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Immigrant and extrinsic hybrid inviability contribute to reproductive isolation between lake and river cichlid ecotypes

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Understanding how reproductive barriers evolve and which barriers contribute to speciation requires the examination of organismal lineages that are still in the process of diversification and the study of the full range of reproductive barriers acting at different life stages. Lake and river ecotypes of the East African cichlid fish *Astatotilapia burtoni* show habitat-specific adaptations, despite different levels of genetic differentiation, and thus represent an ideal model to study the evolution of reproductive barriers. To evaluate the degree of reproductive isolation between genetically divergent lake and river populations, we performed a mesocosm mating experiment in a semi-natural setting at Lake Tanganyika. We assessed reproductive isolation in the presence of male–male competition by analyzing survival and growth rates of introduced adults and their reproductive success from genetic parentage of surviving offspring. The genetically divergent river population showed reduced fitness in terms of survival, growth rate, and mating success in a lake-like environment. Hybrid offspring between different populations showed intermediate survival consistent with extrinsic postzygotic reproductive barriers. Our results suggest that both prezygotic (immigrant inviability) and postzygotic reproductive barriers contribute to divergence, and highlight the value of assessing multiple reproductive barriers acting at different stages and in natural contexts to understand speciation mechanisms.

KEY WORDS: **Cichlid, hybrid inviability, immigrant inviability, local adaptation, Lake Tanganyika, reproductive isolation.**

Despite continuous interest in the topic (Darwin 1859; Sobel et al. 2010; Seehausen et al. 2014), fundamental questions in speciation research remain open. For example, although one of the main goals of speciation research is to identify the magnitude and order of appearance of isolating barriers that contribute to speciation, there is disagreement as to how this can be accomplished (Sobel et al. 2010). A promising strategy to properly address this question is to examine the full range of potential isolating barriers in incipient species, since only by studying recently diverged taxa is it possible to distinguish the isolating barriers that have actually contributed to speciation from those that have accumulated after speciation is complete (Schemske 2010; Sobel et al. 2010; Nosil 2012). However, while a large body of literature on isolating mechanisms exists, relatively few studies have explored the relative contribution of several potential mechanisms acting at different life stages to total reproductive isolation, and these studies have mostly been conducted in plants (e.g., Ramsey et al. 2003; Kay 2006; Richards and Ortiz-Barrientos 2016). Studies of isolating barriers that use replicate population pairs with different level of genetic divergence (from nascent to young species) are particularly powerful for directly testing which forms of reproductive barriers act at different time-points during speciation, and how rapidly their intensity changes with increasing genetic distance along the so-called "speciation continuum" (Schemske 2010; Nosil et al. 2017).

Reproductive isolation is generally defined as the product of all barriers to gene flow between divergent populations that are in contact (Mallet 2006). Based on their timing in the life cycle of an organism, reproductive barriers can be classified into premating-prezygotic, postmating-prezygotic, and postzygotic barriers (Coyne and Orr 2004). One important, yet long neglected, class of prezygotic reproductive barriers is immigrant

inviability, that is reduced fitness of immigrants relative to locally adapted individuals (Nosil et al. 2005; Ingley and Johnson 2016). Immigrant inviability directly leads to the reduction of interpopulation matings relative to intrapopulation matings, due to reduced survival of immigrants prior to mating in the less suitable (foreign) habitat. Moreover, immigrant females that survive and mate may still perish before they have produced offspring, further reducing genetic exchange via hybrid progeny (Nosil et al. 2005).

Extrinsic or "environment-dependent" postzygotic barriers occur when hybrids suffer reduced fitness in the dominant parental environment, for example because they fall between the parental niches (Coyne and Orr 2004; Nosil 2012). On the other hand, intrinsic postzygotic barriers occur when hybrids suffer reduced viability and/or fertility due to intrinsic genetic incompatibilities independent of the environment (e.g., Bateson-Dobzhansky-Müller incompatibilities) (Dobzhansky 1936; Coyne and Orr 2004; Seehausen et al. 2014). The presence and strength of many potential reproductive barriers, such as natural and sexual selection against migrants and hybrids, can only be experimentally evaluated if divergent populations are brought into secondary contact with each other and/or the divergent environment in natural settings, such as in enclosures in the wild or in mesocosms (Hanson et al. 2016). While some reproductive barriers, such as immigrant inviability, have been assessed in multiple systems, the reduced reproductive success of immigrants has only rarely been estimated in experiments that try to mimic natural conditions (Porter and Benkman 2017; Svensson et al. 2017).

The Haplochromini is the most species-rich and ecologically most diverse tribe of African cichlid fish, well known for multiple adaptive radiations in different lakes in Africa, making it an exceptionally rewarding model system in speciation research (Turner 2007; Maan et al. 2016). Among them, the generalist species *Astatotilapia burtoni* (Günther 1893) that inhabits Lake Tanganyika and affluent rivers (Fig. 1) is an excellent model to study the early phases of adaptive divergence. Lake and river ecotypes of *A. burtoni* show habitat specific adaptations, despite varying levels of genetic differentiation among them (Theis et al. 2014; Egger et al. 2017; Pauquet et al. 2018). Adjacent lake and river environments differ in both abiotic and biotic conditions including water chemistry, habitat structure, and prey composition (Theis et al. 2014). River fish have shallower bodies, associated with the flow regime in the river habitat, whereas lake fish have a superior mouth position, longer gill rakers, and more slender lower pharyngeal jaw bones. The shifts in trophic structures correspond to different diets: while the lake ecotype feeds predominantly on plant/algae and zooplankton, the river ecotype preys upon snails, insects, and plant seeds (Theis et al. 2014).

Previous studies found no or weak reproductive isolation between genetically close lake and river populations ($F_{ST} < 0.06$; Egger et al. 2017) (Theis et al. 2014; Rajkov et al. 2018). Here, we investigate reproductive barriers between populations that exhibit strong genomic differentiation ($F_{ST} > 0.4$; Egger et al. 2017) to achieve a more general understanding of adaptive divergence in this system. We performed a mesocosm experiment in replicate lake-like environments to assess reproductive isolation between genetically divergent lake and river *A. burtoni* populations. Males and females from three different populations (local lake – sampled next to the experimental site, foreign lake, and foreign river – sampled at the opposite coast of Lake Tanganyika, see Fig. 1A) were placed in outdoor mesocosms and their surviving offspring were genotyped to assign parentage. We estimated fitness components and potential reproductive barriers acting at different stages, including survival, growth rate, mating success, F1 offspring survival, and fecundity. Our expectation was that, if there was strong local adaptation, the local lake population would perform the best in the mesocosms with lake-like environment, followed by the foreign lake population, and the foreign river population would show the lowest performance.

Material and Methods

STUDY SYSTEM

Astatotilapia burtoni (Fig. 1B) exhibits a lek-like polygynandrous mating system, where only dominant males gain access to territories and females (Fernald and Hirata 1977). Males are known to be highly aggressive toward conspecifics (Fernald 1980), and a size difference of even less than 10% body length has been shown to provide a significant advantage to the larger opponent in territorial combats (Alcazar et al. 2014). After spawning, females protect a brood of more than 30 developing eggs in their mouth for approximately two weeks and guard the fry for several weeks after releasing them (Fernald and Hirata 1979). Females typically do not feed during the entire period of mouthbrooding (Grone et al. 2012), and mouthbrooding is generally known to cause a loss of body mass in cichlids (Balshine-Earn 1995; Smith and Wooton 1995). Multiple paternity in *A. burtoni* has been detected in mate choice experiments under laboratory conditions in ~7% of genotyped broods (Theis et al. 2012). In the present study, we test for reproductive isolation between two lake populations and one river population. We used a lake population from the estuary of the Kalambo River (KaL) on the east coast of Lake Tanganyika, a lake population from the west coast (NdL), and a genetically divergent river population from the west coast – the Lufubu River (LfR) (referred to as Lf2 in Theis et al. 2014, 2017; Egger et al. 2017; Pauquet et al. 2018) (Fig. 1A).

STUDY DESIGN

The mesocosm experiment was designed to address pre- and postzygotic extrinsic and intrinsic forms of reproductive

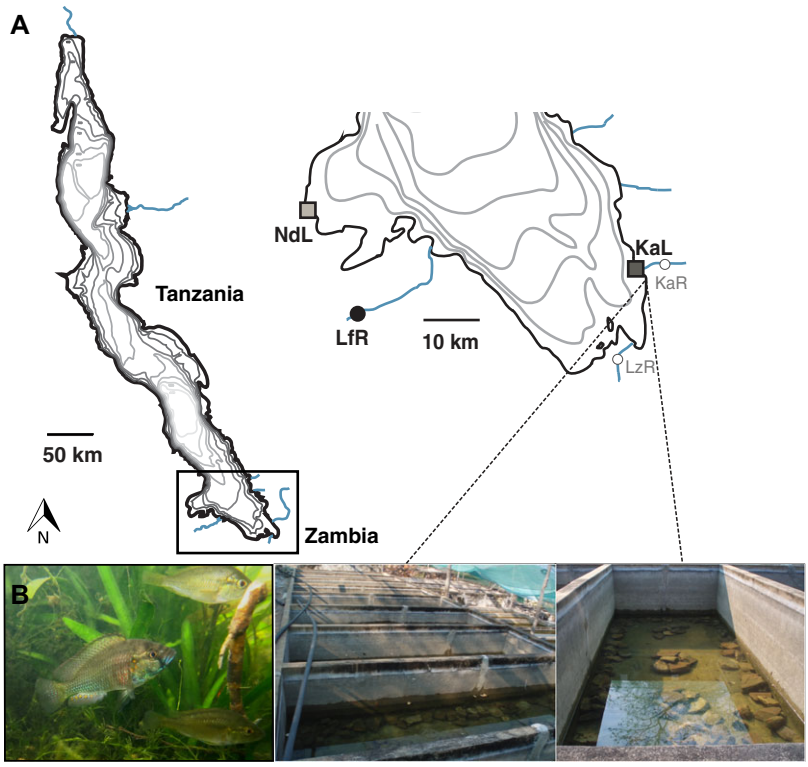

Figure 1. ***Astatotilapia burtoni*** **populations used in this study: KaL – Kalambo lake, NdL – Ndole lake, LfR – Lufubu river, and in previous studies: KaR – Kalambo river, LzR – Lunzua river (A); adult male and two females and location of the experimental mesocosms (B).**

isolation among the three populations. The experiment was carried out between November 2015 and August 2016 in six outdoor concrete ponds (mesocosms) (Fig. 1) at Kalambo Lodge, Zambia, near the location where the KaL population was sampled, under study permits nr. 003376, 004264, and 004266 issued by the Lake Tanganyika Research Unit, Department of Fisheries, Republic of Zambia.

Adult fish were caught at the source locations using hook and line fishing and kept in concrete ponds with lake water for ~10 days before the start of the experiment. After this acclimation period, wild-caught adults were anaesthetized with clove oil, photographed, measured (±0.5 mm), weighed (±5 mg), sexed by visual inspection of external coloration and the genital papilla, fin-clipped, and tagged with visible implant elastomer tags (VIE, Northwest Marine Technology). Each individual received a population tag (KaL–front left side of the dorsal fin, NdL–front right, LfR–middle right) to enable subsequent sorting, size matching, and counting of individuals. In all cases, individuals returned to normal activity within a few minutes after tagging. Males were selected for size to achieve a similar size distribution between the three populations within each mesocosm. Each mesocosm (dimensions: 3.2 m × 1.4 m × 0.5 m; length × width × water depth) was stocked with three females and three males from each of the three populations ($n_{\text{total}} = 108$). In mesocosm 6, one KaL male was wrongly sexed and one NdL female from mesocosm 4 was accidentally relocated during the experiment. This resulted in mesocosm 6 having two KaL females, four KaL males, and four NdL females at the end of the experiment. Numerous evenly distributed rocks provided territories for males and shelter for females and offspring. Our experimental setup included male–male competition, which represents the natural situation in species that live in social groups with strong dominance hierarchy. Ponds, located in the sun, were supplied with lake water. Algae cover formed on the walls and rocks over the course of the experiment, serving as a food source and mimicking the lake environment. Fish were fed with a supplement of commercial flake food that was not fed ad libitum (~0.3 g per pond per day) to ensure survival and a successful experiment. After a period of eight months, we emptied the mesocosms and collected all remaining adult fish (72 out of initially 108) and all surviving offspring and eggs. Fish weighing more than 1 g were photographed, measured, fin-clipped, and sexed if possible. Unfortunately, due to (i) logistic constrains imposed by the location in a remote area in Africa with no facilities that would enable construction of an experimental environment with flowing river water; and (ii) the presence of crocodiles and hippos in the riverine environment, no reciprocal control experiment in river environment could be performed. However, we can evaluate the effect of genetic differentiation between populations on reproductive isolation by using the previous experiment with a very similar setup performed in the same ponds with lake water

as a comparison (Theis et al. 2014). In this experiment the same lake population was used (KaL), together with two more closely related river populations from the Lunzua (LzR) and Kalambo (KaR) river.

PARENTAGE ANALYSES

Genomic DNA from incubated eggs and fin clips was extracted using a 5% Chelex solution (Casquet et al. 2012). The samples were genotyped at five microsatellite loci (Ppun5, Ppun7, Ppun21, UNH130, and Abur82) following the methods described in Theis et al. (2014). We genotyped all adults ($n_{\text{adult\_total}} = 108$ introduced + 73 surviving), all free-swimming juveniles and six individuals from each brood collected from mouthbrooding females. Some fry were expelled from the mouth during handling and those individuals were all genotyped (leading to some broods having more than six juveniles per brood genotyped) ($n_{\text{offspring\_total}} = 693$). Samples from all tagged individuals taken at the beginning and at the end of the experiment were matched using the R package Allelematch (Galpern et al. 2012) to identify the introduced adults. 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 counted as a single mating event, except if they belonged to different size classes (free-swimming young *vs.* fry). If more than one father was detected in broods collected from mouthbrooding females, these were treated as separate mating events. Mating patterns were inferred from parentage data by conservatively assuming a single mating event for each batch of full siblings of the same size class assigned to a particular parent pair.

BODY SHAPE

Photographs of the left side of each individual were taken using a Nikon D5000 camera, under standardized lighting conditions with a ruler for scale. To aid in digital landmark placement, we used three metal clips to spread the fins at the anterior insertions of the dorsal and anal fin, and at the insertion of the pectoral fin. We used geometric morphometrics to estimate body shape (Zelditch et al. 2004). In total, the photographs of 271 individuals (Table S1C) were used for geometric morphometric analyses. Using TPSDIG2 (v.2.26; Rohlf 2016) we placed 17 homologous landmarks on the image of each fish (Fig. S1). A tps file containing x and y coordinates was used as input for the program MORPHOJ (v.1.06d; Klingenberg 2011) and superimposed with a Procrustes generalized least squares fit (GLSF) algorithm to remove all nonshape variation (Rohlf and Slice 1990). Principal component analysis (PCA) and Canonical variate analysis (CVA, Mardia et al. 1979) were used to assess shape variation among different populations of introduced adults at the beginning and at the end of the experiment, and all types of offspring crosses. To correct for allometric size effects, the CVA was performed on the residuals of the regression of shape on centroid size. The statistical significance of pairwise differences in mean shape distances of CVA was obtained using permutation tests (10,000 permutations).

DATA ANALYSIS

Adults survival and growth rate

We assessed introduced adult survival among the three experimental populations using binomial generalized linear-mixed effect models (GLMMs) with survival as a binary-dependent variable and population, sex, initial standard length, size deviation (deviation in initial mass from the mean mass per mesocosm) as fixed predictors. The replicated mesocosms were set as a random effect. We calculated specific growth rates as $\text{SGR} = \frac{100}{\text{time}} \ln(\frac{m_{\text{final}}}{m_{\text{initial}}})$ for survivors. To correct for individual differences in mass at the beginning of the experiment, specific growth rates were regressed on initial mass. The residual SGR values (rSGR) were used as a measure of relative growth performance (following Barber 2005; Scharsack et al. 2007). We assessed growth rates among the three experimental populations using linear-mixed effect models (LMMs) with rSGR as a dependent variable, population, sex, and sex: population interaction as fixed predictors. The replicated mesocosms were set as a random effect. Since females often show signs of weight loss during mouthbrooding male and female growth rates were analyzed separately.

Reproductive success

We scored reproductive success among the three experimental populations via: male mating status as binary variable (mated *vs.* unmated) (i), proportion of the total number of mating events in each mesocosm per male, that is the proportion of broods completely or partly sired by a male (ii), and number of surviving offspring per mating event (iii). Male mating success was analyzed using binomial GLMMs with either mating status (i), or proportion of mating events (ii) as a dependent variable and population, size deviation (deviation in initial standard length from the mean male length per mesocosm), and male initial size as fixed predictors. The male identity and replicated mesocosms were set as random effects. The models were run using the complete dataset (including males that reproduced during the experiment but did not survive until the end of the experiment) and the survivors (all males that survived until the end of the experiment) only. We assessed offspring survival (iii) using GLMM with the number of surviving free-swimming offspring as a dependent variable and the type of cross (female population × male population), male initial size, and female initial size as fixed predictors. We used Poisson probability distribution for the count variable number of surviving offspring. Female identity, male identity, and replicated mesocosms were set as random effects.

GLMMs and LMMs were calculated using the R package lme4 (Bates et al. 2015). Significance level for the fixed effects

was determined by type II χ^2-based likelihood-ratio tests using the drop1 function of the lme4 package for GLMMs and with type II ANOVAs with Kenward–Roger correction for *F*-statistics and degrees of freedom (d.f.) using lmerTest package (Kuznetsova et al. 2017) for LMMs. We checked all GLMMs for overdispersion and included observation level as a random effect to account for the extravariance in the data, with one random effect level for each observation (male and female identity) (Harrison 2014). Tukey–Kramer post hoc tests were applied to test for significance of pairwise comparisons between populations using the lsmeans package (Lenth 2016). All statistical analyses were performed in R version 3.3.2 (R Core Team 2016). For detailed information on sample sizes see Table S1.

Results

ADULT SURVIVAL

Adult survival depended on population of origin and sex (population $\chi^2_{\text{d.f.}=2} = 31.745$, $P < 0.001$; sex $\chi^2_{\text{d.f.}=1} = 4.123$, $P = 0.042$, Fig. 2A, Table S2) and was higher in lake than in river fish (post hoc test: KaL – LfR, $P < 0.001$, NdL – LfR, $P < 0.001$, KaL – NdL, $P = 0.642$). When males and females were analyzed separately, population had an effect on male (population $\chi^2_{\text{d.f.}=2} = 24.075$, $P < 0.001$) and on female survival (population $\chi^2_{\text{d.f.}=2} = 11.396$, $P = 0.003$). Survival was higher in lake males than in river males (post hoc test: KaL – LfR, $P = 0.005$, NdL – LfR, $P = 0.006$), and in local lake females than in river females (post hoc test: KaL – LfR, $P = 0.010$). There was no difference in survival between lake males (post hoc test: KaL – NdL, $P = 0.996$), lake females (KaL – NdL, $P = 0.500$), and no significant difference between foreign lake and river females (NdL – LfR, $P = 0.096$).

ADULT GROWTH

When the whole dataset was analyzed, relative growth rate was affected by sex and population:sex interaction (sex $F_{1,63.1} = 540.270$, $P < 0.001$, population:sex $F_{1,63} = 18.040$, $P < 0.001$, Fig. 2B, Table S3). The interaction between population of origin and sex resulted from the inverse population growth patterns in males and females due to some lake females losing weight while mouthbrooding (Fig. S2), as they incubated more broods than river females (see below). When the sexes were analyzed separately, population of origin had an effect on growth in females (population $F_{2,24.8} = 25.429$, $P < 0.001$) and in males (population $F_{2,34.7} = 4.789$, $P = 0.015$). Local lake males grew faster than river males (post hoc test: KaL – LfR, $P = 0.009$), whereas river females grew faster than lake females (post hoc test: KaL – LfR, $P < 0.001$, NdL – LfR, $P < 0.001$) and foreign lake females grew faster than local lake females (post hoc test: KaL – NdL, $P < 0.044$). There was no significant difference in growth between foreign lake and river males (post hoc test: NdL – LfR, $P = 0.073$).

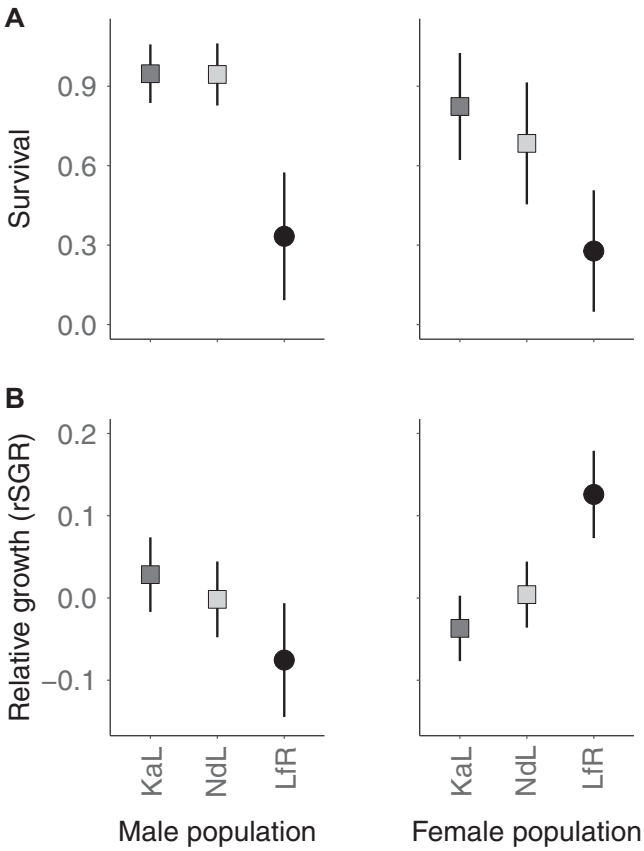

Figure 2. Survival (expressed as the average proportion of surviving individuals ± CI 95%) (A) and relative growth performance (least square means of the full models ± CI 95%) (B) of adults introduced in the mesocosms per population for males and females. Rectangles represent lake populations (KaL – Kalambo lake, NdL – Ndole lake) and circles river population (LfR – Lufubu river).

MATING EVENTS

Of the 693 genotyped offspring 80% (552) could be identified as F1 offspring of the introduced adults and were used to analyze mating patterns. The rest of the genotyped offspring were F2 or backcrosses with introduced adults (125) – collected as eggs from the mouth of F1 females, or unassigned (16). Due to the sharing of alleles between sibling F1 males, it was often not possible to unambiguously assign paternity for F2 offspring to a specific father with the five microsatellite loci used for parentage analyses. Therefore, in the remainder we discuss mating patterns inferred using the F1 offspring only.

Most females had mated with more than one male. When considering free-swimming F1 offspring only, 27 of 42 broods had multiple (up to four) fathers (64%; mean number of fathers per brood 2.0 ± 0.1 across all broods). When only genotyped (F1) eggs were considered, 16 of 25 broods had multiple (up to three)

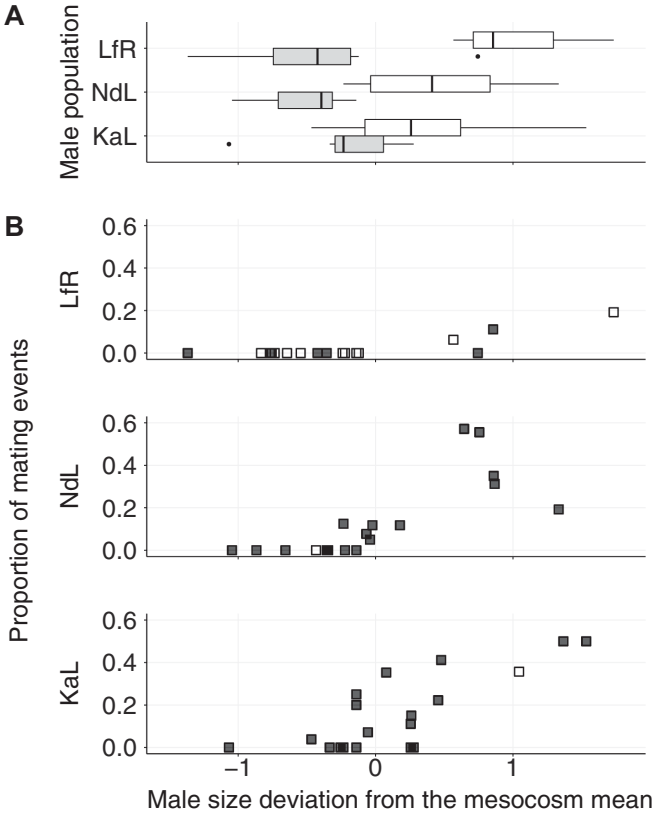

Figure 3. Male mating success depending on population and deviation in size (initial standard length) from the mesocosm mean. Mating status (white – mated, gray – unmated) (A) and number of mating events (percentage of mesocosm total) (filled signs – survivors, hollow signs – individuals that died) (B).

fathers (64%; 1.9 ± 0.8). As expected from a previous mesocosm experiment (Theis et al. 2014) there was higher variation in mating success of males than of females, with only 3–5 males mating per pond, and 6–8 females. All surviving females mated, except one river individual (Table S1). KaL females raised the highest number of broods (1.69 ± 0.13), followed by NdL (1.62 ± 0.14) and LfR females (1 ± 0.31). The highest number of mating events was detected between KaL males and KaL females (29), followed by other types of lake-lake crosses (19–21) and lake-river crosses (2–6) (Fig. S3A). We did not detect any mating events between a river male and a river female.

MALE MATING SUCCESS

Male mating success depended on male population and size deviation from the mean male size per mesocosm, both in terms of mating status (population $\chi^2_{\text{d.f.}=2} = 8.968$, $P = 0.011$; size deviation $\chi^2_{\text{d.f.}=1} = 12.477$, $P < 0.001$, Table S4, Fig. 3A) and with regard to the proportion of the total number of mating events (population $\chi^2_{\text{d.f.}=2} = 18.578$, $P < 0.001$; size deviation $\chi^2_{\text{d.f.}=1} = 19.837$, $P < 0.001$, Table S5, Fig. 3B). When survivors were analyzed separately, only size deviation had a significant effect on male mating success and the population effect was marginally significant (model2, Tables S4 and S5). For males that had mated ($n = 25$), population had the largest effect on the proportion of mating events (population $\chi^2_{\text{d.f.}=2} = 15.270$, $P < 0.001$; size deviation $\chi^2_{\text{d.f.}=1} = 11.761$, $P < 0.001$, Table S5).

OFFSPRING SURVIVAL

The number of surviving offspring per parent pair was calculated considering the free-swimming juveniles only. There was no asymmetry in the number of surviving hybrid offspring depending on which population was the mother or the father (model 1, post hoc test, all $P \geq 0.999$, Table S5, Fig. S4). Therefore, reciprocal crosses were subsequently pooled and the number of surviving offspring was analyzed with five levels of cross type instead of eight as an explanatory variable (model 2, Table S7). In both models the number of surviving offspring was explained by cross type (model 1: cross type $\chi^2_{\text{d.f.}=7} = 27.919$, $P < 0.001$; model 2: cross type $\chi^2_{\text{d.f.}=4} = 27.489$, $P < 0.001$, Table S7). The highest number of offspring per parent pair survived when both parents were from KaL (8.3 ± 1.6, max 29) followed by crosses where one parent was KaL and the other NdL (6.3 ± 1.1) and when both parents were from NdL (3.9 ± 1.1) (Fig. 4A). The lowest number of offspring survived when one of the parents was a river fish (LfR.NdL 2.2 ± 0.6, LfR.KaL 1.9 ± 0.4). There was a significant difference in number of surviving offspring between local lake cross and both types of lake-river crosses (post hoc test: KaL.KaL – KaL.LfR, $P < 0.001$; KaL.KaL – NdL.LfR, $P = 0.003$), local lake cross and foreign lake cross (KaL.KaL – NdL.NdL, $P = 0.023$) and lake-lake hybrids and both types of lake-river hybrids (KaL.NdL – KaL.LfR, $P = 0.001$; KaL.NdL – NdL.LfR, $P = 0.016$; Table S6).

BODY SHAPE

The CVA of body shape revealed a significant differentiation between groups (different populations of introduced adults at the beginning and at the end of the experiment, and all types of offspring crosses) and an overlap between both populations of lake adults at the end of the experiment, between both types of lake-river crosses, and between lake-lake hybrids and each of the pure parental lake crosses (Fig. 4B, Table S7). The first two CV axes explained 49% and 35% of the variance in the data, whereas the next eight axes together explained only 16% of the total variance. CV1, which described shape changes in terms of body height (Fig. S5), and CV2, which described shape changes in terms of mouth position (Fig. S5), separated river and lake populations. Whereas mean adult body shape at the end of the experiment overlapped for both lake populations, the river population displayed a distinct shape that did not converge to the lake body shape during the eight-month experiment. PCA showed

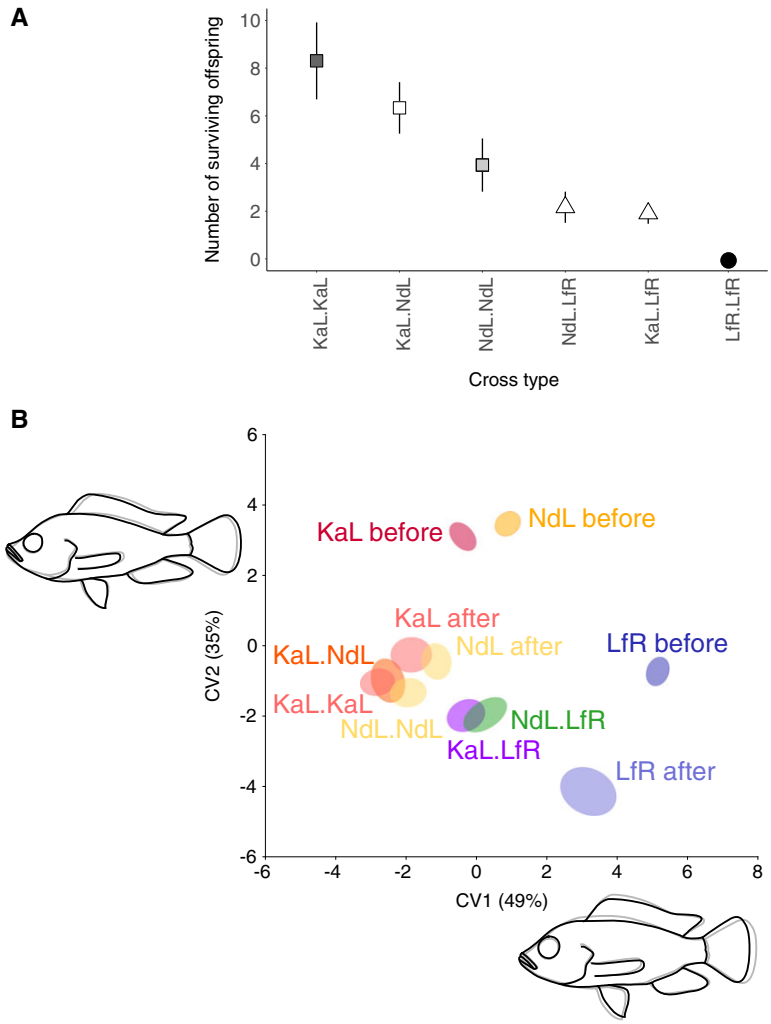

Figure 4. Mean numbers of surviving offspring per brood ± SE depending on the type of cross (A): dark gray – KaL.KaL, light gray – NdL.NdL, black - LfR.LfR, white – hybrids; rectangles represent pure lake crosses, triangles lake-river hybrids, and circle river-river cross. CVA of body shape (B), adult body shape changes during the experiment: red – Kalamabo lake (KaL), yellow – Ndole lake (NdL), blue – Lufubu river (LfR); offspring body shape: red – KaL.KaL, yellow – NdL.NdL, orange – KaL.NdL, purple — LfR.KaL, green — LfR.NdL. CV shape change outlines are for illustration purposes only, from gray to black outlines with increasing values, scaling factor 10 by default.

similar results (Fig. S6), where PC1, that described shape changes in terms of mouth and eye size, separated introduced adults from the offspring; PC2, that described shape changes in terms of head size, separated lake adults from river adults, and the offspring; and PC3, that described shape changes in terms of body height and mouth position, separated lake, and river adults.

Discussion

The goal of this study was to test for reproductive isolation between genetically divergent lake and river populations of *A. burtoni* under seminatural conditions and to evaluate the relative contributions of different reproductive barriers. Estimation of pre- and postzygotic extrinsic barriers revealed substantial reproductive isolation between the divergent Lufubu River population and the two lake populations. River fish suffered from immigrant inviability (in terms of lower adult survival and growth rate) and from lower mating success. Moreover, the complete lack of offspring from river parents and the very small number of surviving lake-river hybrid offspring further support immigrant inviability and extrinsic inviability of lake-river hybrids in the lake environment.

ENVIRONMENT DEPENDENT REPRODUCTIVE ISOLATION (IMMIGRANT INVIABILITY)

The experimental setup used in this study enabled us to simulate lake conditions and to follow the fitness of introduced fish at different stages. The mesocosms were supplied with lake water of the same chemical composition and provided similar food availability as what the local lake (KaL) population is normally exposed to. River and lake habitats substantially differ with

respect to water parameters (e.g., conductivity, pH) (Table S9) and in aquatic environments parasite risk often varies between lake and river environments (Scharsack et al. 2007), as well as between different locations within a lake (Raeymaekers et al. 2013). Moreover, the water parameters also differ between different locations in the lake, and in particular lake water close to the estuary of the Lufubu River has very different chemical properties–closer to the riverine than to the lacustrine environment. The algae cover on the rocks in the mesocosms made the mesocosms an even more suitable environment for the lake populations, which feed mostly on algae and plant material in the wild, unlike the river population that also feeds on macro-invertebrates (Theis et al. 2014). Taken together, all these factors likely contributed to the relatively higher fitness of the local lake population, followed by the foreign lake population and the river population, matching the predictions of the local adaptation hypothesis.

Similar to the results of a previous transplant experiment that compared fitness of wild-caught lake and river fish from the Kalambo river system in cages in the lake (Rajkov et al. 2018), we found lower fitness of river individuals in a lake-like environment. Moreover, the highest number of surviving F1 offspring of local lake parents, followed by the offspring of foreign lake parents, and a lack of offspring of foreign river parents, supports the role of immigrant inviability in reproductive isolation.

Adult growth rates showed opposite patterns in males and females. Local lake males grew the fastest, followed by the foreign lake males and the river males. The reverse pattern in females was likely due to longer total mouthbrooding duration in lake females that raised more broods than river females, leading to higher weight loss in lake females.

MATING PATTERNS

The results of the present study suggest that in general smaller males were outcompeted by larger dominant males (Fig. 3, Tables S4 and S5). In *A. burtoni*, size and dominance are positively correlated (Fernö 1987) and dominant males are much more likely to reproduce. However, some local KaL males succeeded to mate even when they were among the smallest in their mesocosm, and river males only succeeded to mate if they were very large (Fig. 3A), and even then participated only in a small proportion of mating events (Fig. 3B).

The high frequency of multiple paternity observed resembles estimates for other haplochromines from lakes Malawi and Victoria (Kellogg et al. 1995; Parker and Kornfield 1996; Maan et al. 2004; Tyers and Turner 2013) and is very similar to estimates from a similar experimental setup using the Lake Victoria haplochromine species *Pundmilia nyererei* (Maan et al. 2004) (64%; mean number of fathers per brood 2.0 ± 0.1 across all broods in this study *vs.* 68%; 1.8 ± 0.1).

EXTRINSIC POSTZYGOTIC ISOLATION

The only river female that was mouthbrooding when the experiment was terminated incubated 33 larvae with absorbed egg yolk (of which at least six were sired by a NdL male). This suggests that the fecundity of river females—in terms of the number of produced eggs—was probably not reduced. This goes against the argument of lower fecundity of river individuals in a foreign environment but in favor of lower survival of their offspring (extrinsic hybrid inviability). Furthermore, both types of hybrids (KaL.NdL and river.lake) show an intermediate performance in terms of survival, between the pure offspring of their parental types, supporting extrinsic postzygotic isolation (Hatfield and Schluter 1999). Likewise, a recent study in stickleback fish found extremely strong genetic effects on the relative survival and condition of the juveniles in a mesocosm experiment (Best et al. 2017).

BODY SHAPE

Data from this and the previous mesocosm experiment (Theis et al. 2014) demonstrated a clear separation between river and lake ecotypes along the CV that describes shape changes in terms of body height and mouth position (CV3, Fig. S7). However, unlike genetically close river populations that showed a high degree of plasticity (Rajkov et al. 2018) and converged towards the lake body shape in the mesocosms with standing lake water after six months (Theis et al. 2014), the body shape of adults from the genetically divergent LfR population did not change to the lake body shape after eight months under mesocosm conditions. This suggests less plasticity in body shape of LfR individuals compared to previously tested genetically closer river populations (Theis et al. 2014). Furthermore, the body shape of the LfR individuals could be one of the factors contributing to their low survival in a setup with standing lake water and algae as main food source. Other traits that were not investigated in this study, such as gut length, tooth morphology, and immune system in response to parasites could potentially also contribute to the observed differences in performance. Taken together, our data demonstrate that the genetically most divergent and putatively ancestral river population (Pauquet et al. 2018) has low fitness in the lake environment and is less plastic in comparison to other river populations that are more recently derived from adjacent lake populations (Egger et al. 2017).

NO INTRINSIC POSTZYGOTIC BARRIERS DETECTED

We detected F2 offspring from one male of KaL.LfR cross, suggesting that there is not complete hybrid sterility in lake-river hybrids, even between the most divergent populations. Nevertheless, with the data at hand, we cannot completely exclude that some intrinsic incompatibilities could also be reducing hybrid fitness. The apparent lack of intrinsic postzygotic isolation observed here is not surprising, as it is known to accumulate with time, and

very young species display little or no intrinsic postzygotic isolation (Bolnick and Near 2005; Mallet 2006; Schemske 2010). In cichlids, premating isolation accumulates fast initially but then changes little with increasing genetic distance between species (Stelkens et al. 2010). In contrast, intrinsic postzygotic isolation between closely related species is negligible but then accumulates relatively fast, resulting in complete hybrid inviability after 4.4–18.4 million years (Stelkens et al. 2010).

STRENGTH OF REPRODUCTIVE ISOLATION CORRESPONDS TO GENETIC DIVERGENCE

As data on reproductive isolation for *A. burtoni* lake-river pairs begin to accumulate (Theis et al. 2014; Rajkov et al. 2018; this study) it becomes possible to compare the importance of different barriers and the levels of reproductive isolation between lake-river population pairs with different levels of genetic divergence. These experiments used population pairs from different lake-river systems for which estimated divergence times vary from ∼13,000 (Kalambo lake-river pair) to ∼180,000 years (Lufubu lake-river pair) (Egger et al. 2017). While it is admittedly difficult to compare the results of different experiments, it is still possible to draw some general conclusions, as some of the experiments were performed in a similar setup. In a previous mesocosm experiment that used the same mesocosms and tested genetically closer populations from Kalambo and Lunzua rivers, no difference in survival of introduced adults or F1 offspring was detected, and all possible mating combinations occurred (Theis et al. 2014). In addition, the differences in survival and growth rate of introduced adults between the two lake populations and the foreign river population observed in the present study were much higher than the differences observed using wild-caught juvenile fish from two genetically closer populations from the Kalambo River system in a transplant experiment (Rajkov et al. 2018). Furthermore, lake-river F1 hybrids from the Kalambo River system showed equally high fitness as the pure crosses in the lake environment (Rajkov et al. 2018). Taken together, these results suggest that across all investigated population pairs both prezygotic and postzygotic isolation increase with genetic distance between lake and river populations.

RELATIVE IMPORTANCE OF DIFFERENT BARRIERS

Reproductive barriers that first come into play early in life history are of particular importance as subsequent barriers can only prevent gene flow that remains after the effects of earlier-acting barriers (Sobel et al. 2010). As immigrant inviability (prezygotic) acts earlier in the ontogeny than hybrid inviability (postzygotic), it likely has a greater relative contribution to limiting gene flow, ultimately leading to speciation. In speciation driven by divergent selection, extrinsic postzygotic, and prezygotic barriers evolve first and frequently interact to mediate reproductive isolation, and intrinsic postzygotic barriers usually evolve later in the speciation process (Seehausen et al. 2014). In cichlids, prezygotic behavioural reproductive barriers, in particular female preference for male coloration, are known to be an important component of reproductive isolation (Kocher 2004; Kraaijeveld and Pomiankowski 2004; Maan and Sefc 2013; Selz et al. 2014).

A. burtoni lake-river populations represent the first cichlid model that allows direct comparison to what is known from probably the best-studied speciation continuum in fish—the stickleback lake-river system (McKinnon and Rundle 2002; Berner et al. 2009; Kaeuffer et al. 2011; Lucek et al. 2013; Stuart et al. 2017). In stickleback, premating isolation evolves before postmating isolation, and extrinsic isolation is far stronger than intrinsic isolation (Hatfield and Schluter 1999; Vamosi and Schluter 2009; Lackey and Boughman 2016). Here, we detected extrinsic barriers in a cichlid species that act before and after zygote formation and include selection against immigrants and their offspring. Importantly, 111 detected mating events did not result in a single surviving pure river F1 individual. All analyzed fitness parameters suggest that local adaptation to the lake environment contributed to the higher performance of the lake ecotype.

LIMITATIONS

Unlike some laboratory experimental setups such as the "partial partition method" (Turner et al. 2001) that allow for female choice to be investigated independent of male competition, our experimental setup did not allow us to distinguish between female choice and male dominance. However, our setup is closer to the situation in nature, where female *A. burtoni* are surrounded by dominant males, and thus we are confident that this kind of setup provides us with more accurate information with respect to the barriers that are actually important for reproductive isolation in nature. Our experimental design also does not enable detection of mating events with no surviving offspring and thus did not allow us to differentiate whether the lack of offspring from specific individuals resulted from a failure of fish to spawn or through embryo and juvenile mortality. Furthermore, high mortality of the river population decreased the power of our analysis of their mating success. However, studying all the reproductive barriers acting sequentially in a semi-natural setup enabled us to accurately estimate the cumulative effect of all the investigated barriers–on the basis of the total number of surviving free swimming F1 juveniles from each cross type (Fig. S2B).

CONCLUSION

We tested river and lake ecotypes of *A. burtoni* for reproductive barriers that reduce gene flow between them. This is one of the very few studies investigating environment dependent (extrinsic) components of reproductive isolation in cichlids. We found strong prezygotic (selection against immigrants) and postzygotic

barriers (selection against hybrids) in line with local adaptation. As prezygotic barriers are not complete, postzygotic barriers cause a substantial reduction in gene flow between ecotypes. Our results highlight the value of assessing multiple reproductive barriers acting at different stages in natural contexts as well as the importance of postzygotic barriers in addition to prezygotic barriers even during the early stages of speciation. Future studies should try to disentangle the role of female mate choice *vs*. male competition in this system, as well as early inviability *vs.* missed mating opportunities using controlled laboratory setups.

AUTHOR CONTRIBUTIONS

J.R., W.S. and B.E. conceived the study; all coauthors contributed to the experimental design; J.R., A.A.T.W., and B.E. conducted the fieldwork; J.R. conducted the molecular laboratory work, analyzed the data, and wrote the article, with feedback from all coauthors.

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DATA ARCHIVING

The doi for our data is https://doi.org/10.5061/dryad.5fn863j.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Position of 17 landmarks used for body shape analysis.

Figure S2. Absolute growth rates (mg/day) of adults introduced in the mesocosms per population: males (a) and females (b): dark grey – Kalambo lake (KaL), light grey – Ndole lake (NdL), black – Lufubu river (LfR); and photos of typical female from each population at the end of the experiment (c) showing signs of weight loss in mouthbrooding lake females.

Figure S3. Total number of detected mating events (a) and total number of surviving free-swimming F1 juveniles (b) from all replicated mesocosms per type of cross (female population. male population): KaL – Kalambo lake, NdL – Ndole lake, LfR – Lufubu river; dark grey – KaL.KaL, light grey – NdL.NdL, white – hybrid crosses.

Figure S4. Mean numbers of surviving offspring per brood ± SE per type of cross (female population. male population): KaL – Kalambo lake, NdL – Ndole lake, LfR – Lufubu river; dark grey – KaL.KaL, light grey – NdL.NdL, black – LfR.LfR, white – hybrid crosses.

Figure S5. Body shape transformation grids from Canonical variate analysis (CVA) along the CV1 and CV2 axes presented in Figure 4.

Figure S6. PCA of body shape. Adult body shape changes during the experiment: red – Kalamabo lake (KaL), yellow – Ndole lake (NdL), blue – Lufubu river (LfR); offspring body shape: red – KaL.KaL, yellow – NdL.NdL, orange – KaL.NdL, purple - LfR.KaL, green - LfR.NdL.

Figure S7. CVA of body shape from this (2016) and previous experiment (2014) described in Theis et al. 2014 that included the same Kalabo lake population (KaL) as well as two populations from the Kalambo (KaR) and the Lunzua (LzR) River.

Table S1. Sample size details.

Table S2. Generalized linear mixed models of survival of adult *A. burtoni* introduced to the mesocosms.

Table S3. Analyses of variance tables of mixed effect models on relative growth (rSGR).

Table S4. Generalized linear mixed models of mating status (1: mated, 0: unmated) of male *A. burtoni* introduced to the mesocosms.

Table S5. Generalized linear mixed models of mating success (proportion of all mating events in the mesocosm) of male *A. burtoni* introduced to the mesocosms.

Table S6. Results of Tukey-Kramer *post hoc* tests on the least square means of the full models for number of surviving offspring per pair from Table S7.

Table S7. Generalized linear mixed models of reproductive success (number of surviving free-swimming F1 offspring) of *A. burtoni* introduced to the mesocosms.

Table S8. Pairwise body shape differentiation among groups: Procrustes (upper triangular matrix) and Mahalanobis (lower triangular matrix) distances from the CVA (Fig. 4b): b – before the experiment, a – after the experiment.

Table S9. Environmental data for lake and river *A. burtoni* sampling sites.