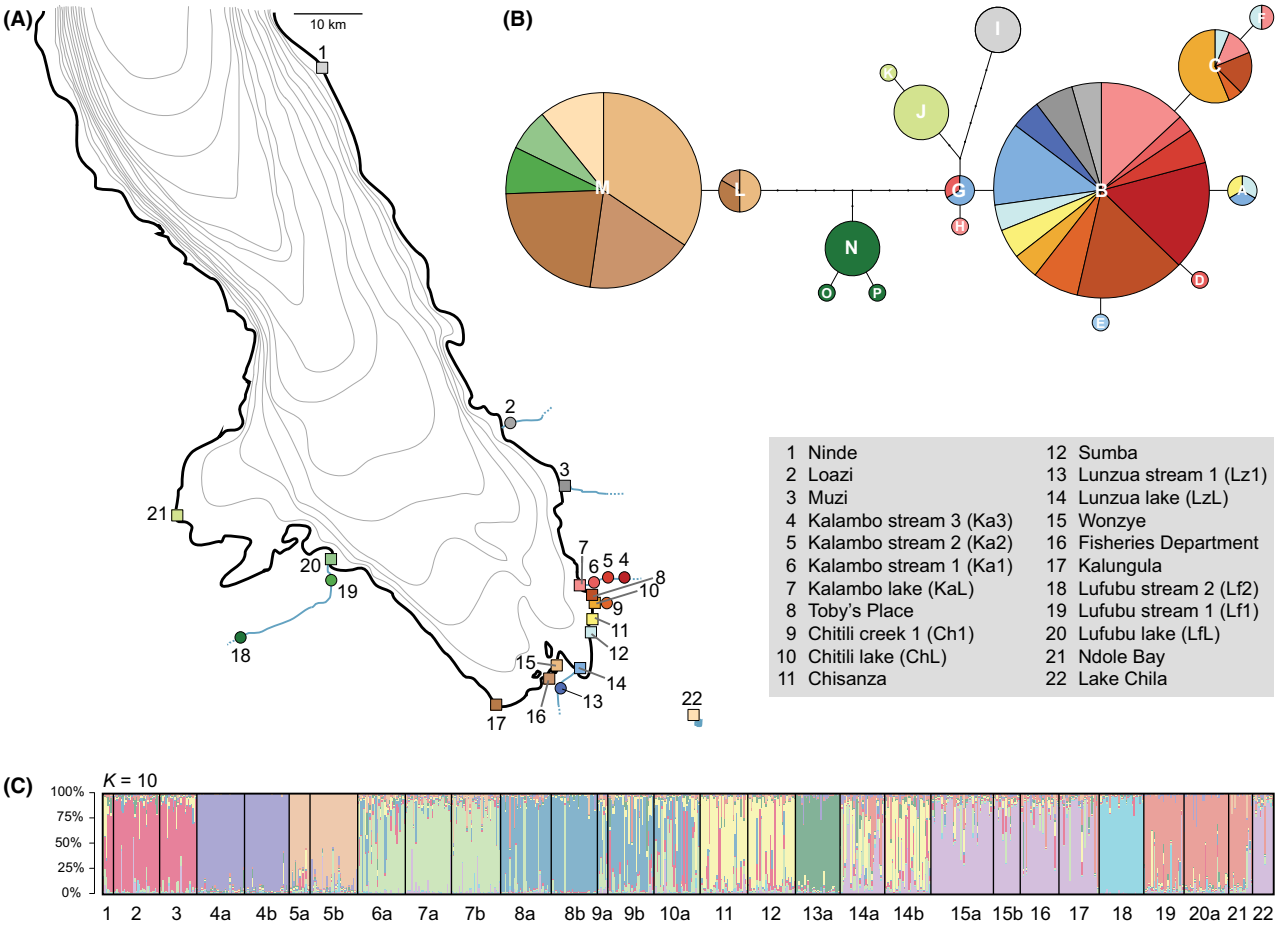

Fig. 1 Sampling locations and genetic differentiation among all populations revealed by microsatellite and mtDNA analyses. (A) The 22 sampling localities indicated by numbers on the southern part of LT (squares represent lake and circles stream populations; bathymetric lines are placed at every 100 m water depth, after Coulter 1991). Names of localities are listed in the grey box. (B) Haplotype genealogy based on mtDNA showing the 16 haplotypes (A–P) and the deep split between eastern (populations 2–14; haplotypes A–H) and western (populations 15–17, 19–20; haplotypes L and M) populations. Each colour represents a locality, which correspond to the colours on the map. (C) Structure plot based on nine microsatellite loci for all populations: the 29 population samples from 22 localities (names in the grey box; 'a' and 'b' refer to different sampling years, note that not all sampling years were analysed) group in 10 genetic clusters ($K = 10$; colours representing these clusters are decoupled from the population colours in the map). LT, Lake Tanganyika.

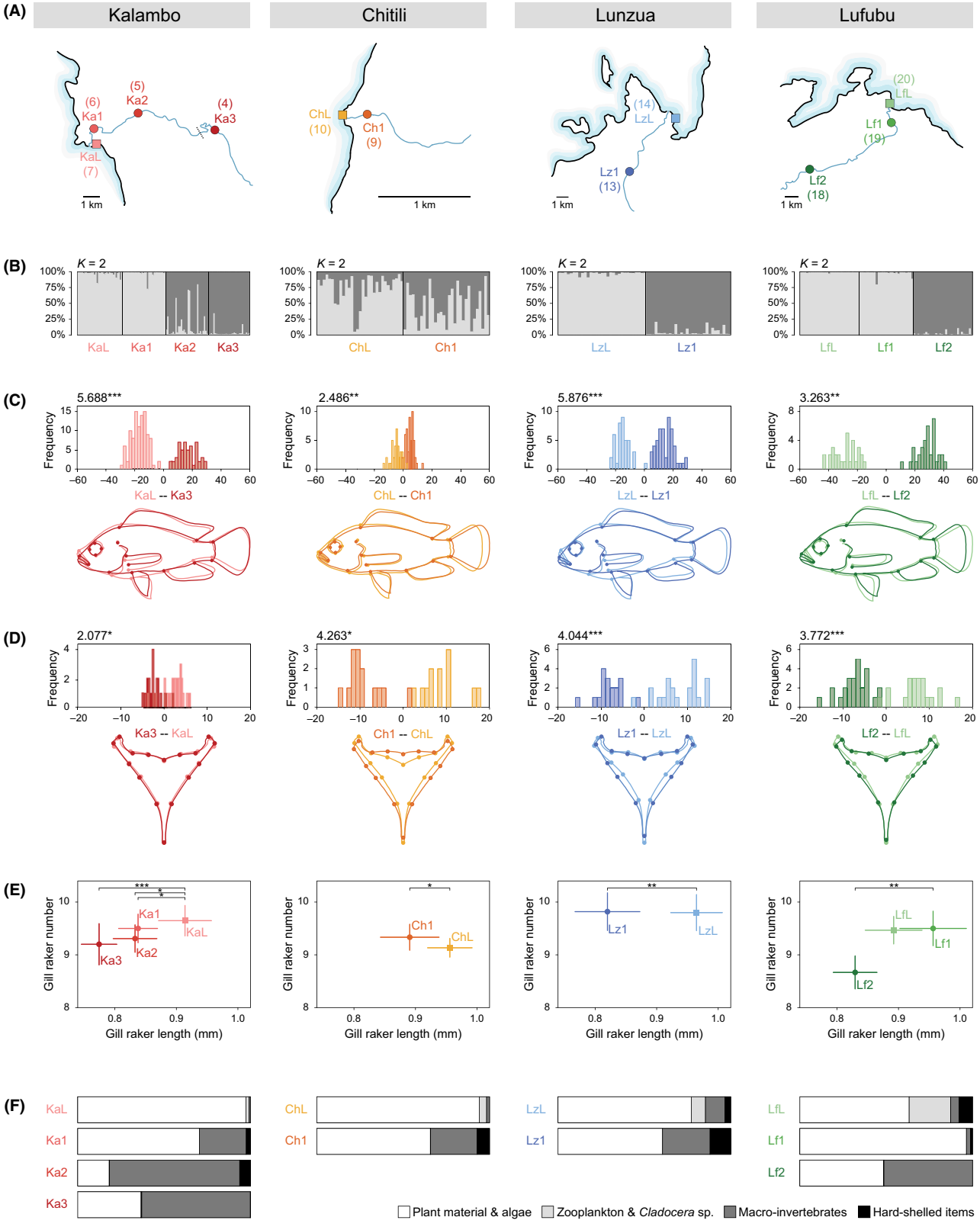

Fig. 2 Divergence between lake and stream habitats in four systems. (A) Maps showing sampling localities for each lake–stream system (see grey box in Fig. 1 for full names of localities). (B) Structure plots for each lake–stream system (shades of grey represent different genetic clusters; K = number of genetic clusters). (C) Discriminant scores of body shape comparisons and corresponding landmark shifts from the discriminant function analyses (DFA) between the lake population and the most upstream population for each lake–stream system show that lake fish generally have a deeper body and a more superior mouth position compared with stream fish. DF differences are always increased threefold in the outlines, which are drawn for illustration purposes only. DFA results are indicated with Mahalanobis distances on top of the DF score plots. (D) Discriminant scores of lower pharyngeal jaw (LPJ) shape comparisons and corresponding landmark shifts from the DFA between the lake population and the most upstream population for each lake–stream system show that lake fish generally have a slender and more elongated LPJ compared with stream fish. (E) Differences in size corrected male gill raker length and number between populations within each lake–stream system. Error bars represent 95% confidence intervals of the means. Lake fish generally have longer gill rakers compared with stream fish (Table S6, Supporting information). (F) Averaged proportions of the different stomach content categories for each population. Generally, lake fish feed more on softer and smaller food particles, whereas stream populations feed more on hard-shelled and larger food items. Significance levels: $*P < 0.05$, $**P < 0.01$ and $***P < 0.0001$.

Water current measurements

Surface water current and microhabitat current (measured directly where the fish were sighted) were determined at 10 sampling sites in July 2013. The flow regime differs between dry and wet season; however, relative differences between sampling sites are likely to be consistent. Surface current was estimated by measuring the time a float (0.5 L plastic bottle filled with 0.25 L water) travelled 10 m downstream. Measurements were taken five times at each site, and the velocity was calculated from the average of these measurements. For microhabitat current, we determined the relative level of water motion in lake and stream habitats as a proxy. To this end, we used Life Savers candies (wint-o-green flavour, individually wrapped variety; $N = 5$) to measure the relative rate of dissolution (which is directly related to water current), following the method described by Koehl & Alberte (1988). Life Savers were either tied to plants or were hand-held into the underwater habitat using a stick and line and left to dissolve for 6 min. Additionally, a baseline dissolution rate was determined by placing a candy in a bucket filled with water from the respective site (no current) for 6 min. We determined the weight of each candy before and after treatment (dried at ambient temperature for at least 2 h) to calculate the mass (g) lost relative to the baseline.

Genetics

Total DNA was extracted from fin clips preserved in ethanol applying a proteinase K digestion followed by either a high-salt (Bruford *et al.* 1998) or a MagnaPure extraction using a robotic device (MagnaPure LC; Roche Diagnostics), following the manufacturer's protocol (Roche, Switzerland). We first determined the DNA sequence of a 369-bp segment of the mitochondrial control region for 5–40 samples per location (total $N = 359$, Table S1, Supporting information) using published primers (Kocher *et al.* 1989; Salzburger *et al.* 2002). The PCR fragments of the control region were purified using ExoSAP-IT (USB), directly sequenced with the BigDye sequencing chemistry (Applied Biosystems) and analysed on an ABI 3130*xl* genetic analyzer (Applied Biosystems). Mitochondrial DNA sequences were aligned using CODONCODE ALIGNER (v.3.5; CodonCode Corporation). A maximum-likelihood analysis, using the GTR + G + I as suggested by JMODELTEST (Posada 2008), was carried out in PAUP*4.0b10 (Swofford 2002) to construct an unrooted mitochondrial haplotype genealogy following the method described in Salzburger *et al.* (2011).

A total of 786 individuals (Table S1, Supporting information) were genotyped at the following nine microsatellite loci: Ppun5, Ppun7, Ppun21 (Taylor *et al.* 2002), UNH130, UNH989 (Lee & Kocher 1996), Abur82 (Sanetra *et al.* 2009), HchiST46, HchiST68 (Maeda *et al.* 2009) and Pzeb3 (Van Oppen *et al.* 1997). Fragment size calling was carried out on an ABI 3130*xl* genetic analyzer (Applied Biosystems) in comparison with the LIZ 500(−250) internal size standard. Genotypes were determined manually using PEAK SCANNER (v.1.0; Applied Biosystems). Microsatellite scoring data were examined and rounded to valid integers using TANDEM (Matschiner & Salzburger 2009). The microsatellite data were used to calculate population pairwise F_{ST} values in ARLEQUIN (v.3.5.1.2; Schneider *et al.* 1999) and D_{EST} (Jost 2008) using the package DEMETICS (Gerlach *et al.* 2010) in R (v.3.1.0; R Development Core Team 2014). STRUCTURE (v.2.3.3; Pritchard *et al.* 2000) was then used to infer population structure. First, all 29 populations (22 localities, seven of which were sampled twice in different years) were run in a joint analysis (Markov chain Monte Carlo simulations were run for 500 000 replications, burn in = 50 000, admixture and correlated allele frequency options). Ten replicated simulations were performed for $K = 1$–16, and the most likely number of genetic clusters was inferred using the ΔK method (Evanno *et al.* 2005) implemented in the software HARVESTER (Earl & von Holdt 2012). Then, each lake–stream system

was analysed separately using the same parameters as described above and K = 1–10 for Kalambo, K = 1–6 for Lufubu, Chitili and Lunzua.

To test for isolation by distance, we conducted a simple Mantel test in R (package ecodist, Goslee & Urban 2007) using the genetic distance (pairwise F_{ST} values) and the geographic distance in metres between sites measured along the shoreline on Google Earth. For this analysis, only populations from the LT shoreline were used (N_{pop} = 13) and all riverine populations (2, 4–6, 9, 13, 18, 19; see Fig. 1) and the population from Lake Chila (22) were excluded.

Body shape

The photographs of 791 individuals (Table S1, Supporting information) were used for geometric morphometric analyses by recording the coordinates of 17 homologous landmarks (Fig. S1A, Supporting information; for details see Muschick *et al.* 2012) using tpsDIG2 (v.2.11; Rohlf 2008). The x and y coordinates were transferred to the program MorphoJ (v.1.05f; Klingenberg 2011) and superimposed with a Procrustes generalized least squares fit (GLSF) algorithm to remove all nonshape variation (Rohlf & Slice 1990). Additionally, the data were corrected for allometric size effects using the residuals of the regression of shape on centroid size for further analyses. Canonical variate analyses (CVA; Mardia *et al.* 1979) were used to assess shape variation when several populations were compared, and discriminant function analyses (DFA) were performed for comparisons between two populations only (i.e. within some lake–stream systems). The mean shape distances of CV and DF analyses were obtained using permutation tests (10 000 permutations). Although males and females show strong body shape differences, the pooled data revealed the same results as the separate analyses for each sex (data not shown), presumably because intersexual within-population differences are smaller than intrasexual differences among populations (Fig. S2, Supporting information). Therefore, both sexes were combined in the analyses presented.

In a first step, we conducted a CVA for 20 populations and another one for the 11 shoreline populations only to test whether the clustering in morphospace shows signs of isolation by distance. Further tests for morphological isolation by distance were conducted with a simple Mantel test in the ecodist package in R using the morphological (Mahalanobis) and the geographic distance (measured in metres along the shoreline). In a second step, the lake–stream populations were tested within each system as well as in a combined data set.

Finally, we also performed a CVA focusing on the mouth position (landmarks 1, 2, 7 and 12, capturing mouth angle; Fig. S1A, Supporting information). We only used male individuals here, as this trait shows a much stronger sexual dimorphism compared with, for example, body shape.

Gill raker morphology

Following Berner *et al.* (2008), we counted gill raker number and measured the length of the 2nd, 3rd and 4th gill raker of the right first branchial arch and calculated the mean for each of 281 individuals collected from the four lake–stream systems (Table S1, Supporting information). As average gill raker length correlated positively with standard length (SL) in both sexes (males: regression, $R^2 = 0.8432$, $P < 0.0001$; females: regression, $R^2 = 0.5477$, $P < 0.0001$), mean gill raker length was regressed to SL for size correction. The individual residuals from the common within-group slope were then added to the expected gill raker length at grand mean SL (male = 0.879 mm, female = 0.783 mm) to maintain the original measurement unit. These values represent a size-independent gill raker length and were used for the comparisons between populations within each lake–stream system separately applying an ANOVA. For the Kalambo and Lufubu systems, for which we had more than two populations, a TukeyHSD was performed to adjust for multiple testing. Male (N = 155) and female (N = 126) data were analysed separately because size corrected gill raker length differed between the sexes (gill rakers are longer in females; ANOVA using size corrected values, P = 0.0095), and the sex ratios differed among populations. As we obtained similar results for males and females, we present the results of male data only. All statistical analyses were conducted in R.

Lower pharyngeal jaw morphology

Geometric morphometric analyses were applied on 224 lower pharyngeal jaw bones (LPJ) from the four lake–stream systems (Table S1, Supporting information). Pictures of the cleaned jaws were generated using an office scanner (EPSON perfection V30/V300, resolution: 4800 dpi) with a ruler on every scan to maintain size information. Following Muschick *et al.* (2012), x and y coordinates of eight homologous landmarks and 20 semilandmarks plus the image scales were acquired in tpsDIG2. After a sliding process with tpsRELW (Rohlf 2007), we reduced the initial data set to 16 landmarks consisting of eight true landmarks and eight semilandmarks (Fig. S1C, Supporting information; for details see Muschick *et al.* 2012). The symmetric components of the procrustes-aligned coordinates (GLSF algorithm) were then regressed against centroid size to correct for

allometry. The residuals of the regression were used to perform DFA for each lake–stream system by comparing each lake population with the geographically most distant stream population. Further, we conducted several CVAs comparing multiple populations within each system and over all populations of the lake–stream systems. The significance levels of the obtained mean shape distances were computed using permutation tests (10 000 permutations). As we found smaller intersexual within-population differences in LPJ shape than intrasexual differences among populations (Fig. S2, Supporting information), all analyses were conducted with pooled sexes. Statistical analyses of the morphometric data were performed in MORPHOJ.

Stomach and gut content

To investigate whether the populations differ with respect to food resource use, we inspected gut and stomach contents. To this end, the intestines of 102 male individuals (Table S1, Supporting information) were opened under a binocular (LEICA, $MZ7_5$) and the content was separated into the following five categories: plant material and algae, sand, macro-invertebrates (insects and insect larvae), hard-shelled items (mollusc shells and plant seeds), and zooplankton and micro-invertebrates (mainly small shrimps of the LT endemic genus *Limnocaridina*, cladocerans and copepods). The volume (in %) of each category was determined by comparison with serial volume units. For the illustration of the proportions of food items only, the category 'sand' was excluded.

Testing for associations between genetic differentiation, morphometric traits and environment

Partial Mantel tests were applied to compare pairwise differences of morphometric traits (Mahalanobis distances for body shape, mouth position and LPJ, metric measurements for gill rakers) from lake–stream populations with the corresponding F_{ST} values, while correcting for geographic distances. In a second step, the influences of several environmental parameters (microhabitat current, proportion of hard-shelled food items and proportion of macro-invertebrates) and geographic distance on the same morphometric differences were analysed with a multiple regression on distance matrices (MRM). MRM is an extension of the partial Mantel analysis and allows multiple regression of the response matrix on any number of explanatory matrices (Lichtstein 2007). Of 10 000 permutations were performed, as recommended by Jackson & Somers (1989). All analyses were performed using the package ecodist in R. Note that we had to exclude Lf1 in these analyses due to the lack of environmental data.

Testing for reproductive isolation and trait plasticity

We evaluated reproductive isolation among lake and stream *A. burtoni* populations in triadic mating trials. The common garden setting of this pond experiment also allowed us to test for plasticity in body shape and gill raker morphology in F1 offspring.

The experiment was carried out between July 2013 and January 2014 in five concrete ponds at Kalambo Lodge, Zambia. Experimental ponds (dimensions: 3.2 × 1.4 × 0.5 m) were stocked with seven females and four males each from two stream populations (Ka3 and Lz1) and one lake population (KaL). Wild-caught adults were photographed and fin-clipped before starting the experiment. Males were selected for size to achieve a similar size distribution among the three populations within each pond. Concrete ponds were supplied with lake water; fish were fed with commercial flake food two times a day.

After a period of six months, we collected and fin-clipped all offspring plus all remaining adult fish (55 out of 165 initially introduced) from the ponds. Fish weighting more than 1 g were photographed and measured. We then genotyped all putative parental individuals and 593 offspring (i.e. all free living juveniles plus 5 individuals from each brood within a females' mouth) at five microsatellite loci (Ppun5, Ppun7, Ppun21, UNH130 and Abur82), following the methods described above. Parentage was inferred using the software CERVUS (Kalinowski *et al.* 2007), with no mismatch allowed. Offspring that were assigned to the same mother and father were combined as a single mating event, except if they belonged to different size classes (free-swimming young vs. wrigglers). In case of the detection of more than one father in broods collected from mouthbrooding females, these were treated as two mating events. Multiple paternity in *A. burtoni* has been detected previously in mate choice experiments under laboratory conditions in ~7% of genotyped broods (Theis *et al.* 2012).

We then used F1-offspring to test for a heritable component of body shape ($N = 130$) and gill raker ($N = 132$) morphology. F1 individuals were categorized as offspring resulting from the following mating combinations: KaL-KaL, Ka3-Ka3, Lz1-Lz1, Ka3-Lz1, KaL-Ka3 and KaL-Lz1 (Table S2B, Supporting information). Body shape was analysed using the same methods as described above. Due to low sample size in some of the crosses, we reduced the number of landmarks to 6 (landmarks 1, 2, 8, 12, 14 and 15; Fig. S1A, Supporting information). We first conducted CVAs for the three interpopulation crosses (KaL-Ka3, KaL-Lz1, Lz1-Ka3) and their corresponding within-population crosses (KaL-KaL, Ka3-Ka3, Lz1-Lz1) separately to test

whether (i) within-population crosses are differentiated and (ii) whether interpopulation crosses show intermediate body shape with respect to within-population crosses. Additionally, within-population F1 offspring were analysed in a CVA together with their corresponding wild-type populations to detect plastic shifts in body shape induced by the common garden setup. Moreover, we conducted a CVA to compare body shape of introduced specimens before and after the experiment, to test for plastic responses in adults. Gill raker length and number of F1 offspring were measured and analysed using the same methods as described above for wild populations. Mean gill raker length correlated positively with SL ($R^2 = 0.58$, $P < 0.0001$) and was corrected for body size. As with body shape, the three interpopulation crosses (KaL-Ka3, KaL-Lz1 and Lz1-Ka3) and their corresponding within-population crosses (KaL-KaL, Ka3-Ka3 and Lz1-Lz1) were first analysed separately. Then, within-population crosses were compared with their corresponding wild-type populations after applying a common size correction.

Results

Water current measurements

Water current was generally stronger at upstream localities, with the exception of Kalambo (water current was stronger at Ka2 than Ka3; see Table 1A for values and Appendix S1, Supporting information for habitat descriptions). As surface and microhabitat current are significantly correlated ($R^2 = 0.6155$, $P = 0.0072$), we used only microhabitat current for further analyses.

Genetics

Sequencing of the mitochondrial control region of 359 specimens revealed the presence of 16 haplotypes. The haplotype genealogy (Fig. 1B) indicates a deep split between the eastern (1–14, haplotypes A–I) and the western (15–17, 19–20, haplotypes L and M) populations. Moreover, the most upstream Lufubu population (18) comprises three haplotypes (N–P), which are clearly distinct from all other lineages. The haplotypes found at the western shoreline of LT at Ndole Bay (21, haplotypes J and K) group with the ones from the northernmost population at the eastern shoreline of LT at Ninde (1, haplotype I). The Lake Chila fish (22) contain the major mtDNA haplotype of the western haplotype lineage (haplotype M).

The analysis of nine microsatellite loci revealed moderate to strong differentiation between populations, even within lake–stream systems (Table S3A, Supporting information for population pairwise F_{ST} and D_{EST}). F_{ST} and D_{EST} values are highly congruent, and P-values (F_{ST}) and confidence intervals (D_{EST}) indicate significant differentiation between most population pairs except for some geographically adjacent populations (15 and 16 for both F_{ST} and D_{EST}, 16 and 17 for F_{ST} but not D_{EST}) and some of the populations sampled twice in two different years (4a and 4b, 7a and 7b, 15a and 15b). Based on F_{ST} and D_{EST} values, population 22 (Lake Chila) and 16 (Fisheries Department, LT) are not significantly differentiated.

Bayesian clustering with STRUCTURE of the entire data set resulted in a most likely number of $K = 10$ (Fig. 1C). The three Tanzanian populations (1–3) cluster together, despite rather large geographic distances between them.

Table 1 Microhabitat current as well as stomach and gut content information. (A) Microhabitat current (represented by dissolution rate in mg/s) at the localities from the lake–stream systems with 95% confidence intervals in brackets. (B) Average values with corresponding 95% confidence intervals in brackets for the proportions of the different stomach content categories (plant and algae, zooplankton, sand, macro-invertebrates, and hard-shelled items)

A		B					
Locality	Microhabitat current: dissolution rate (mg/s)	Population	Plants and algae	Zooplankton	Sand	Macro-invertebrates	Hard-shelled items
KaL	0.032 (±0.039)	KaL ($N = 10$)	0.954 (±0.036)	0.018 (±0.015)	0.020 (±0.037)	0.008 (±0.006)	0 (±0)
Ka1	0.280 (±0.356)	Ka1 ($N = 10$)	0.605 (±0.120)	0 (±0)	0.148 (±0.070)	0.228 (±0.095)	0.019 (±0.017)
Ka2	4.842 (±0.986)	Ka2 ($N = 10$)	0.179 (±0.090)	0.001 (±0.002)	0.009 (±0.018)	0.749 (±0.102)	0.061 (±0.031)
Ka3	2.962 (±0.888)	Ka3 ($N = 10$)	0.359 (±0.098)	0.004 (±0.005)	0.018 (±0.017)	0.618 (±0.105)	0.001 (±0.001)
ChL	1.029 (±0.223)	ChL ($N = 5$)	0.877 (±0.101)	0.039 (±0.021)	0.069 (±0.094)	0.015 (±0.010)	0 (±0)
Ch1	4.311 (±0.542)	Ch1 ($N = 10$)	0.613 (±0.148)	0.001 (±0.001)	0.064 (±0.046)	0.253 (±0.138)	0.069 (±0.053)
LzL	0.094 (±0.096)	LzL ($N = 10$)	0.565 (±0.226)	0.027 (±0.034)	0.313 (±0.227)	0.087 (±0.096)	0.008 (±0.009)
Lz1	2.749 (±0.685)	Lz1 ($N = 10$)	0.441 (±0.091)	0 (±0)	0.259 (±0.121)	0.224 (±0.099)	0.076 (±0.036)
LfL	0.693 (±0.604)	LfL ($N = 10$)	0.628 (±0.233)	0.240 (±0.257)	0.007 (±0.007)	0.047 (±0.061)	0.077 (±0.081)
Lf1	n/a	Lf1 ($N = 7$)	0.935 (±0.039)	0 (±0)	0.031 (±0.026)	0.023 (±0.031)	0.011 (±0.011)
Lf2	4.261 (±0.763)	Lf2 ($N = 10$)	0.433 (±0.164)	0.001 (±0.002)	0.117 (±0.053)	0.450 (±0.156)	0 (±0)

Along the Zambian shoreline, several 'pure lacustrine populations', that is populations not being adjacent to a river, cluster together, even when being separated by large sandy bays (16 and 17, separated by Mbete Bay; 12 and 14, separated by Chituta Bay). The population from Lake Chila (22) belongs to the same genotypic cluster as populations 15, 16 and 17 from LT. Specimens from the same population but sampled in different years always cluster together (indicated by 'a' and 'b' in Fig. 1C).

There was a strong pattern of isolation by distance for populations sampled along the shoreline (Mantel-$R = 0.5539$, $P = 0.0164$).

The separate STRUCTURE analyses for each of the four lake–stream systems are depicted in Fig. 2B. The most likely number of genetic clusters was $K = 2$ for all systems (Fig. S3, Supporting information). Note, however, that it is not possible to infer ΔK for $K = 1$.

Body shape

The CVA of body shape of the 20 sampled populations revealed a significant differentiation between all populations (Fig. S4A; Table S3B, Supporting information). The main body shape changes are described by canonical variate 1 (CV1, accounting for 32% of the variance), which shows a change in body depth, mouth position as well as in head size, and CV2 (accounting for 17% of the variance) describing additional changes in caudal peduncle and eye size.

No pattern of isolation by distance was detected regarding body shape for populations sampled along the shoreline (Mantel-$R = 0.2116$, $P = 0.1415$). The CVA plot of all shoreline populations (Fig. S4B, Supporting information) does not show closer positions in morphospace of more closely located populations, but rather indicates stronger clustering of pure lacustrine populations (of LT and Lake Chila) compared with the more scattered shoreline populations that are adjacent to streams.

When analysing each lake–stream system separately, and comparing each lake population with the most distinct corresponding stream population, it becomes apparent that lake fish generally have a deeper body and a more superior mouth position compared with stream fish. This body shape change, together with clearly partitioned discriminant scores, was found in the systems Kalambo (KaL and Ka3), Lunzua (LzL and Lz1) and Lufubu (LfL and Lf2). The lake and river populations of the Chitili system (ChL and Ch1) showed an overlap of the discriminant scores of the DFA and therefore smaller but still significant changes in body shape (Fig. 2C).

The pattern is more complex when body shape is compared within the river systems for which more than two populations have been sampled (Kalambo and Lufubu River). Three of the four Kalambo populations (KaL, Ka1 and Ka3) show a continuous shift from lake towards more upstream populations, with lake fish having a deeper body and a more superior mouth. The remaining Kalambo population (Ka2) clustered separately (Fig. S5A; Table S4A, Supporting information). The two downstream populations of the Lufubu system (LfL and Lf1) displayed a similar differentiation in body shape compared with the distinct upstream population (Lf2), again in the form of a more superior mouth position (Fig. S5A; Table S4B, Supporting information).

All populations of the lake–stream systems together show little congruence in CV1–CV2 morphospace occupation and only the populations from the two lake populations of the similar rivers Kalambo and Lunzua clustered together (KaL and LzL in Fig. 3A) and one of the Kalambo populations overlapped substantially with the first two Lufubu populations (Ka2, LfL and Lf1 in Fig. 3A). The body shape changes, however, followed similar trajectories between river and lake populations throughout all systems, as evidenced by similar unidirectional shifts in CV1 (illustrated by a bar in Fig. 3A). In all four river systems, lake fish had deeper bodies and a more superior mouth along CV1 (accounting for 45% of the variance in the CVA) (Fig. 3A and Table S5A, Supporting information).

Gill raker morphology

ANOVA detected significant differences in gill raker length between male lake and stream fish in all populations, with generally longer gill rakers in lake populations and raker length decreasing with increasing geographic distance from the lake (Fig. 2E; Table S6, Supporting information). In more detail, the lake population from the Kalambo system (KaL) showed significantly longer gill rakers compared with each of the stream populations (Ka1, Ka2 and Ka3), which did not differ significantly among each other. In the Chitili and the Lunzua system, we found a significant difference between the lake and stream populations. In Lufubu, the lake population (LfL) showed no differences in raker length compared with the first upstream population (Lf1), but gill rakers of Lf1 fish were longer compared with the most upstream population (Lf2). However, gill raker number did not differ between lake and stream fish in any of the four lake–stream systems. The results for females, which showed the same trend of longer gill rakers in lake populations compared with stream populations, are shown in Fig. S5C and Table S6 (Supporting information).

Lower pharyngeal jaw morphology

We also detected differentiation between lake and stream fish in the morphology of the LPJ (Fig. 2D). For

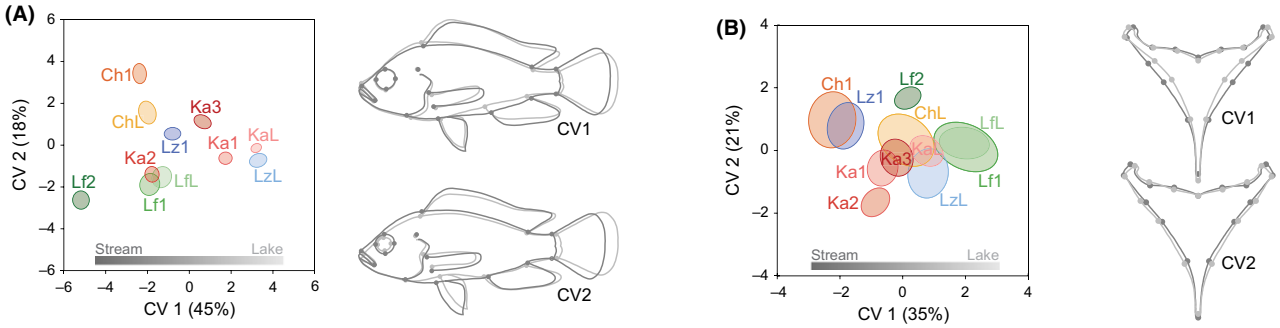

Fig. 3 Body shape and lower pharyngeal jaw (LPJ) shape differentiations of all populations from the lake–stream systems. Canonical variate analyses (CVA) plots illustrate the distribution of the populations on CV1 and CV2 (ellipses represent the 95% confidence intervals of the means) and the shifts are represented in the outline drawings (outlines are always drawn for illustration purposes only, from dark to light grey with increasing values, scaling factor 10 by default; abbreviations of locality names are defined in the grey box in Fig. 1). (A) Shifts in body shape between each lake population and their corresponding stream populations are unidirectional on the axis of CV1 (represented with the bar), indicating that lake fish have deeper bodies and a more superior mouth (Table S5A, Supporting information). (B) For LPJ morphometrics, all lake populations cluster together and show unidirectional shifts along CV1 towards their corresponding stream populations. Lake fish generally have slender and more elongated LPJ compared with stream fish (Table S5B, Supporting information).

each system, we compared the lake population to the stream population with the largest geographic distance to the lake. The Kalambo lake (KaL) and the most upstream population (Ka3) showed a minor overlap in discriminant scores and only a small but still significant difference in LPJ shape, with broader LPJ in stream fish compared with lake fish. In the Chitili, Lunzua and Lufubu systems, we found similar, yet more pronounced shifts in LPJ width. In the Chitili system, an additional shift towards a more convex posterior curve and shorter posterolateral horns in stream fish was detected. Although the underlying shape changes differed among the systems, there was a consistent shift in width of the jaws with broader LPJ in stream fish compared with lake fish.

The system specific CVA of the Kalambo River populations showed a continuous increase in LPJ width and an increasing angle of the posterolateral horns from the lake population (KaL) to the first and the second upstream populations (Ka1 and Ka2). The fourth Kalambo population (Ka3) clustered with the first upstream population (Ka1). In the Lufubu system, we found a considerable overlap in CV1 and CV2 of the lake population (LfL) and the adjacent stream population (Lf1), but a distinct LPJ shape in the furthermost upstream population (Lf2) having broader and shorter LPJ (Fig. S5B; Table S4C,D, Supporting information).

The CVA with all 11 lake–stream populations included showed a significant difference (based on Mahalanobis distances) in LPJ shape among all populations except between LfL and Lf1 (Fig. 3B; Table S5B, Supporting information). CV1 (accounting for 35% of the variance) represented mainly a change in broadness and length of the LPJ, whereas CV2 (accounting for 21% of the variance) described an additional change in angle of the posterolateral horns. In the CV1–CV2 morphospace, all lake populations clustered together, indicating similar LPJ shapes in the lake populations. All systems show a shift in LPJ shape along CV1 with broader and shorter LPJ in stream fish compared with lake fish (illustrated by a bar in Fig. 3B). Along CV2, the lake populations showed a consistent shift in angle of the posterolateral horns (except for the Kalambo system, where the shift was in the opposite direction).

Stomach and gut content

Stomach and gut content analyses revealed that *A. burtoni* is a generalist, feeding on a mixed diet composed of plant material, algae, insects, insect larvae, molluscs and planktonic components (Fig. 2F). The diet composition differed between lake and stream habitats, whereby lake fish feed more on softer and smaller food particles (plants and algae, zooplankton) and stream fish more on hard-shelled and bigger prey items (mollusc shells, plant seeds, insects and insect larvae).

In all four systems, we found a plant, algae and zooplankton-biased diet in lake fish and a parallel increase in the proportion of macro-invertebrates with increasing distance to the lake (Table 1B). In addition, the proportion of hard-shelled food items was generally higher in river populations, except for the Lufubu lake population, where a considerable proportion of hard-shelled food items has been found.

Testing for associations between genetic differentiation, morphometric traits and environment

The partial Mantel tests revealed that none of the morphometric trait differences correlated with genetic distance (F_{ST} values; Table 2A). Genetic differentiation at neutral markers therefore does not seem to be the determining factor for the observed differences among the lake and stream populations. The MRM including environmental parameters showed that the differences rather arise by the effect of environmental conditions: body shape was significantly influenced by both geographic distance and by water current. Mouth position correlated with current and was also influenced by feeding (proportion of macro-invertebrates). While gill raker length correlated with the proportion of macro-invertebrates, LPJ shape tends to be influenced by feeding on hard-shelled food items and correlated with microhabitat current (Table 2B).

Testing for reproductive isolation and trait plasticity

A total of 55 (of 165 initially introduced) wild-caught adult individuals and 593 F1 offspring were recovered from the experimental ponds. Loss of individuals was most likely due to aggressive and territorial behaviour of males. At the time the experiment was terminated, at least one female per population had survived in each pond, and in three of five ponds, at least one male per population had survived (Table S2A, Supporting information). Parentage analyses revealed that across the five ponds, all possible mating combinations occurred, but were not evenly distributed among the replicates (see Appendix S2, Supporting information for details). A qualitative inspection of the data indicated no assortative mating with respect to population but revealed that only 2–5 males reproduced per pond. Further, reproducing males were predominantly large males based on SL measurements taken at the beginning and at the end of the experiment. In *A. burtoni*, size and dominance are positively correlated (Fernö 1987), and dominant males are much more likely to reproduce. Accordingly, the observed pattern is likely a result of biased mating with respect to male size and dominance. This is also supported by comparing our observed data with a simulation assuming random mating with respect to population, but an increased mating probability of large males (see Appendix S2, Supporting information for details).

The morphometric analyses in F1 offspring revealed that while purebred (i.e. intrapopulation crosses) differed among each other in body shape in CV1 (accounting for 62–88% of the variance), between-population crosses were intermediate (Figs 4A and S6; Table S7A, Supporting information). A CVA including F1 offspring and wild populations demonstrates shifts in body shape under common garden conditions and a closer clustering of within-population crosses as compared to the corresponding wild populations (Fig. S7A; Table S8A, Supporting information). Interestingly, the body shape of introduced adult specimens also converged during the experimental period, with the stream populations (Ka3 & Lz1) becoming more like the lake population (KaL) (Fig. S7B; Table S8B, Supporting information). (Note that the experimental set-up in ponds resembles more the lake situation.)

Gill rakers were significantly longer in within-lake population offspring compared with within-stream population offspring, and intermediate in the interpopulation crosses (Fig. 4B; Table S7B, Supporting information). No difference in gill raker number was detected. Within-population offspring from the common garden experiment show a shift towards longer gill rakers compared with the corresponding wild populations (Fig. S7C; Table S8C, Supporting information).

Discussion

Phylogeography and population structure of Astatotilapia burtoni in southern LT

Overall, our study revealed an unexpectedly high degree of genetic and morphological diversity and

Table 2 Testing for associations between genetic differentiation, morphometric traits, and environment. (A) Genetic distances (F_{ST}) were correlated with morphological distances (Mahalanobis) using a partial Mantel test including geographic distance as a correction factor. (B) Combined multiple regression on distance matrices (MRM) between morphological and ecological distances

A Morphometric trait	Genetic distance (F_{ST})	B Morphometric trait	Microhabitat current	Hard-shelled items	Macro-invertebrates	Geographic distance
Overall body shape	0.268 (Mantel- $R = 0.133$)	Overall body shape	0.0042**	0.2717	0.4323	0.0253*
Mouth position	0.825 (Mantel- $R = -0.226$)	Mouth position	0.0157*	0.1793	0.0175*	0.8627
Gill raker length	0.496 (Mantel- $R = -0.005$)	Gill raker length	0.4182	0.4504	0.0373*	0.2270
LPJ shape	0.762 (Mantel- $R = -0.186$)	LPJ shape	0.0219*	0.0587	0.4712	0.3425

LPJ, lower pharyngeal jaw.
Significance levels: *$P < 0.05$ and **$P < 0.01$.

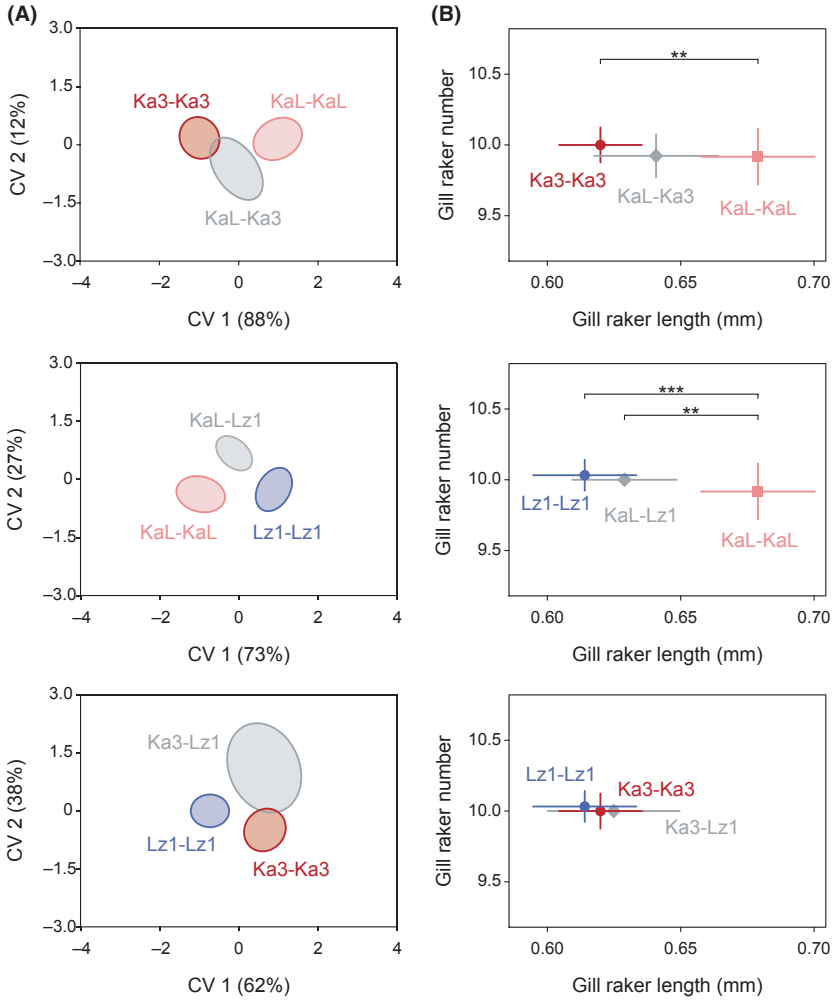

Fig. 4 Body shape (A) and gill raker comparisons (B) of each interpopulation cross with the corresponding within-population crosses from the pond experiment (Fig. S6, Supporting information for corresponding CV outlines and Table S7, Supporting information for distance and significance values).

extensive population structure in *A. burtoni* from southern LT (Figs 1, 2 and S4A, Supporting information). Notably, we identified two main mtDNA control region haplotype lineages in *A. burtoni* that are separated by 10 mutations (Fig. 1B). The genetic diversity in *A. burtoni* is thus similar to, or even exceeds the diversity observed in the same marker in the entire haplochromine cichlid assemblage of Lake Victoria (Verheyen *et al.* 2003). It has long been recognized that substantial differences exist in inter- and intraspecific genetic variation in mtDNA within different East African cichlid radiations and that the degree of differentiation reflects the respective age of a lineage rather than morphological disparity (Sturmbauer & Meyer 1992). The great diversity in mtDNA in *A. burtoni*, even across small geographic scales, thus suggests a deep coalescence time and, consequently, the presence of this species in the study area over long time periods. This is in line with a previous multispecies study that detected deep coalescence times in the only analysed *A. burtoni* population (collected in the area of our Ka3 site) based on microsatellite markers (Elmer *et al.* 2009).

The data at hand indicate that while mtDNA clearly separates the populations into an eastern (1–14) and a western clade (15–20; with the exception of population 21, see below) (Fig. 1B), such a clear-cut barrier to gene flow is not evident in the nuclear DNA markers (Fig. 1C): The population assignment tests with STRUCTURE suggest some gene exchange between populations 14 and 15, and the pairwise differences in F_{ST} and D_{EST} between populations 14 and 15 are among the smallest detected (nevertheless significant), fitting the isolation-by-distance scenario among the lacustrine populations. Similarly, while population 21 is clearly distinct in its mtDNA from the geographically nearest populations 19 and 20 (Fig. 1B), some level of gene flow between these populations is indicated based on the nuclear DNA markers (Fig. 1C). Such a pattern could be explained by male-biased dispersal along the shoreline of LT (Stiver *et al.* 2007). Male-biased dispersal and the preference

for shallow, sandy habitats would also explain why—in contrast to lake cichlids occurring in the rocky shoreline habitat of LT (e.g. Koblmüller *et al.* 2011)—long stretches of sandy shorelines do not seem to act as strong barriers to gene flow in *A. burtoni* (see e.g. 1–3, 12 and 14, 16 and 17, 20 and 21).

Recent migration along the shoreline cannot, however, explain the distribution of the main mtDNA haplotype lineages in *A. burtoni* (i.e. the clear-cut separation into an eastern and a western haplotype clade and the distinctiveness of populations 18 and 21). The bathymetry of the southern LT basin together with periodically occurring and climatically induced fluctuations in the lake level of LT (see e.g. Sturmbauer *et al.* 2001, 2005; Koblmüller *et al.* 2011) might provide one explanation for the overall structure of the mtDNA haplotype genealogy (Fig. 1B). The deep split between the eastern and the western haplotype lineages could, for example, be directly related to an underwater ridge in exactly the area between populations 14 and 15 (see fig. 1 of Koblmüller *et al.* 2011), which might have acted as migration barrier at times of low lake level stands, especially for a species associated to rivers, estuaries and shallow waters such as *A. burtoni*. Low lake level might also permit migration across what is at present two opposite shorelines of LT (see e.g. Sturmbauer *et al.* 2001; Baric *et al.* 2003), thus explaining the close relationship between population 21 from the western (Zambian/Congolese) part of LT to the eastern (Tanzanian) populations 1–3.

The close relatedness of the Lake Chila population (22) to populations sampled around Mpulungu (15–17), and especially to population 16 (Table S3A, Supporting information), is somewhat puzzling. Lake Chila is a small and shallow lake about 20 km southeast of LT, and connected to LT through a small outflow draining into LT near Sumba (population 12). However, there is no faunistic association between Lake Chila and LT, except for *A. burtoni*, and we could only detect elements of a fish fauna in Lake Chila, which is otherwise typical for the Chambeshi, Zambesi and the Zambian/Congo watersheds (*Serranochromis angusticeps*, *S. robustus*, *S. thumbergi*, *Pseudocrenilabrus* cf. *philander* and *Tilapia sparmanii*) (Skelton 1993). As Lake Chila's *A. burtoni* are genetically indistinguishable from population 16, yet distinct from population 12, and because there are reports of a recent stocking of this small lake (L. Makasa, Fisheries Department Mpulungu, personal cummunication), a human-induced translocation is the likely source of the current Lake Chila *A. burtoni* stock (despite records of the presence of *A. burtoni* in that lake more than 50 years ago as evidenced by a collection by M. Poll from 1949 deposited in the Royal Museum for Central Africa in Tervuren, Belgium).

In summary, we show that *A. burtoni* occurs along a lake–stream environmental gradient in southern LT and that several lake–stream systems have been colonized independently. One of these systems, the Lufubu, is genetically very distinct from the other three (Kalambo, Chitili and Lunzua), especially with respect to mtDNA. However, we can, at present, not infer the precise colonization history of *A. burtoni* in southern LT. In particular, we cannot assess whether any of the surveyed river populations is the source of *A. burtoni* in the area or whether all the river systems have been colonized from LT. A more thorough analysis including a denser sampling across a much larger geographic area would be necessary to fully understand the phylogeographic history and population structure of *A. burtoni*.

Adaptive divergence between lake and stream habitats in Astatotilapia burtoni

Integrative studies of fish species that occur along an environmental gradient have provided important insights into speciation (Hendry *et al.* 2000; Seehausen *et al.* 2008; Berner *et al.* 2009; Roesti *et al.* 2012). Our survey of *A. burtoni* in the southern part of LT reveals that this species occurs along a lake–stream environmental gradient and is present, in high abundance, in every suitable habitat ranging from truly lacustrine environments to river estuaries, larger rivers and small creeks draining into LT (Figs 1A and 2A). Importantly, we show that populations inhabiting the same environment tend to be morphologically similar, irrespective of their genetic background (Figs 2, 3 and S4B, Supporting information). For example, among populations sampled within LT, there is a closer morphological resemblance between the truly lacustrine populations (i.e. the populations away from any river) and between the populations near river estuaries (Fig. S4B, Supporting information). Interestingly, the only sampled lacustrine *A. burtoni* population outside from LT (from Lake Chila) clusters closely in morphospace with the truly lacustrine populations from LT (Fig. S4B, Supporting information) (note, however, that this resemblance might also be due to recent introduction; see above). In addition, while there is a strong signal of isolation by distance with respect to genetics along the shoreline of LT, this is not the case for body morphology, suggesting that similar environmental pressures, but not relatedness, mediate the emergence of similar body shapes in *A. burtoni*.

This pattern becomes even more evident when comparing the body shape between lake and stream populations from the four lake–stream systems studied in detail. Generally, we find that lake fish exhibit deeper

bodies and a more superior mouth compared with stream fish (Figs 2C and 3A) and that mouth position is correlated with feeding mode (Table 2B). In addition, we detected a significant correlation between body shape and water current (Table 2B), which is in line with adaptations to different flow rates as predicted by hydrodynamic theory (Webb 1984). However, these changes in morphology only partially agree with those found in other lake–stream systems in fishes. In sockeye salmon, for example, beach residents, too, have deeper bodies compared with their riverine counterparts (Hendry *et al.* 2000). In Canadian three-spine stickleback, on the other hand, lake fish tend to have more slender bodies compared with stream fish due to shifts in feeding modes (e.g. Schluter & McPhail 1992; Berner *et al.* 2008, 2010; Ravinet *et al.* 2013).

In addition to the body shape differences, we also detected significant shifts in trophic morphology across the lake–stream transition in *A. burtoni* (Fig. 2D,E and 3B). The morphological trajectory of the gill raker apparatus along this habitat gradient resembles that in other groups of fishes. Just as in sticklebacks (Berner *et al.* 2008; Ravinet *et al.* 2013), gill rakers are shorter in *A. burtoni* stream fish compared with lake fish. Gill rakers are an important trophic trait in fishes, and believed to function as a cross-flow filter to concentrate particles inside the oral cavity and to transport particles towards the oesophagus (Sanderson *et al.* 2001). In stickleback and other fishes, divergence in gill raker morphology is driven by differential prey resource use (e.g. Bentzen & McPhail 1984; Robinson & Wilson 1994; Skulason & Smith 1995; Berner *et al.* 2008). Likewise, in *A. burtoni*, shorter gill rakers are associated with the consumption of larger food items and longer gill rakers with smaller food particles. However, there were no significant differences in gill raker numbers between lake and stream populations. Divergence in gill raker length accompanied by stasis in gill raker number has also been found in European stickleback lake–stream population pairs, which was explained by the insufficient time for divergence and differences in the genetic architecture compared with Canadian lake–stream populations (Berner *et al.* 2010). While our population-genetic analyses based on mtDNA suggest a deep coalescence time among the major haplotype lineages in *A. burtoni*, little is known about the timing of splitting events among the studied lake–stream populations. Generally, gill raker number varies considerably among LT cichlid species (M. Rösti, personal observation), but it may be less prone to environmentally induced phenotypic variation than other morphological traits such as gill raker length and the LPJ (Lindsey 1981). We also detected sexual dimorphism in gill raker length, with females having longer gill rakers compared with males. In addition, there appears to be a sexual dimorphism in head shape, with females showing more slender yet larger heads (Fig. S1B, Supporting information). Both might be explained by functional differences due to the female mouthbrooding behaviour characteristic for haplochromines.

Trophic divergence between *A. burtoni* lake–stream populations is also evident from differences in LPJ morphology between habitats. The morphology of the oral and pharyngeal jaws is highly diverse in cichlids (Fryer & Iles 1972; Liem 1973; Salzburger 2009; Muschick *et al.* 2012) and related to functional feeding ecology (Liem 1980; Muschick *et al.* 2012, 2014). Experimentally induced, plastic changes in cichlid pharyngeal jaws have been shown to be due to the mode of feeding rather than differences in nutritional composition. For example, Nicaraguan Midas cichlids (*Amphilophus citrinellus*) fed on whole snails developed heavier and more hypertrophied LPJs compared with individuals fed on either crushed whole snails or snail bodies without shells (Muschick *et al.* 2011). Similar shifts in LPJ morphology along with different resource use are known from natural cichlid populations (Meyer 1990; Hulsey *et al.* 2008). In line with these studies, the broader and shorter LPJs of *A. burtoni* stream fish compared with lake fish may pose an adaptation to the shift in diet towards harder food items such as seeds, snails and other hard-shelled invertebrates found in stomachs of stream populations (Fig. 2F; Table 1B). In our analyses, we found that LPJ morphology tends to correlate with the proportion of hard-shelled food items, but there is also a correlation between LPJ and water current (Table 2B). This latter correlation could be due to the method used to infer LPJ shape, which might be influenced by more general shifts in head morphology across the lake–stream gradient.

Phenotypic plasticity constitutes an alternative outcome to speciation in the face of divergent selection (West-Eberhard 2005; Pfennig *et al.* 2010). The generalist species *A. burtoni* dwells in many different habitats, which could result in the evolution of highly plastic populations expressing a variety of phenotypes. On the other hand, speciation could also be initiated via plastic responses to novel environments followed by genetic assimilation (e.g. Waddington 1942; West-Eberhard 2003). Our common garden experiment demonstrated that both plastic and genetic components influence body shape and gill raker length in *A. burtoni*. The F1 offspring from the within-population matings generally show significant differentiation with respect to both body shape and gill raker length, and interpopulation crosses generally display intermediate phenotypes. This pattern, together with the conserved higher body shape and shorter gill rakers of the lake population offspring

(KaL-KaL), compared with the within-stream population crosses speaks for a genetic component underlying trait differentiation (Fig. 4). However, shifts in F1 offspring in both traits under common garden conditions compared with wild populations indicate that trait plasticity also contributes to the detected differences (Fig. S7, Supporting information). Whether these patterns also hold with regard to LPJ morphology and to what extent plasticity and heritability contribute to the detected differences in body shape and trophic traits remains to be tested in future experiments.

We did not find any evidence for assortative mating with regard to population in our mating experiment. All possible mating combinations occurred, and male dominance effects seemed to determine the observed mating patterns (Appendix S2, Supporting information). The absence of reproductive barriers in spite of strong genetic and morphological differentiation has also been reported from lake and stream stickleback (Raeymaekers *et al*. 2010). However, a transplant experiment later indicated that selection against immigrants, together with various other factors, might be contributing to reproductive isolation in this system (Räsänen & Hendry 2014). Similarly, we cannot rule out that barriers, which we did not detect in our experiment, could contribute to reproductive isolation among lake and stream populations. In *A. burtoni*, with its lek-like polygynandrous mating system, only dominant males gain access to territories as well as (several) females and are therefore able to reproduce (Fernald & Hirata 1977). Although no bias in dominance among populations was evident from our data, possible male aggression biases (and probably undetected female preferences) should be tested under more controlled conditions in the future (see Theis *et al.* 2012). As a next step, it would be interesting to test whether the genetically most distinct populations, for example Lf2 vs. KaL, are reproductively isolated.

Evidence for (ecological) speciation is often inferred via a positive correlation between the levels of (adaptive) divergence in phenotypic traits and the levels of neutral genetic differentiation between populations, when controlled for geographic distance ('isolation by adaptation', Nosil 2012). In *A. burtoni*, we did not find correlations between any morphological trait measured and F_{ST} values (Table 2A). This gene-flow approach based on neutral markers does have several caveats, though (see Nosil 2012), and a lack of signal does not necessarily exclude the possibility of (ecological) speciation. Due to the geographic isolation of some populations (e.g. populations located above waterfalls or geographically very distant populations), differentiation at neutral loci might occur without barriers to gene flow caused by divergent selection in *A. burtoni*, resulting in a failure to detect isolation by adaptation. Note that there was also no pattern of isolation by distance detectable if only lake–stream populations were included in the analysis, as opposed to the pattern detected along the shoreline (see above). However, lake and stream populations from the four lake–stream systems (and populations within systems) appear to rest at different stages of the speciation continuum. In the Chitili system, for example, the lake and stream populations are geographically close, genetically admixed and also less differentiated in body shape and gill rakers compared with the pairwise comparisons from the Kalambo, Lunzua and Lufubu systems shown in Fig. 2. Although there are several outliers in our data (e.g. relatively pronounced LPJ differentiation within the Chitili system compared with very little LPJ differences between the clearly genetically distinct populations KaL and Ka3), lake and stream populations belonging to distinct genetic clusters generally show more differentiation in morphological traits (Fig. 2).

Taken together, our study revealed the presence of multiple divergent lake–stream populations in the southern LT drainage. Phenotypic divergence between populations from the four independent lake–stream systems follows similar trajectories: Divergence in body shape is associated with different flow regimes in lake and stream habitats, whereas shifts in trophic structures are linked to differential resource use. We did not detect a signal for isolation by adaptation; however, more powerful genetic data such as genome scans may clarify the interplay between levels of gene flow and phenotypic divergence in these systems. A first test for reproductive isolation among the more closely related lake and stream populations did not reveal any population-assortative mating patterns. Importantly, analyses of F1 offspring reared under common garden conditions indicate that the detected trait differences among *A. burtoni* populations do not reflect pure plastic responses to different environmental conditions, but that these differences also have a genetic basis.

The *A. burtoni* lake–stream system constitutes a valuable model to study the factors that enhance and constrain progress towards speciation, and offers the unique possibility to contrast replicated lake–stream population pairs at different stages along the speciation continuum in cichlids. In addition, it allows evaluating parallelism across different species, that is lake–stream pairs of stickleback and cichlids. Characterizing potential reproductive barriers and the role of plasticity in phenotypic divergence in more detail, together with studies on genomic differentiation, promises to contribute to understanding the process of speciation in natural populations.

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B.E., W.S., A.T. and F.R. designed the study; B.E., W.S., A.T. and F.R. wrote the manuscript. B.E. produced and analysed the population-genetic data, A.T. produced and analysed body shape data and conducted mantel test and MRM statistics, F.R. produced and analysed data on gill rakers, LPJs, stomach contents and paternity. All authors participated in sampling, were involved in the experimental design of the pond experiment and provided input on the manuscript.

Data accessibility

Mitochondrial DNA sequences: GenBank accessions KM508103–KM508461.

mtDNA sequence alignment, microsatellite genotypes, morphological data, stomach and gut content data, environmental data and common garden experiment data: Dryad doi:10.5061/dryad.pp0q1.

Supporting information

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

Fig. S1 Landmark positions for body shape and LPJ analyses and sex differences in head shape.

Fig. S2 Comparison of intersexual within-population differences and intrasexual differences among populations in morphometric traits (body shape and LPJ).

Fig. S3 Mean likelihood (L(K) ± SD) over 10 STRUCTURE runs assuming K clusters (left); ΔK statistic (right).

Fig. S4 Body shape differentiation among the 20 sampled populations and among the 11 shoreline populations only.

Fig. S5 Body shape and LPJ shape differentiation within systems with more than two populations and gill raker length and number in females.

Fig. S6 Outlines to illustrate the body shape changes in F1 individuals of the pond experiment.

Fig. S7 Plasticity in body shape and gill raker length.

Table S1 Sample size details for each analysis with information about sampling year and geographic coordinates for each locality.

Table S2 Sample size details and result summary of the pond experiment.

Table S3 Pairwise genetic and morphometric (body shape) distances between populations.

Table S4 Pairwise morphometric (body shape and LPJ) distances within systems with more than two populations.

Table S5 Pairwise morphometric (body shape and LPJ) distances of all populations from the lake-stream systems.

Table S6 *P*-values for within system gill raker length comparisons for males and females.

Table S7 Pairwise morphometric (body shape and LPJ) distances between F1 crosses

Table S8 Pairwise morphometric (body shape) distances and *P*-values of gill raker comparisons among different groups of the pond experiment.

Table S9 Microsatellite diversity in populations of *Astatotilapia burtoni*.

Table S10 Genetic diversity of mtDNA sequences.

Appendix S1 Description of river systems.

Appendix S2 Pond experiment—Simulation.