Naturally and sexually selected traits in haplochromine cichlid fishes

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Introduction

Humankind seeks for explanations to describe the evolution of the astonishing biodiversity surrounding us. To understand organismal diversity, we first need to understand the evolutionary processes underlying it. However, we are still struggling with Darwin's 'mystery of mysteries' (Darwin 1859), that is to understand how and why new species form (Coyne & Orr 2004). The establishment of reproductive isolation between divergent populations can evolve through barriers in post- (Snook et al. 2009) and pre-copulatory sexual selection (Darwin 1871). The two fundamental modes of Darwinian sexual selection are contests for mates (intrasexual selection) and mate choice by the opposite sex (intersexual selection) (Darwin 1871). Even though reproductive isolation could arise through sexual selection alone, it was hypothesized that it functions most effectively in conjunction with selection for species recognition or ecological selection (Ritchie 2007). Speciation through ecological selection drives adaptive diversification into a variety of ecological niches, which is described as 'adaptive radiation' in evolutionary groups that have exhibited exceptional extent of diversification (Schluter 2000).

A textbook example of adaptive radiations and, therefore, an ideal system to study diversification are the perciform fishes of the family Cichlidae (e.g. Maan et al. 2006; Seehausen et al. 2008; Salzburger 2009). Their rapid speciation resulted in an estimated number of around 3'000 species (Snoeks 1994; Turner et al. 2001), turning cichlids into the most species-rich family of vertebrates (Salzburger & Meyer 2004; Salzburger 2009). Cichlids are distributed across South and Central America, Africa and parts of India. This distribution suggests a Gondwanian origin of the group (Salzburger 2009). Their centre of diversity, however, lies in the East African Great Lakes, which harbour extremely diverse and species-rich flocks of cichlid fishes and are therefore a prime model system in evolutionary biology (Meyer 1993; Turner et al. 2001; Seehausen 2006). In addition to the extrinsic environmental factors such as geologic and climatic events creating novel ecological niches (Fryer & Iles 1972; Sturmbauer 1998; Sturmbauer et al. 2001), several evolutionary key innovations have been hypothesized to have played a role in their rapid speciation and adaptations to a variety of ecological niches. Of particular importance are the special pharyngeal jaw apparatus (Fryer & Iles 1972; Liem 1973), the highly complex reproductive behaviour (Fryer & Iles 1972; Goodwin et al. 1998; Kornfield & Smith 2000) and the wealth of colour morphs. It was shown that colour and pigmentation patterns seem to play a central role in the explosively radiating cichlid fish lineages in the East African Great Lakes in general, and in haplochromine cichlids in particular (Seehausen et al. 1999; Kocher 2004; Turner 2007; Salzburger 2009). Haplochromines comprise about 80% of East African cichlid species including the entire species flocks of lakes Victoria and Malawi, the tribe Tropheini from Lake Tanganyika and many riverine species (e.g. Turner et al. 2001; Salzburger et al. 2005). Interestingly, all haplochromines are maternal mouthbrooders, with females incubating their offspring – until fully developed – in their buccal cavities (e.g. Fryer & Iles 1972; Salzburger et al. 2005). This special breeding behaviour evolved several times during cichlid evolution (Goodwin et al. 1998), but only the 'modern haplochromines' show a derived polygynous or polygynandrous maternal mouthbrooding system with males displaying the so-called egg-spots on their anal fins (Fryer & Iles 1972; Salzburger et

al. 2005, 2007).

These ovoid markings consist of a transparent outer ring encircling a brightly coloured yellow, orange or reddish centre (Wickler 1962; Fryer & Iles 1972). The conspicuous central area is formed by two chromatophore cell types, xanthophores and iridophores (Salzburger et al. 2007; Santos et al. 2014). Even though this trait is proposed to be a putative key innovation mediating the evolutionary success of haplochromines (Salzburger et al. 2005; Salzburger 2009), their function is not fully understood. Several hypotheses exist that seek to explain the function of egg-spots: Wickler (1962) associated the function of egg-spots with the special mouthbrooding behaviour, and suggested that egg-spots mimic real eggs and function as an attracting signal during courtship and as releasers for egg-uptake and, hence, to maximize fertilization. Support for Wickler's hypothesis was only found with respect to the function in courtship since females of the species *Astatotilapia elegans* and *Pseudotropheus (Maylandia) aurora* preferred to lay batches with males with many egg-spots (Hert 1989, 1991), whereas females of *Pseudotropheus (Maylandia) lombardoi* preferably chose males with an artificially enlarged egg-spot over males with one natural or many egg-spots (Couldridge 2002). However, there was no influence of egg-spots on fertilization rate (Hert 1989). Further doubts about the egg mimicry hypothesis arose because egg-spots often do not resemble size, shape and colour of a species' actual eggs (Jackson & van Lier Ribbink 1975; Goldschmidt 1991). This mismatch between real eggs and egg-spots may be due to a trade-off between attractiveness towards females and conspicuousness for predators (Goldschmidt 1991). An alternative explanation could be that egg-spots serve as species recognition signal (Axelrod & Burgess 1973).

So far, the results from studies that aimed to evaluate the function and selection pressures on egg-spots are scarce, rather inconsistent and raise the necessity for new experimental work on their mode of action and their evolutionary origin. **Part 1** of my thesis is therefore dedicated to the **evolution and function of egg-spots**. The first manuscript focuses on the evolutionary origin of anal fin egg-spots, more specifically, we tested the hypothesis whether **a sensory bias has triggered the evolution of egg-spots in cichlid fishes (1.1)**. Mate choice trials were conducted to see if females of the basal haplochromine *Pseudocrenilabrus multicolor* (naturally showing no true egg-spot on its anal fin) prefer computer-animated photographs of males with an artificially added egg-spot. Additionally, colour preferences (outside a mating context) were tested in a phylogenetically representative set of East African cichlids.

The next two chapters focus on the putative function of egg-spots in sexual selection in the two haplochromine species *Astatotilapia burtoni* **(1.2 The function of anal fin egg-spots in the cichlid fish *Astatotilapia burtoni*)** and *Astatotilapia calliptera* **(1.3 Egg-spot pattern and body size asymmetries influence male aggression in haplochromine cichlid fishes)**, which both exhibit several egg-spots on their anal fin. In both species, mate choice trials were conducted to test if females prefer to lay eggs with males with many egg-spots over males with fewer or no egg-spots. Since carotenoid based colouration can be indicative for the health and strength of its bearer (e.g. Endler 1978, 1980; Hill 1992), egg-spots are

Figure 1 *Astatotilapia burtoni* female (left) and male (right), showing the egg-spots on its anal fin.

also a prime example to examine if there is a function in intrasexual selection. Therefore, male aggression experiments were conducted in both species to test if egg-spots could play a role in the assessment of an opponent's strength.

Visual signals will most probably not only diverge due to sexual selection, but might be influenced by their environment and are therefore expected to evolve to a point where viability costs balance mating advantage (Darwin 1871; Zahavi 1975; Endler 1978; Andersson 1994). To examine how the egg-spot phenotype can be influenced by sexual and ecological selection, the next manuscript examines the **variation of anal fin egg-spots along an environmental gradient in a haplochromine cichlid fish (2.1)**. This project constitutes the first of two studies of **Part 2** describing adaptive **divergence in lake-stream systems** in *A. burtoni*. This species represents an ideal model organism to address questions about adaptive divergence in lake-stream systems in cichlids, since *A. burtoni* is one of only few cichlid species, which inhabits shallow zones of one of the East African Great Lakes as well as rivers and streams surrounding it (Fernald & Hirata 1977; Kullander & Roberts 2011). Populations of lacustrine and riverine habitats of four lake-stream systems were examined with regards to sex- and habitat-specific differences in egg-spot characteristics such as number, size and colouration. Finally, we tested for an association between the conspicuousness of male egg-spots and underwater light environment as well as the status of the immune system.

However, not only visual signals - like egg-spots - can adapt to the respective environmental conditions, but lake-stream systems are also a unique system to study how populations experiencing different environmental conditions may diverge in general. So far, adaptive divergence in cichlids has mainly been investigated within lakes, e.g. along depth or habitat gradients (see e.g. Barluenga et al. 2006; Seehausen et al. 2008). The *A. burtoni* setting should therefore be established as the first lake-stream system in cichlids, which is described in the second study of Part 2 **(2.2 Adaptive divergence between lake and stream populations of an East African cichlid fish)**. Here, we first established phylogeographic relationships and assessed the population structure as well as body shape differences in over 20 *A. burtoni* populations from the southern part of Lake Tanganyika. In a second step, we focused on four lake-stream systems in detail (the same systems as in chapter 2.1) and, in addition to the body shape and population-genetic surveys, we quantified other ecologically relevant traits (gill raker and lower pharyngeal jaw) as well as stomach contents. To test whether the shifts in the examined traits reflect ecologically based adaptive divergence (Berner et al. 2009; Harrod et al. 2010), we tested for an association between morphological variation and environmental factors, such as resource use and water velocity. Finally, a mating experiment was conducted to test for reproductive isolation among lake and stream populations. Adults and offspring from this common garden setting were further used to evaluate levels of phenotypic plasticity in the traits body shape and gill raker morphology.

During the sampling trips for the study mentioned above, we observed a clear-cut barrier for the occurrence of *A. burtoni* in the streams surrounding Lake Tanganyika. At a certain elevation *A. burtoni* was absent and seemed to be replaced by another riverine cichlid, namely a species of the *Pseudocrenilabrus philander* complex. Interestingly, they both were found to co-occur in Lake Chila, a small lake 20 km south of Lake Tanganyika. The first **side project** of **Part 3** concentrates on this *P. philander* complex with the manuscript about the **phylogeographic and phenotypic assessment of a basal haplochromine cichlid fish from Lake Chila, Zambia (3.1)**. Here we report the discovery of a population of the normally riverine *P. cf. philander* in Lake Chila. We examined this lake population for increased morphological variation compared to riverine populations of *P. cf. philander*. With this dataset we wanted to test whether ecological opportunity in the form of a greater number and more diverse ecological niches promotes diversification in lakes compared to rivers (as seen in e.g. Stelkens & Seehausen 2009). The phenotypic variability of this Lake Chila population was evaluated in relation to other lacustrine and riverine populations by quantifying colouration and body shape. Additionally, phylogeographic history was investigated with attention to a case of hybridization of two distinct lineages.

The second side project focuses on a special case of morphological variation, namely mouth

asymmetry, by performing a field based assessment of attack strategy and feeding success in the scale-eating cichlid fish *Perissodus microlepis* **(3.2 A fitness benefit for mouth dimorphism in a scale-eating cichlid fish)**. *Perissodus microlepis* is the most common and perhaps the most specialized lepidophagous cichlid in Lake Tanganyika (Takahashi et al. 2007) and exhibits a pronounced asymmetry with individuals that feature a mouth slightly bent to the right or to the left side in order to optimize feeding successes (Hori 1993). In this study the lateralisation dynamics in *P. microlepis* were reassessed in a semi-natural environment in order to confirm laboratory based findings about asymmetrical attack strategies and to test if dimorphic experimental populations of *P. microlepis* ultimately are more successful and show a higher feeding success than monomorphic experimental populations. All together, we aimed to disentangle causalities in the evolution of this system and to demonstrate the selective advantage of dimorphic mouth opening and attack strategy in scale-eaters. This is necessary to explain how such asymmetries have evolved and can be maintained in natural populations.

In summary, my thesis consists of two main parts and a third part comprising two side projects. **Part 1** investigates the trait egg-spots, which were mentioned to be a key innovation of haplochromines, the most species-rich tribe of cichlids. Three manuscripts deal with their mode of action as well as their evolutionary origin. **Part 2** examines the divergence among lake and stream populations with respect to egg-spots and in a second project with respect to body shape and other ecologically relevant traits. Additionally, the phylogeographic relationships of *A. burtoni* populations from the southern part of Lake Tanganyika were established.

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Part 1

evolution and function of egg-spots

1.1 A sensory bias has triggered the evolution of egg-spots in cichlid fishes

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This project was part of the master thesis of YK. I helped planning the experiment, conducting the fieldwork, analysing the data and was also involved in writing and discussing of the manuscript.

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A Sensory Bias Has Triggered the Evolution of Egg-Spots in Cichlid Fishes

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Abstract

Although, generally, the origin of sex-limited traits remains elusive, the sensory exploitation hypothesis provides an explanation for the evolution of male sexual signals. Anal fin egg-spots are such a male sexual signal and a key characteristic of the most species-rich group of cichlid fishes, the haplochromines. Males of about 1500 mouth-brooding species utilize these conspicuous egg-dummies during courtship – apparently to attract females and to maximize fertilization success. Here we test the hypothesis that the evolution of haplochromine egg-spots was triggered by a pre-existing bias for eggs or egg-like coloration. To this end, we performed mate-choice experiments in the basal haplochromine Pseudocrenilabrus multicolor, which manifests the plesiomorphic character-state of an egg-spot-less anal fin. Experiments using computer-animated photographs of males indeed revealed that females prefer images of males with virtual ('in-silico') egg-spots over images showing unaltered males. In addition, we tested for color preferences (outside a mating context) in a phylogenetically representative set of East African cichlids. We uncovered a strong preference for yellow, orange or reddish spots in all haplochromines tested and, importantly, also in most other species representing more basal lines. This pre-existing female sensory bias points towards high-quality (carotenoids-enriched) food suggesting that it is adaptive.

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Introduction

The haplochromines are the most famous and diverse group of cichlid fishes and widely distributed in Africa. Yet, their center of diversity is located in East Africa, where they constitute, for example, the entire cichlid species flocks of lakes Victoria and Malawi [1,2,3]. The actual species count for haplochromines remains unknown, although it is assumed that at least 1500 species are teeming in the lakes and rivers of East Africa [4,5]. Save a small number of species, all haplochromines exhibit so-called egg-spots, making this trait *the* characteristic feature of haplochromines and a putative key innovation mediating their evolutionary success [1,4]. The exceptions are several derived species that have lost egg-spots secondarily and a few basal species that presumably never had them [1].

Genuine ('true') egg-spots are found on male anal fins and consist of a conspicuous yellow, orange, or reddish inner circle and a transparent outer ring (Figure 1) [3,6,7]. This makes them a costly trait, as fish cannot synthesize carotenoid-based pigments themselves [8,9]. Egg-spots appear to resemble real eggs, which is why it has been proposed that these markings are 'dummies' that mimic freshly laid eggs in order to attract females and to maximize fertilization success [6,7]. All haplochromines are female mouth-brooders, which means that females incubate their offspring – until fully developed – in their oral cavities. Immediately upon spawning, a haplochromine female takes up the eggs into her mouth; the territorial male instantly presents his anal fin egg-spots, to which the female responds in form of snatching, thereby positioning her mouth close to the males' genital papilla that discharges sperm. Wickler's egg mimicry hypothesis [6,7] is disputed, however, as egg-spots often do not resemble size, shape and color of a species' actual eggs (see [10]). Also, it has been shown that fertilization success did not vanish when egg-spots were removed artificially [11,12].

Here, we focus on the evolutionary origin of anal fin egg-spots rather than on their immediate function. More specifically, we test the hypothesis that the exploitation of a pre-existing bias has triggered the evolution of this conspicuous male trait in haplochromine cichlids [10]. The evolutionary origin of sexual signals is largely unknown and a matter of debate [14]. It is commonly accepted, however, that male signals can evolve in response to pre-existing sensory biases in females ('sensory exploitation hypothesis') [13,14,15,16,17,18]. Such a female sensory bias may well be *adaptive*, namely if it evolved in another context than mating and through natural rather than through sexual selection [14,17,18]. Male guppies, for example, seem to mimic fruits that are a valuable food source and females are attracted by both males displaying the trait and by objects with respective colors [19]. Male swordtail characins, on the other hand, possess extended and pigmented opercular paddles that resemble invertebrate prey organisms [20]. Computer simulations also revealed that – at least under some circumstances – foraging preferences may result in increased mating preferences for similarly colored mates [21]. It has further been shown that disruptive female preferences in three-spine sticklebacks

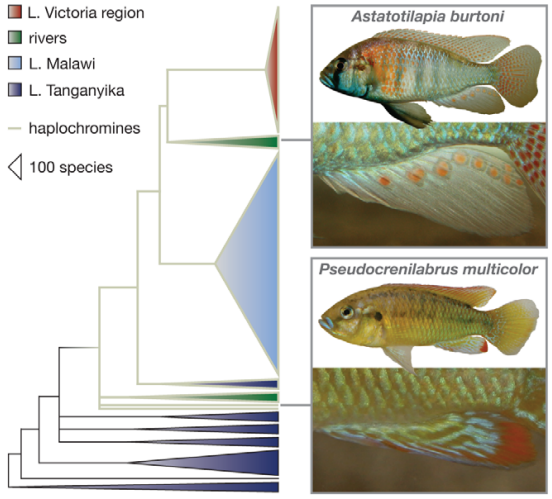

Figure 1. Schematic consensus phylogeny of the East African cichlids based on mitochondrial and nuclear gene segments (after [1,25,36]). The haplochromines (indicated by grey branches) are a derived and species-rich clade. The males of most haplochromine species display anal fin egg-spots, just as exemplified here for *Astatotilapia burtoni*. A few ancestral species, such as *Pseudocrenilabrus multicolor*, do not have egg-spots. Note that *A. burtoni* belongs to a riverine clade and occurs within Lake Tanganyika and surrounding rivers.
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are linked to the visual systems' adaptation to different light regimes [22]. A similar case of 'sensory drive speciation' is reported from Lake Victoria haplochromines, where adaptations to different turbidity levels mediate female mate choice [23]. Finally, a preference for males with elaborated ornaments could also be adaptive in situations where males must ingest carotenoids to display these colors (e.g. [24]).

We find that females of a basal and egg-spot less haplochromine species prefer males with artificial ('*in-silico*') egg-spots and that haplochromines and more basal and non-mouth-brooding cichlid lines prefer color dots resembling egg-spots.

Results

Laboratory mate choice trials

We first tested whether females of the basal and egg-spot-less haplochromine cichlid *Pseudocrenilabrus multicolor* (Figure 1) could discriminate between males of their own species and males of another, more derived and egg-spot bearing haplochromine (*Astatotilapia burtoni*), when presented animated images on a computer screen in front of an experimental tank (Figure 2A). We found that focal females spent significantly more time and interacted significantly more often with the animation showing the conspecific male (related sample t-test; time spent: $N = 12$; $t = 3.13$; $df = 11$; $p<0.01$; number of reactions: $N = 12$; $t = 4.72$; $df = 11$; $p<0.001$; reaction time: $N = 12$; $t = 6.06$; $df = 11$; $p<0.001$) (see Figure 2B; Movie S1). Apart from demonstrating the females' ability to recognize conspecifics, this experiment highlights the usefulness of computer animations in female mate choice experiments with *P. multicolor*.

Pseudocrenilabrus multicolor females did not discriminate between animated images of males and such in which the red fin-fringe had been painted *in- silico* with the anal fin's brownish ground color (related sample t-test; time spent: $N = 15$; $t = -0.17$; $df = 14$; $p = 0.87$; number of reactions: $N = 15$; $t = 0.38$; $df = 14$; $p = 0.71$; reaction time: $N = 15$; $t = 0.38$; $df = 14$; $p = 0.71$; Figure 2C), suggesting that females are not advertent to the red fringe of male anal fins when choosing a mate. We confirmed this using live fish and a two-way choice set-up (time spent: related sample t-test; $N = 15$; $t = 0.04$; $df = 14$; $p = 0.97$; number of interactions: Wilcoxon signed-rank test; $N = 15$; $V = 65$; $p = 0.78$; interaction time: related sample paired t-test; $N = 15$; $t = 0.05$; $df = 14$; $p = 0.96$). This demonstrates that preference tests using computer animations reveal results congruent to mate choice experiments with live fish.

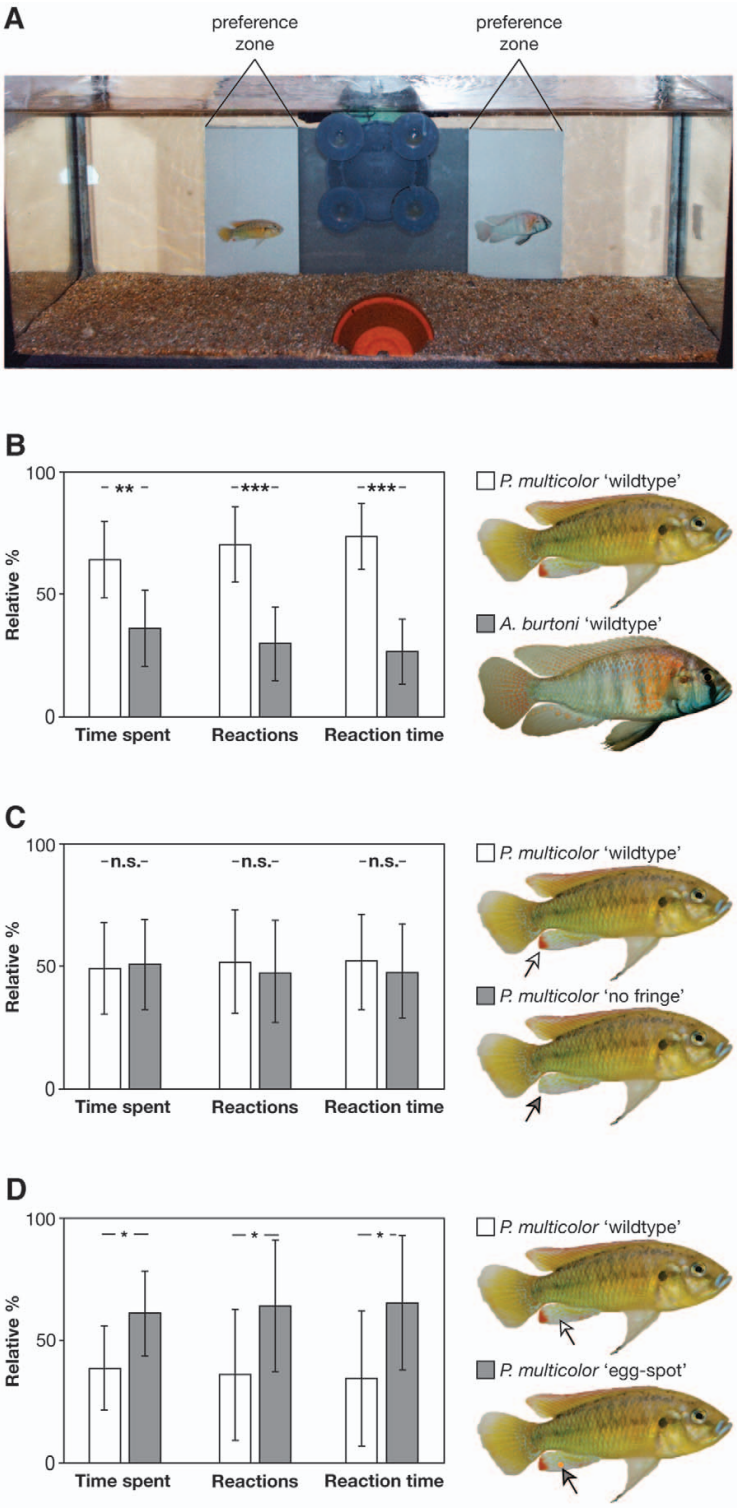
We found, however, that focal females spent significantly more time in front of the image of a male with the artificial egg-spot (Wilcoxon signed-rank test; $N = 20$; $V = 41$; $p = 0.015$); females also reacted more often with the egg-spot bearing male by following its animated movements (related sample t-test; $N = 20$; $t = -2.35$; $df = 19$; $p = 0.029$); and, *P. multicolor* females spent more time reacting with the image of a modified male (Wilcoxon signed-rank test; $N = 20$; $V = 42.5$; $p = 0.020$) (Figure 2D). This clearly indicates that females of an ancestral haplochromine species show a preference for males with the derived character state of egg-spots, which is suggestive for the existence of a pre-existing bias for orange spots.

Color-dot preference tests

In our color-dot experiments in the field, all four tested haplochromine species showed a strong preference for yellow, orange or red dots (Tables S1, S2). Importantly, most other species belonging to basal cichlid lineages did so, too, and only three species showed a weak (*C. frontosa* and *C. leptosoma*) or strong (*O. nasuta*) preference for green. Notably, *C. frontosa* reacted almost as often to orange dots (29 times) as it did to green ones (30 times); a similar situation was observed for *C. leptosoma* between yellow (8 times) and green (11 times). For both species, a clear preference could thus not be determined. Also, with only 20 pecks each in a period of five minutes, *C. leptosoma* and *O. nasuta* showed the by far smallest number of pecks, questioning the strength of their preference for a particular color. In any case, a character state reconstruction on the basis of a molecular phylogeny (Figure 3C) clearly indicates that the preference for red dots existed before the evolution of haplochromines, irrespective of how we coded the preference for *C. frontosa*, *C. leptosoma* and *O. nasuta* (indecisive, orange or green, yellow or green).

In the laboratory experiments using computer animated color dots (Figure 3B, D–F), we detected a non-random distribution of color preferences in all three species tested (Friedman test; *A. burtoni*, $N = 20$; $p<0.001$; *P. multicolor*, $N = 20$; $p<0.001$; *J. marlieri*, $N = 20$; $p<0.001$).

In line with our color preference experiments in the field, all three species showed a preference for egg-spot like colors (yellow, orange and red), while blue and green were hardly ever chosen (Figure 3D–F, Table S3). Importantly, *A. burtoni*, which is the only species that we could test both in the field and in the lab, showed highly congruent responses to the stationary color dots in the pond set-up and the animated color dots in the laboratory experiments. Interestingly our lab experiments uncovered sex-specific differences in the haplochromines: *A. burtoni* females significantly more often pecked at and followed the orange-colored dots (Wilcoxon rank-sum test; $N = 20$; $p = 0.037$) and *P. multicolor* females significantly more often pecked at and followed the red-colored dots than did the males (Wilcoxon rank-sum test; $N = 20$; $p = 0.045$), while *P. multicolor* males reacted more often to yellow


A
preference zone
preference zone
B
100
50
0
Relative %
- ** -
-***-
-***-
Time spent
Reactions
Reaction time
P. multicolor 'wildtype'
A. burtoni 'wildtype'
C
100
50
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Relative %
-n.s.-
-n.s.-
-n.s.-
Time spent
Reactions
Reaction time
P. multicolor 'wildtype'
P. multicolor 'no fringe'
D
100
50
0
Relative %
— * —
— * —
— * —
Time spent
Reactions
Reaction time
P. multicolor 'wildtype'
P. multicolor 'egg-spot'

Figure 2. Female preference tests in *Pseudocrenilabrus multicolor* using computer animated stimuli. (A) The experimental set-up consists of an iMac computer behind an experimental aquarium (60×30×30 cm). Two animations are shown simultaneously (in this case a conspecific male and a heterospecific, *Astatotilapia burtoni*; see [B]). (B) Results from the 'benchmark' experiment, in which *P. multicolor* females were given the choice between a conspecific and a heterospecific (*A. burtoni*) male. The females reacted significantly more often with the animated image showing a conspecific male. (C) Results from the 'red fringe' experiments, in which *P. multicolor* were left the choice between a male with and one without the red fringe on the tip of the anal fin. We could not detect any difference in female response, which is also backed-up by two-way choice experiments with live fish (see Figure S1). (D) Results from the 'egg-spot' experiment, in which *P. multicolor* females could choose between a natural male and a male bearing an *in- silico* egg-spot. Females showed a significant preference for the male with the artificial egg-spot. Arrowheads indicate the minute differences between the images presented to the females.
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dots compared to females (Wilcoxon rank-sum test; $N = 20$; $p = 0.045$).

Discussion

Anal fin egg-spots are a characteristic feature of the most species-rich group of cichlids, the haplochromines [1,4,25]. While several hypothesis exist that seek to explain the function of this conspicuous male trait (see e.g. [6,7,12]), little is known about their evolutionary origin. Here we test the hypothesis that male egg-spots in haplochromines evolved to exploit a pre-existing bias in females [10]. A crucial prerequisite in favor of this hypothesis is that the preference for egg-spots (the sensory bias) is phylogenetically older than anal fin egg-spots themselves [14,18,26,27]. We confirm this prediction in two independent and *per se* complementary experiments.

First, we show that females of the basal haplochromine species *Pseudocrenilabrus multicolor*, which manifests the plesiomorphic character-state of an egg-spot-less anal fin (Figure 1), do show a clear preference for the animated photograph of a male with an artificial egg-spot over an otherwise identical animated photo-

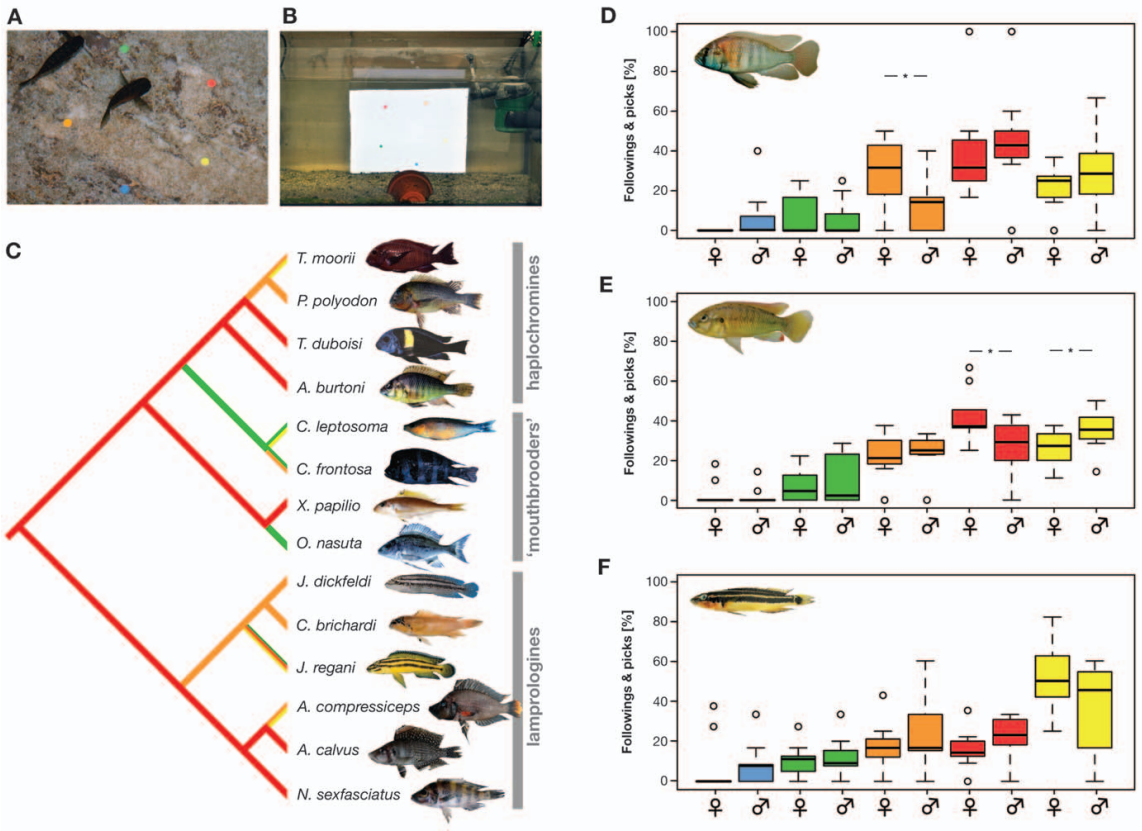

Figure 3. Color preference tests in different East African cichlid species. (A) Set-up of the field experiment at Lake Tanganyika. Fishes were presented five color dots on a transparent foil and we measured the number of pecks towards each dot. (B) Set-up of the laboratory experiments. Individual fishes were presented five color dots on a computer screen. (C) Ancestral character state reconstruction of color preferences in a phylogenetically representative set of cichlids from Lake Tanganyika. Most species clearly preferred orange or red colors. Importantly, also the substrate spawning lamprologines showed such a preference. (D–F) Results from the color-dot preference experiments in the laboratory with the haplochromines Astatotilapia burtoni (D) and Pseudocrenilabrus multicolor (E) and the lamprologine Julidochromis ornatus (F). Significant differences between males and females are indicated.
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graph of a male without an egg-spot (Figure 2D). Obviously, *P. multicolor* females perceive the minute difference between the two computer-animated images of males (i.e. the artificial egg-spot, which spans less than 1% of the lateral area), which seems plausible given the visual capabilities of cichlids [17,18].

Second, our field experiments suggest that a preference for yellow, orange or red dots, which resemble the color and shape of egg-spots, existed before the radiation of the haplochromines. Most East African cichlid species tested and, importantly, the majority of the egg-spot-less species belonging to cichlid lineages basal to the haplochromines, show clear preferences for such egg-spot-like dots over blue and green dots (Table S1, Figure 3). The only three species not showing a clear preference for egg-spot-like colors were indecisive and/or showed very weak preferences overall (as measured by the number of pecks per 5 minute trial). Our character state reconstructions indicate that the preference for egg-spot-like colors was present before the emergence of the first haplochromines and that even the substrate spawning lamprologines show a bias towards yellow, orange or red dots (Figure 3C). These results are backed up by our color preference experiments under laboratory conditions in two haplochromines and one lamprologine (Figure 3D–F).

Taken together, our experiments suggest that sensory exploitation of a pre-existing bias was responsible for the evolution of anal fin egg-spots in haplochromine cichlids. The question is now what could have triggered the bias for egg-spot-like dots in (female) cichlids. Tobler [10] proposed that it is the affinity to detect own eggs as such. This should have evolved in mouth-brooding females as a result of their limited number of relatively large eggs and, consequently, the immediate reduction of fitness when failing to take up all the eggs. This hypothesis is compatible with our mate choice experiments in the basal and egg-spot-less haplochromine *P. multicolor*. Yet, the preference for egg-spot-like dots is prevalent in male and female cichlids and also in substrate spawners basal to haplochromines (which, nevertheless, perform brood care). This, in turn, suggests that it is not the affinity for own eggs that evolved, as males should not show this affinity and substrate spawners have much smaller and less conspicuous eggs. It seems more likely that the observed pre-existing bias in East African cichlids points towards high quality – e.g. carotenoid-rich – food like shrimps, algae and, notably, fish eggs. A preference for carotenoid-enriched diets is known from several taxa (e.g. [28,29,30]), and the heritability of algal-foraging ability in guppies suggests that, in this case, females might actually benefit from preferring males with a pronounced carotenoid-based coloration indicative of their foraging skills [29,31]. Such a pre-existing bias towards yellow, orange or reddish dots that resemble food could reasonably well explain why yellow, orange or reddish egg-spots (i.e. convergently evolved blotches on the fins of other cichlids [4,10,25]) have evolved multiple times in addition to and outside the haplochromines.

Methods

Laboratory mate choice trials

All laboratory mate choice experiments were performed at the Zoological Institute of the University of Basel under the permission of the Cantonal Veterinary Office, Basel, Switzerland (permit number 2403). Live cichlids were kept in isolation and under standardized conditions (12 h dark/12 h light; 25°C).

Before turning towards our central question, we had to assess the usefulness of computer animations in experiments with the haplochromine cichlid species *Pseudocrenilabrus multicolor*. While computer-animated stimuli are frequently used in West African cichlids [32,33], little is known about how haplochromines react to it. Finally, there is a technical component too, as it has been shown that the reaction to a stimulus may vary depending on the computer screen used [34]. Therefore, we first tested three different computer screens: a SONY® 17″ CRT display, and two Apple® iMac computers with a dull 17″ and a bright 21″ LCD display, respectively. In our set-up, females reacted most when presented images on the 17″ iMac G5 (pers. observation). We also evaluated still and animated photographs of males and found that female *P. multicolor* reacted most to the following animations: 7 seconds upwards movement, 2 seconds remaining in still position, 7 seconds downward movement, 2 seconds remaining in still position (pers. observation; the animations were created with Apple® Keynote® software and exported as Quicktime® movies).

As a benchmark, we tested whether *P. multicolor* females can discriminate between a conspecific and a heterospecific (*Astatotilapia burtoni*) male. To this end, we positioned an iMac (17″ iMac G5 running Mac OSX version 10.5.7; chip model ATY Radeon×1600, 1400×900 pixels, 32 Bit color) directly behind a glass aquarium (60×30×30 cm) so that it covered about 2/3 of the aquarium's width (Figure 2A). On the very left and the very right of the iMac, there was a 10.5 cm neutral zone not covered by the screen. These areas plus the two sides were covered with visual barriers, so that only the front panel remained transparent. Thus, we could video-tape each experimental trial with a SONY® DCR-HC90E Handycam® (note that all computer- animated experiments were performed in a closed compartment to avoid interference of the experimenter). The bottom of the aquarium was covered with sand, and in the front center, right below the filter, we placed half a flower-pot to provide shelter to the focal female. For the animations, the screen was divided into two 10.5 cm wide outer parts (where the actual animations were shown) and an 18 cm central part that remained grey (Figure 2A). In this experiment, twelve *P. multicolor* females were exposed to two size-matched images of a male *P. multicolor* and a male *A. burtoni*, which were animated to move up and down in an infinite loop (see above for animation settings); the images of the males were pasted into a neutral grey background (R: 149, G: 149, B: 149). Each female was tested twice, once in the morning and once in the afternoon (with at least 5 h between experiments), and the stimuli were switched between the two rounds (with the morning set-up being chosen randomly). At the beginning of each experiment, the female was allowed to habituate for 10 minutes before the parallel animations started. Beginning from the first reaction of the focal female to the animation (i.e. the female swimming towards the animation, stopping in front of the monitor, facing the stimulus and swimming along with the animation), we recorded the following three – not mutually exclusive – behavioral parameters for a period of ten minutes (based on the video-taped material): (*i*) '*time spent*' (in seconds) as the time that a female spent in front of each animation (practically, we started counting when 50% of the female body entered the preference zone, i.e. the 10.5 cm grey zone of each animated male, and stopped when 50% of the female body left this zone); (*ii*) '*number of reactions*' (integer) in how often a given female would follow the up- or downward-movement of a stimulus male; and (*iii*) '*reaction time*' as the time (in seconds) that a female would actively follow the up- or downward-movement of a stimulus male. For statistical analyses, the counts from the two rounds of experiments with each focal female were averaged. To account for individual differences in the total time spent and the number of reactions among females, we used individual percentages of the total number of observations as response variables [32,34]. All data were analyzed with the software R (vers. 2.8.1).

In a second round of experiments, we focused on the red fringe on the anal fin of male *P. multicolor*, as we could not exclude the possibility that this trait is the target of female choice in this basal haplochromine species. We used the same parameters as before, except that this time we gave females the choice between two images of a male, of which one retained the natural phenotype, whereas the other was modified *in-silico* so that its red fringe was replaced by the brownish ground color of the rest of the anal fin (using Adobe® Photoshop®). We tested fifteen focal females and recorded the very same behavioral parameters as mentioned above.

We then repeated this experiment with live fish using a dichotomous set-up (Figure S1A): six pairs of size-matched males of *P. multicolor* were formed to avoid bias. The red fringe on the anal fin of one male of each size-matched pair was removed by fin-clipping. On the other male a piece of dorsal fin was cut to control for possible treatment effects (Figure S1B). The size-matched males of each pair were randomly placed in one of the two outer tanks (40×24×24 cm) adjacent to a central tank (60×30×30 cm). The males were allowed to habituate for several days; during this period the males were inspected for signs of stress. Then, a focal female was placed into the central tank. We recorded the following parameters during 10-minute trials starting with the first interaction: (*i*) '*time spent*' (in seconds) as the time that a female spent in a preference zone (12 cm adjacent to each male tank); (*ii*) '*interactions*' as the number of independent visits to a preference zone; and (*iii*) '*interaction time*' as the time (in seconds) that a female spent in front of an interacting male.

Finally, we tested for a pre-existing bias for egg-spots in females of *P. multicolor* using computer animated stimuli. We presented females two identical male images, except that one of them had an artificial egg-spot. This single egg-spot was designed to resemble real *P. multicolor* eggs in color and average size. Therefore, we photographed and measured 46 eggs and determined the average size (1.86 mm) and color hue (R: 255, G: 150, B: 45). This 'average' egg-spot was then pasted onto the anal fin of a male image using Photoshop®.

Color-dot preference tests

Pond experiments. The preference tests for egg-spot-like dots were carried out in February and March 2010 at 'Kalambo Lodge' at the shore of Lake Tanganyika, East Africa (Zambia; S 8.6232 E 31.2). Wild-caught individuals from 14 cichlid species were kept in ponds (ca. 1×2 m) filled with lake water (ca. 50 cm high). We tested four egg-spot bearing haplochromine species (*Astatotilapia burtoni*, *Petrochromis polyodon*, *Tropheus duboisi* and *T. moorii*) and ten species belonging to other, more basal cichlid lineages including mouth-brooding (*Cyphotilapia frontosa*, *Cyprichromis leptosoma*, *Ophthalmotilapia nasuta* and *Xenotilapia papilio*) and substrate spawning (*Altolamprologus calvus*, *A. compressiceps*, *Chalinochromis brichardi*, *Julidochromis dickfeldi*, *J. regani* and *Neolamprologus sexfasciatus*) representatives. Each pond contained between 11 and 75 individuals, depending on the size of the fish and the sampling success of the local fishermen. All ponds were stocked with a mix of female and male individuals. As most species under study do not show sexual dimorphisms, the exact sex ratio could not be determined. To the 14 species, we presented five conspicuous color dots (yellow, orange, red, green, and blue), which were arranged in a pentagonal shape on a transparent foil (Figure 3A). Two sets of foils with different arrangements of dots were used. After placing the foil on the ponds' grounds, we waited until the first individual approached and pecked at one of the dots. Four observers then counted the number of pecks for a period of five minutes. If one individual stayed at one spot and pecked at it repeatedly, it was counted as one strike only. We first performed a goodness-of-fit test to examine the existence of a preference for certain colors within species (all species preferred some colors over others; $p<0.001$; Table S1). The color preference within each species was then determined using a series of binomial tests (Table S2) and subjected to an ancestral character state reconstruction. To this end, we used a phylogenetic tree derived from a maximum likelihood analysis based on mitochondrial sequence data (NADH Dehydrogenase Subunit II gene; 1047 bp; [1,35]). Preference for the colors blue, green, yellow, orange and red were coded as numbers and we allowed for multiple characters states in species that did not show a significant preference for only one color. Ancestral color preferences were reconstructed with parsimony as implemented in Mesquite (vers. 2.74, [36]). We would like to note here that it is essentially impossible to perform such an experiment within the lake itself, as there are too many species and interactions between species; also, we would never find so many individuals of the same species together. It is also important to note that we were not able to test *P. multicolor* in the wild, as this species does not occur within Lake Tanganyika.

Laboratory experiments. Since the color-dot preference tests in the field could potentially be influenced by pseudo-replication within ponds, we repeated this experiment in the lab using three available lab strains and computer animations. Three species (*Pseudocrenilabrus multicolor*, 10 males and 10 females; *Astatotilapia burtoni*, 11 males and 9 females; *Julidochromis ornatus*, 9 males and 11 females) were tested for color preference under controlled laboratory conditions, allowing assessing individual fish and males and females separately. To this end, five colored spots (yellow, orange, red, green, and blue; diameter: 1 cm) were arranged circularly on neutral grey background in a computer animation, displaying a simultaneous circular movement. Two animations were designed to randomize the initial position of the five color dots. The focal fish was introduced into an aquaria tank (60×30×30 cm) and left for 30 min before the start to acclimatize. Then the animation was presented to the focal fish via a computer screen (see above), placed in front of the experimental tank. The behavior of the focal fish was recorded for 1 hour with a video-camera and analyzed with the software iMovie®. Thirty minutes of behavior after the first reaction were analyzed and two parameters were recorded: the number of times the focal fish pecked each colored dot and number of times the focal fishes followed each colored dot. The percentage data was angular-transformed and analyzed with the statistics software R, applying a Friedman test and a series of Wilcoxon signed-rank tests (with and without Bonferroni correction; Table S3). Sex differences were tested through Wilcoxon rank-sum tests.

Supporting Information

Figure S1 Two-way choice tests in *Pseudocrenilabrus multicolor*. (A) Scheme of the experimental set-up consisting of two outer tanks (40×24×24 cm) adjacent to a central tank (60×30×30 cm). Each male tank (outer tanks) was equipped with a plastic perforated shelter, while the central female tank was equipped with three shelters: two shelters were placed next to each outer male tank and one shelter was placed in the middle of the tank. In this setup the females had the possibility to communicate visually with the two different males at the left and right extreme of their central tank (12 cm preference zone). Only visual communication was permitted. (B) Results from the 'fin-clipping' experiment, in which *P. multicolor* females were given the choice between a male where the red fringe at the anal fin was removed

by fin-clipping and a size-matched control male that was fin-clipped at the dorsal fin. Females did not show any preference.
(PDF)

Table S1 Color-dot preference tests in ponds. Preferred colors for each species are indicated.
(PDF)

Table S2 Color-dot experiments in ponds. P-values resulting from binomial tests.
(PDF)

Table S3 Laboratory color-dot preference test. P-values were calculated from percentage data with arcsine transformation and are presented with and without Bonferroni correction for *Astatotilapia burtoni* (A), *Pseudocrenilabrus multicolor* (B) and *Julidochromis ornatus* (C).
(PDF)

Movie S1 Female choice experiments in *Pseudocrenilabrus multicolor* using computer animations.
(MOV)

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Author Contributions

Conceived and designed the experiments: BE YK AT WS. Performed the experiments: BE YK AT. Analyzed the data: BE YK AT WS. Wrote the paper: BE YK AT WS.

Table S1 Color-dot preference tests in ponds. Preferred colors for each species are indicated.
doi:10.1371/journal.pone.0025601.s002

species	sample size	yellow	orange	red	blue	green	goodness of fit
Altolamprologus calvus	45	6	21	162	0	1	p<0.001
Altolamprologus compressiceps	50	77	104	11	2	10	p<0.001
Astatotilapia burtoni	36	11	43	154	0	0	p<0.001
Chalinochromis brichardi	45	13	31	10	7	2	p<0.001
Cyphotilapia frontosa	43	14	29	11	6	30	p<0.001
Cyprichromis sp. leptosoma	50	8	0	1	0	11	p<0.001
Julidochromis dickfeldi	30	3	75	33	0	9	p<0.001
Julidochromis regani	27	11	13	20	0	9	p<0.001
Neolamprologus sexfasciatus	18	3	2	62	0	24	p<0.001
Ophtalmotilapia nasuta	12	2	4	0	0	14	p<0.001
Petrochromis polyodon	11	17	48	16	1	2	p<0.001
Tropheus duboisi	70	67	169	250	6	18	p<0.001
Tropheus moorii	30	22	32	4	0	2	p<0.001
Xenotilapia papilio	75	1	8	31	0	1	p<0.001

Table S2 Color-dot experiments in ponds. P-values resulting from binomial tests.
doi:10.1371/journal.pone.0025601.s003

binomial test	*A. calvus*	*A. compressiceps*	*A. burtoni*	*C. brichardi*	*C. frontosa*	*C. sp. leptosoma*	*J. dickfeldi*	*J. regani*	*N. sexfasciatus*	*O. nasuta*
yellow vs.orange	0.0059	0.0530	< 0.0001	0.0096	0.0315	0.0078	< 0.0001	0.8388	1	0.6875
yellow vs. red	< 0.0001	< 0.0001	< 0.0001	0.6776	0.6900	0.0391	< 0.0001	0.1496	< 0.0001	0.5000
yellow vs. blue	0.0313	< 0.0001	0.0010	0.2632	0.1153	0.0078	0.2500	0.0010	0.2500	0.5000
yellow vs. green	0.1250	< 0.0001	0.0010	0.0074	0.0226	0.6476	0.1460	0.8238	< 0.0001	0.0042
orange vs. red	< 0.0001	< 0.0001	< 0.0001	0.0015	0.0064	-	< 0.0001	0.2962	< 0.0001	0.1250
orange vs. blue	< 0.0001	< 0.0001	< 0.0001	0.0001	0.0001	-	< 0.0001	0.0002	0.5000	0.1250
orange vs. green	< 0.0001	< 0.0001	< 0.0001	< 0.0001	1	0.0010	< 0.0001	0.5235	< 0.0001	0.0309
red vs. blue	< 0.0001	0.0225	< 0.0001	0.6291	0.3323	-	< 0.0001	< 0.0001	< 0.0001	-
red vs. green	< 0.0001	1	< 0.0001	0.0386	0.0043	0.0063	0.0003	0.0614	< 0.0001	0.0001
blue vs. green	-	0.0386	-	0.1797	< 0.0001	0.0010	0.0039	0.0039	< 0.0001	0.0001

Table S3 Laboratory color-dot preference test. P-values were calculated from percentage data with arcsine transformation and are presented with and without Bonferroni correction for *Astatotilapia burtoni* (A), *Pseudocrenilabrus multicolor* (B) and *Julidochromis ornatus* (C).
doi:10.1371/journal.pone.0025601.s004

A1 ***Astatotilapia burtoni*** **- males and females (n = 20)**

Friedman test

parameter	p-value
pecks & chases	4.38 *10-8

Wilcoxon signed-rank test

parameter	p-value	significant? (without Bonferroni correction)		significant? (with Bonferroni correction)	
pecks					
red-blue	0.0005199	< 0.05	yes	< 0.05/10	yes
yellow-blue	0.0006637	< 0.05	yes	< 0.05/9	yes
red-green	0.0006807	< 0.05	yes	< 0.05/8	yes
yellow-green	0.000832	< 0.05	yes	< 0.05/7	yes
orange-green	0.00909	< 0.05	yes	> 0.05/6	no
orange-blue	0.01379	< 0.05	yes	> 0.05/5	no
orange-red	0.04538	< 0.05	yes	> 0.05/4	no
orange-yellow	0.09316	> 0.05	no	> 0.05/3	no
red-yellow	0.6085	> 0.05	no	> 0.05/2	no
blue-green	1	> 0.05	no	> 0.05/1	no
chases					
red-green	0.001075	< 0.05	yes	< 0.05/10	yes
red-blue	0.004363	< 0.05	yes	< 0.05/9	yes
red-yellow	0.02075	< 0.05	yes	> 0.05/8	no
orange-red	0.03242	< 0.05	yes	> 0.05/7	no
orange-blue	0.07065	> 0.05	no	> 0.05/6	no
yellow-blue	0.1091	> 0.05	no	> 0.05/5	no
orange-green	0.1815	> 0.05	no	> 0.05/4	no
orange-yellow	0.3271	> 0.05	no	> 0.05/3	no
blue-green	0.3523	> 0.05	no	> 0.05/2	no
yellow-green	0.673	> 0.05	no	> 0.05/1	no
pecks & chases					
yellow-blue	0.0003143	< 0.05	yes	< 0.05/10	yes
red-green	0.0003173	< 0.05	yes	< 0.05/9	yes
red-blue	0.0004447	< 0.05	yes	< 0.05/8	yes
yellow-green	0.002072	< 0.05	yes	< 0.05/7	yes
orange-blue	0.008514	< 0.05	yes	> 0.05/6	no
orange-red	0.009677	< 0.05	yes	> 0.05/5	no
orange-green	0.02185	< 0.05	yes	> 0.05/4	no
red-yellow	0.1146	> 0.05	no	> 0.05/3	no
orange-yellow	0.5319	> 0.05	no	> 0.05/2	no
blue-green	0.2195	> 0.05	no	> 0.05/1	no

A2 ***Astatotilapia burtoni*** **- females only (n = 9)**

Friedman test

parameter	p-value
pecks	0.002
chases	3.90*10-4
pecks & chases	2.61*10-4

Wilcoxon signed-rank test

parameter	p-value	significant? (without Bonferroni correction)		significant? (with Bonferroni correction)	
pecks					
yellow-blue	0.02154	< 0.05	yes	> 0.05/10	no
yellow-green	0.02154	< 0.05	yes	> 0.05/9	no
red-blue	0.03552	< 0.05	yes	> 0.05/8	no
red-green	0.03552	< 0.05	yes	> 0.05/7	no
orange-blue	0.05791	> 0.05	no	> 0.05/6	no
orange-green	0.05791	> 0.05	no	> 0.05/5	no
orange-yellow	0.4974	> 0.05	no	> 0.05/4	no
red-yellow	0.6845	> 0.05	no	> 0.05/3	no
orange-red	1	> 0.05	no	> 0.05/2	no
blue-green	NA	> 0.05	no	> 0.05/1	no
chases					
red-blue	0.008849	< 0.05	yes	> 0.05/10	no
orange-blue	0.02154	< 0.05	yes	> 0.05/9	no
red-yellow	0.02225	< 0.05	yes	> 0.05/8	no
red-green	0.03552	< 0.05	yes	> 0.05/7	no
orange-yellow	0.05149	> 0.05	no	> 0.05/6	no
orange-green	0.2016	> 0.05	no	> 0.05/5	no
yellow-blue	0.1003	> 0.05	no	> 0.05/4	no
blue-green	0.1003	> 0.05	no	> 0.05/3	no
orange-red	0.4461	> 0.05	no	> 0.05/2	no
yellow-green	0.7525	> 0.05	no	> 0.05/1	no
pecks & chases					
red-blue	0.00909	< 0.05	yes	> 0.05/10	no
yellow-blue	0.01379	< 0.05	yes	> 0.05/9	no
orange-blue	0.02201	< 0.05	yes	> 0.05/8	no
red-green	0.02249	< 0.05	yes	> 0.05/7	no
orange-green	0.05149	> 0.05	no	> 0.05/6	no
yellow-green	0.05906	> 0.05	no	> 0.05/5	no
blue-green	0.09751	> 0.05	no	> 0.05/4	no
red-yellow	0.1056	> 0.05	no	> 0.05/3	no
orange-yellow	0.2719	> 0.05	no	> 0.05/2	no
orange-red	0.5294	> 0.05	no	> 0.05/1	no

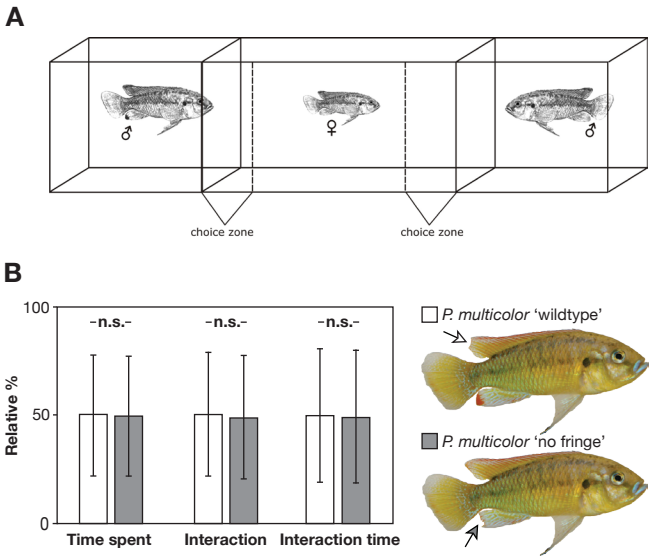
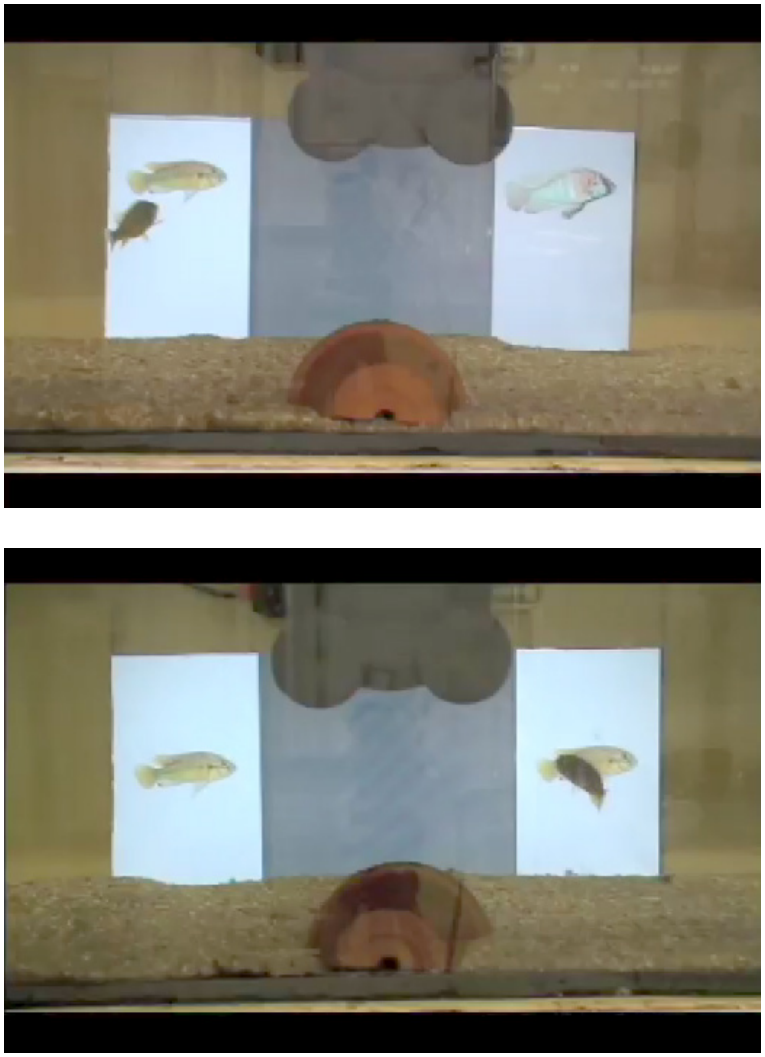


Figure S1 Two-way choice tests in *Pseudocrenilabrus multicolor.* (A) Scheme of the experimental set-up consisting of two outer tanks (40×24×24 cm) adjacent to a central tank (60×30×30 cm). Each male tank (outer tanks) was equipped with a plastic perforated shelter, while the central female tank was equipped with three shelters: two shelters were placed next to each outer male tank and one shelter was placed in the middle of the tank. In this setup the females had the possibility to communicate visually with the two different males at the left and right extreme of their central tank (12 cm preference zone). Only visual communication was permitted. (B) Results from the 'fin-clipping' experiment, in which *P. multicolor* females were given the choice between a male where the red fringe at the anal fin was removed by fin-clipping and a size-matched control male that was fin-clipped at the dorsal fin. Females did not show any preference.
doi:10.1371/journal.pone.0025601.s001



Movie S1 Female choice experiments in *Pseudocrenilabrus multicolor* using computer animations. doi:10.1371/journal.pone.0025601.s005

1.2 The function of anal fin egg-spots in the cichlid fish *Astatotilapia burtoni*

Theis A, Salzburger W and Egger B
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1.2.1 Manuscript: p. 35 - 42
1.2.2 Supporting information: p. 43

This project was conducted partly during my master thesis and partly during my early PhD. I planned, conducted and analysed the experiments under the supervision of BE. I wrote the first draft of the manuscript, which was refined together with BE and WS.

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The Function of Anal Fin Egg-Spots in the Cichlid Fish *Astatotilapia burtoni*

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Abstract

Color and pigmentation patterns of animals are often targets of sexual selection because of their role in communication. Although conspicuous male traits are typically implicated with intersexual selection, there are examples where sex-specific displays play a role in an intrasexual context, e.g. when they serve as signals for aggression level and/or status. Here, we focus on the function of a conspicuous male ornament in the most species-rich tribe of cichlid fishes, the haplochromines. A characteristic feature of these ca. 1500 species are so-called egg-spots in form of ovoid markings on the anal fins of males, which are made up of carotenoid based pigment cells. It has long been assumed that these yellow, orange or reddish egg-spots play an important role in the courtship and spawning behavior of these maternal mouth-brooding fishes by mimicking the eggs of a conspecific female. The exact function of egg-spots remains unknown, however, and there are several hypotheses about their mode of action. To uncover the function of this cichlid-specific male ornament, we used female mate choice experiments and a male aggression test in the haplochromine species *Astatotilapia burtoni*. We manipulated the number and arrangement of egg-spots on the anal fins of males, or removed them entirely, and tested (*1*) female preference with visual contact only using egg-traps, (*2*) female preference with free contact using paternity testing with microsatellites and (*3*) male aggression. We found that females did not prefer males with many egg-spots over males with fewer egg-spots and that females tended to prefer males without egg-spots over males with egg-spots. Importantly, males without egg-spots sired clutches with the same fertilization rate as males with egg-spots. In male aggression trials, however, males with fewer egg-spots received significantly more attacks, suggesting that egg-spots are an important signal in intrasexual communication.

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Introduction

Since the publication of Charles R. Darwin's second most famous book 'The Descent of Man' in 1871 [1], sexual selection has been recognized as being important for speciation because it can mediate reproductive isolation [2,3]. Darwin differentiated between two fundamental modes of sexual selection: (*i*) competition between members of the same sex (often males) for reproductive opportunity ('intrasexual'), and (*ii*) active mate choice of members from one sex (often females) for certain members from the other sex ('intersexual'). Particularly in the latter mode, mate choice is often based on visual ornaments, although color traits can serve with respect to both inter- and intrasexual communication and, hence, inter- and intrasexual selection [4,5]. Moreover, there are instances where the role of ornaments was altered from a function in female choice to one in male-male competition or *vice versa* [6].

Color and pigmentation patterns seem to play a central role in the explosively radiating cichlid fish species in the East African Great Lakes in general, and in the haplochromine cichlids in particular [7,8,9,10]. Haplochromines contain the vast majority of East African cichlid species with the entire species flocks of lakes Victoria (ca. 700 species) and Malawi (ca. 700 species), the tribe Tropheini from Lake Tanganyika (ca. 25 species) and most riverine East African cichlids (ca. 200 species) (see e.g. [11,12]). Therefore haplochromines are not only the – by far – most species-rich tribe of cichlid fishes but also a model of radiating species. A prominent feature of the haplochromines is their wealth of color morphs and their sexual color dimorphism, which is what led many authors to postulate an important evolutionary role of sexual selection via female mate choice [13,14,15,16]. Interestingly, all haplochromines are maternal mouthbrooders where females incubate the eggs in their buccal cavities (see e.g. [12,17]). Mouthbrooding evolved from substrate spawning several times during cichlid evolution [18], but only the 'modern haplochromines' show a derived polygynous or polygynandrous maternal mouthbrooding system with males carrying egg-spots on their anal fins [12,17,19]. These ovoid markings consist of a transparent outer ring and a brightly colored yellow, orange or reddish center [17,20,21]. The conspicuous central area is formed by xanthophores – a pigment cell type containing carotenoids and pteridines [19,22].

Egg-spots appear to be important in the courtship and spawning behavior of haplochromines [20,21,23,24] (Figure 1C). The exact function of egg-spots is unknown, however, and several hypotheses exist that seek to explain their mode of action and their evolutionary origin.

Wickler [20,21] suggested that egg-spots on the male's anal fin mimic real eggs of a species and therefore function as signal

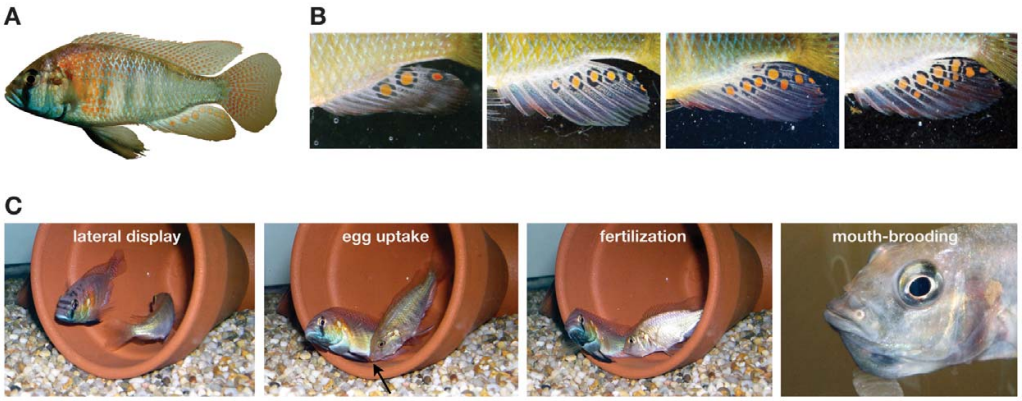

Figure 1. The egg-spots of haplochromine cichlids as exemplified in *Astatotilapia burtoni*. (A) A male of *A. burtoni* showing egg-spots on its anal fin. (B) Natural variation of egg-spots in *A. burtoni*. All these fish were caught and photographed at the south-eastern part of Lake Tanganyika in Zambia. (C) A typical courtship and mating cycle of a haplochromine starts with a lateral display of the male, to which the female responds; she then lays a clutch of eggs and immediately takes them up into her mouth. The male then presents the egg-spots on the anal fin; the female seemingly nuzzles at these egg-spots and the male releases sperm so that the eggs are fertilized within the females' mouth. The eggs and larvae then stay in the buccal cavity of the female for a period of several days to a few weeks. The arrow points to the location of an egg that the female is taking up into her mouth.
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('releaser') during courtship and to maximize fertilization rates. The egg mimicry hypothesis is primarily based on the putatively similar appearance in shape and coloration of egg-spots and the eggs of the respective species [20,21,25]. However, egg-spots and eggs often do not match in size, shape and coloration, which is inconsistent with the mimicry hypothesis [26,27]. Still, this mismatch between real and 'dummy' eggs may be due to a trade-off between attractiveness towards females and conspicuousness for predators [24]. Hert [23] was the first to experimentally test the egg-dummy scenario. She showed that in the species *Astatotilapia elegans* there were no differences in fertilization rates between males without and males with intact egg-spots, which at least partly contradicted the mimicry hypothesis. On the other hand, males with intact egg-spots fertilized twice as many clutches compared to males without egg-spots. Further mate choice trials revealed that females always chose males with egg-spots and preferred males with four egg-spots over males with one egg-spot. In *Pseudotropheus aurora* (now *Maylandia aurora*), females spawned more frequently with males displaying more egg-spots and male egg-spot number correlated significantly with the number of fertilized clutches [28]. Hert [23,28] concluded that egg-spots serve as sexual advertisement and that disruptive selection on male egg-spots may have contributed to reproductive isolation and, hence, speciation. In mate choice trials with *Pseudotropheus lombardoi* (now *Maylandia lombardoi*), a Lake Malawi cichlid in which males display a single egg-spot, females preferred males with one egg-spot over males with an artificially added second one [29]. Couldridge [29] suggested that female preference maintains the single egg-spot in *P. lombardoi* and that egg-spots may be linked to species recognition.

Previous hypotheses regarding the function of egg-spots involve female choice as the main explanation for the maintenance of this conspicuous male trait (see above). Interestingly, however, essential sequences of courtship behavior like quivering and lateral display are also used in male-male interactions. When males fight, which happens frequently in territorial haplochromines, they quiver, move back and forward and attack sideways [30]. So, why shouldn't egg-spots play a role in male-male competition, too? There are several arguments that would implicate egg-spots with intrasexual selection. Importantly, egg-spots are, most likely, an honest signal of male quality, as carotenoids cannot be synthesized *de novo* by animals (pteridines, on the other hand, can be synthesized; yet, this process appears to also be costly) [31,32]. There is evidence that dominant males often display more egg-spots [33,34]. Moreover, competition among males appears as yet another important component of color evolution in cichlids [35,36].

To understand the function of egg-spots in the haplochromine cichlid *Astatotilapia burtoni*, we conducted three experiments. First, females had a choice based on visual cues only between two size-matched males differing in egg-spot number (one trial with naturally varying numbers of egg-spots (experiment 1.1) and one trial with manipulated numbers of egg-spots (experiment 1.2)). Second, we performed a female four-way choice experiment in a partial partition set-up (see e.g. [16]), in which females had the choice between four size-matched males with manipulated egg-spot numbers; we measured fertilization rate and genotyped the offspring in order to assess female preference by determining paternity (experiment 2). Finally, we conducted male aggression trials to test for a potential role of egg-spots in male-male competition (experiment 3).

Results

Experiment 1: female two-way choice

In our female two-way choice experiments two size-matched males were presented to a focal female in two outer tanks arranged on both sides of the central female tank (Figure 2A). In experiment 1.1, 7 females laid more eggs in front of the male with many egg-spots and 11 females laid more eggs in front of the male with fewer egg-spots. Males with many egg-spots were not more likely to receive more eggs from females than males with fewer egg-spots (GLMM, $n = 18$, $z = -0.892$, $p = 0.373$; Figure 3A). In experiment 1.2 only 6 females laid more eggs in front of the male with egg-spots and 15 females laid more eggs in front of the male without egg-spots. Thus, females tended to lay eggs preferentially close to

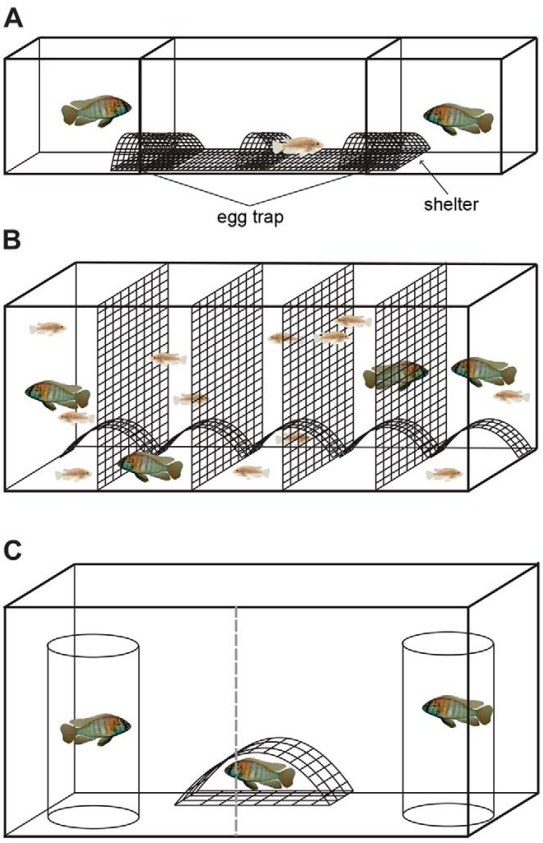

Figure 2. Experimental set-up (schematic view). (A) The two-way choice set-up showing the egg-traps and the shelters, which are permeable to eggs. (B) The four-way choice set-up ('partial partition' method) with semi-permeable grids, passable for females but not for males. (C) The set-up for the male aggression trials with stimulus males in plastic cylinders and the focal male hiding in the shelter.
doi:10.1371/journal.pone.0029878.g002

males without egg-spots (GLMM, $n = 21$, $z = -1.897$, $p = 0.058$; Figure 3B). Most females laid the whole clutch in front of a single male but in 7 out of 18 trials in experiment 1.1 and in 3 out of 21 trials in experiment 1.2 the females laid eggs in front of both males.

Experiment 2: female four-way choice

The four-way choice set-up consisted of a large tank with five equally sized compartments ('partial partition' design; Figure 2B). Once a female was mouthbrooding, we removed the eggs or larvae to determine the fertilization rate and to test for paternity using microsatellites. In the first replicate, the three males with egg-spots fertilized 75% or more of the offspring in 5 clutches and the male without egg-spots fertilized 25% or more of the offspring in 18 clutches. Therefore, females preferred the male without egg-spots (Binomial test, $n = 23$, $p<0.001$). In replicate 2 and 3, however, the males without egg-spots (fertilizing 25% or more of the offspring in 2 and 10 clutches, respectively) were not significantly preferred over males with egg-spots (fertilizing 75% or more of the offspring in 12 and 21 clutches, respectively; replicate 2: Binomial test, $n = 14$, $p = 0.540$; replicate 3: Binomial test, $n = 31$, $p = 0.401$; Figure 4). Due to the observation that within each replicate the three egg-spot bearing males weren't more attractive to females than males without egg-spots, we did not test for differences in the number of offspring sired by the three egg-spot bearing phenotypes. Fertilization rate was close to 100% in all cases, also in broods fathered by the male without egg-spots. A definitive female choice was found in 58 out of the 68 broods genotyped (93%), in which only one male sired the whole clutch. Multiple paternity was detected in 10 out of the 68 broods genotyped (7%). One out of these 10 broods was fathered by 3 different males, whereas 2 fathers were detected in the remaining 9 broods.

Experiment 3: male aggression

To test the role of egg-spots in aggressive behavior, two size-matched males, one with unaltered and one with removed egg-spots, were placed in transparent cylinders and presented to a territorial male of similar size (Figure 2C). Out of 13 focal males, 10 showed a higher attack rate (bites, butts and quivers) towards stimulus males without egg-spots, 2 showed a higher attack rate towards stimulus males with egg-spots and 1 attacked both stimulus males at a same rate. Taken together, focal males attacked the stimulus males without egg-spots more often than the stimulus males with egg-spots (GLMM, $n = 13$, $z = -2.218$, $p = 0.027$; Figure 3C).

Discussion

It is widely recognized that secondary sexual traits are targets of inter- or intrasexual selection, or both [4,5,16,37,38]. Sexual selection is often considered a central driving force in the evolution of the exceptionally colorful and species-rich haplochromine cichlid fishes endemic to rivers and lakes in East Africa [7,9,10]. One particular feature of haplochromines (at least of the derived 'modern haplochromines') is their possession of true egg-spots on the anal fins of males [12]. While previous work on the function of egg-spots solely focused on intersexual selection (female choice), we also tested, for the first time, for a putative role of egg-spots in intrasexual selection (male-male competition). Here, we focus on the haplochromine cichlid *Astatotilapia burtoni*, which is widely used in various kinds of experiments and whose genome has recently been sequenced (see e.g. [39,40]).

The two-way choice experiments revealed that there is no female preference for many egg-spots in *A. burtoni* and that females even tended to prefer males without egg-spots. In these experiments, we used egg-traps to quantify if a focal female laid more eggs towards one of the naturally distinct males (experiment 1.1) or towards one of the males with an artificial difference in egg-spot number (experiment 1.2), as the actual egg-laying activity towards a male appears to be a better predictor of female preference than *e.g.* time-spent (see e.g. [41]). Our four-way choice experiment, with free contact in combination with paternity testing, corroborates that there is no preference for many egg-spots in *A. burtoni* females. While the different replicates did not reveal conclusive results with respect to a female preference for a certain number or arrangement of male egg-spots, this experiment clearly demonstrates that anal fin egg-spots are not required to attract females and to fertilize eggs.

The results from our female two- and four-way choice experiments contradict previous studies on the role of egg-spots in mate choice [23,28] that did, however, not use the more accurate methods of egg-traps (two-way choice set-up) or paternity testing (four-way choice set-up). One biological explanation for the discrepancy between our results and previous ones might lie in the observation that egg-spot number correlates with the size of a male

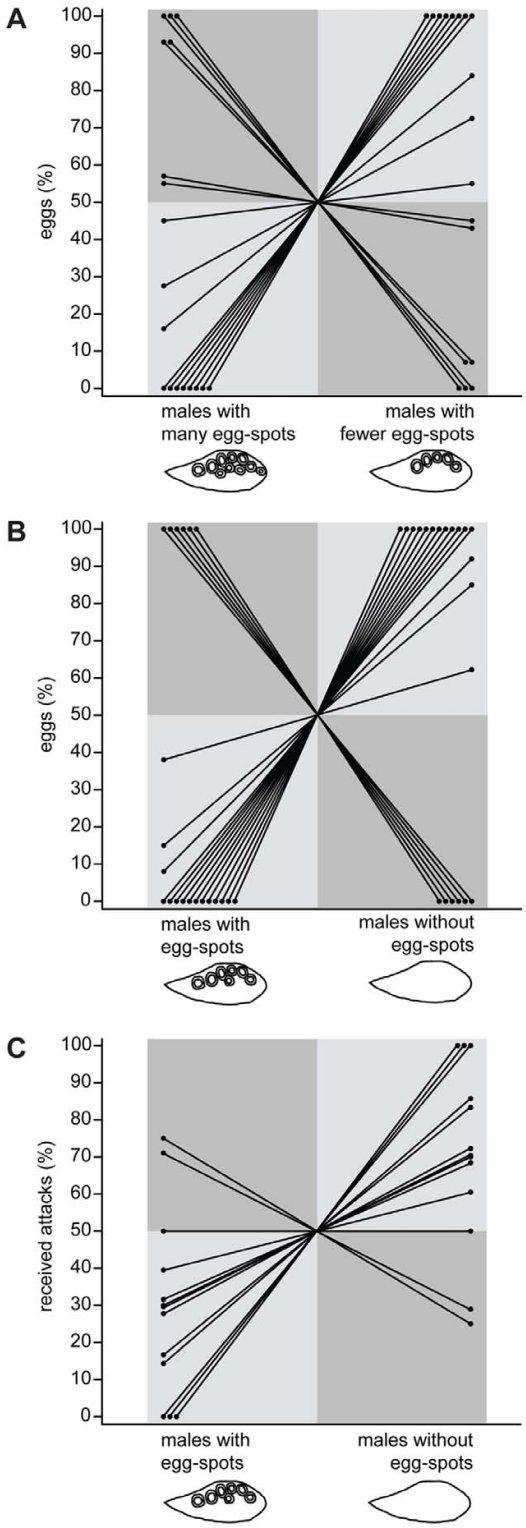

Figure 3. Percentage of eggs and attacks that males received in experiments 1 and 3. Dots connected through lines indicate male pairs used in experiments. Data points in dark shaded squares were coded as 1 (the male with more egg-spots received more than 50% of the eggs of a clutch laid by a female or more than 50% of attacks of the focal male), data points in light shaded squares were coded as 0 (the male with more egg-spots received 50% or less of the eggs of a clutch laid by a female or 50% or less of the attacks of the focal male) for further analyses with generalized linear mixed models (GLMMs). (A) In experiment 1.1, females showed no preference for males with many or fewer egg-spots. (B) In experiment 1.2, females tended to prefer males without egg-spots over males with egg-spots. (C) In experiment 3, focal males attacked stimulus males without egg-spots at a higher rate compared to stimulus males with egg-spots.
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so that larger males generally display more egg-spots in the wild [42]. Females of *Astatotilapia elegans*, which prefer males with many egg-spots to males with fewer spots, also prefer larger males [43], whereas *A. burtoni* females prefer dominant yet smaller males that are more active during courtship [44].

The four-way choice experiment was also designed to detect possible differences in the number of fertilized eggs per clutch (fertilization rate) between the four males with distinct anal fin phenotypes. Obviously, there is no effect of number and arrangement of egg-spots on fertilization rate, as the number of fertilized eggs was 100% in nearly every clutch, which is in line with previous experiments in *A. elegans* [23]. Possibly, female haplochromines have fixed the snapping behavior towards the male anal fin after egg-laying and -uptake, so that egg-spots no longer act as visual triggers (*sensu* Wickler [20,21]) – at least in those species tested so far. This is further corroborated by the relative position of the egg-spots on male anal fins, as they often occur at the terminal end rather than close to the genital opening. Moreover, intraspecific variation in egg-spot number (Figure 1B) would not be likely to prevail if egg-spots were necessary for a successful fertilization [34].

The genotyping of offspring and candidate parents in the four-way choice experiments allowed us to test for extra-pair fertilization in *A. burtoni*. Multiple paternity is rather common in haplochromines. A study on several lekking rock- and sand-dwelling species from Lake Malawi, for example, uncovered multiple paternity in almost every brood [45]. Here we show that multiple paternity also occurs in *A. burtoni* (at least under laboratory conditions), but that its frequency is low (10 out of 68 broods) compared to the natural situation in Lake Malawi cichlids.

Taken together, our female mate choice experiments demonstrate that egg-spots in *A. burtoni* do not serve as recognition pattern (at least on short distances) or attraction signal for females, and that they do not maximize fertilization rates. Still, egg-spots are present in most haplochromine species, indicating an important function additionally to the one in intersexual selection. It has previously been suggested that the honesty and genetic variance of a trait are actually easier maintained through male-male competition than through female choice [6]. Our new results indeed point towards a function of egg-spots in an intrasexual rather than an intersexual context: in a combat situation, males without egg-spots suffered from increased attack rates compared to males with intact egg-spots (Figure 3C), suggesting that egg-spots are an honest signal of male quality used in male-male competition (as carotenoid based ornaments [19,22], egg-spots are likely to display health status or aggressiveness). It is important to note that this effect could only be observed when size-matched males were used; overall, the effect of body size, and possibly body weight, outbalances differences in egg-spot number [34]. Differences in

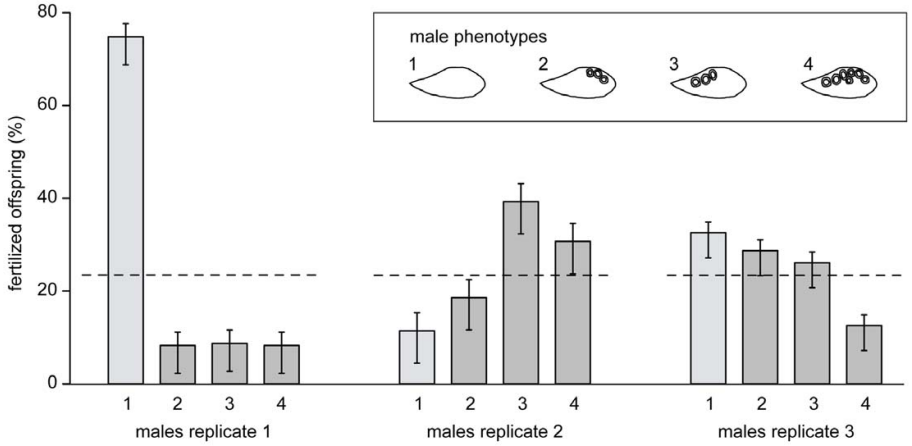

Figure 4. Results of experiment 2 (four-way choice). In replicate 1, females preferred the male without egg-spots (male phenotype 1) over the egg-spot bearing males (phenotype 2, 3 and 4), whereas a more balanced distribution of fertilized eggs was found in replicates 2 and 3. The dotted line indicates the 25% limit, indicating the expected distribution of fertilized offspring under random mating.
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egg-spot number might still have a profound effect in the wild, as males are likely to primarily encounter opponents of similar size. In nature, only 10–30% of the males are territorial [46] resulting in the situation that only the largest sized males are capable of establishing a territory and gain access to females. If those males fight, morphological or behavioral traits other than size – such as the colorful egg-spots – may become important. It has been shown in *A. burtoni* that the attack readiness of males was influenced by specific body patterns - e.g. the head pattern in form of a black bar increases and the orange patch on the cheek decreases number of bites from a competitor [47]. In our study, the stimulus males displayed all patterns known to indicate territorial status (e.g. territorial body coloration, black bar and orange patch) but the lack of egg-spots increased attacks of a competitor.

There are, in fact, other examples from cichlid fishes where coloration or pigmentation signals have an intimidating effect in male combats. Fights in the North American cichlid *Thorichthys meeki* (formerly known as *Cichlasoma meeki*), for example, became more violent when the ornament in form of an eye-spot had been removed [48]. In the Lake Victoria haplochromine genus *Pundamilia*, the advantage of more intensely colored males to win a fight (under white light) vanished under green light conditions masking the carotenoid-based red coloration [49]. Egg-spots in *A. burtoni* appear to exert a similarly intimidating effect on the competitor during threatening and fighting.

Females of several species are known to prefer males with increased carotenoid coloration, which indicates health status: in sticklebacks, for example, the red belly coloration functions as a threat signal in intrasexual competition but also in female mate choice [50]. Note that there are reverted examples, too: in red-collared widowbirds, females select against the male carotenoid display, which also has a dominance function in male-male competition [51], indicating that the display of male dominance and aggressiveness can also have intimidating effects on females. In general, however, such male displays are thought to aid keeping the attack levels in intrasexual competition within limits, as the aggression level and (health) status of rivals may be judged upon these displays [52,53,54]. With respect to the egg-spots of haplochromine cichlids it thus seems likely that the males with more (or more conspicuous) egg-spots are ranked as stronger competitors. These males are then the ones to establish a territory, which in turn gives them opportunity to mate, as social status and mating success are typically correlated in most cichlids [40,55]. That way, females would choose males with more or brighter egg-spots indirectly by choosing males that can pay the competitive cost of gaining a high-quality territory.

Taken together, the involvement of egg-spots in female choice [23,29] and in male aggression (our study) point towards multiple functions of egg-spots in haplochromine cichlids.

Methods

Study species

The cichlid species *Astatotilapia burtoni*, a maternal mouthbrooder, is a generalist living in the estuaries and affluent river systems of Lake Tanganyika, East Africa. As is typical for polygynous mating systems, the species shows sexual dimorphism: males are larger, more intensively colored and their egg-spots are much more pronounced and show, in contrast to female egg-spots, a hyaline circle, which is characteristic for 'true egg-spots' [56]. Phylogenetically, *A. burtoni* is member of a group of riverine haplochromines that are the sister group to the species flock of Lake Victoria region, and, together with the latter, the sister group to the Lake Malawi species assemblage [12,57].

The female test animals were kept in a pure female tank (100×50×50 cm) and the males in mixed-sex stock tanks (100×50×50 cm), from which they were transferred into smaller individual tanks before testing. All tanks provided standardized conditions of constant water temperature of 26°C, pH 7, and a 12:12 h light:dark cycle. Flake food was fed twice a day and frozen artemia was given once a day. Our aquaria strain population, which was used in most of the experiments, originated from an inbred line. The wild caught specimens used in the four-way choice experiment were imported from the Kalambo region in Zambia in 2009.

All laboratory mate choice experiments were performed at the Zoological Institute of the University of Basel under the permission of the Cantonal Veterinary Office, Basel, Switzerland (permit numbers: 2356, 2403). Manipulations on anal fin egg-spots were

performed under clove oil anesthesia (2–3 drops clove oil per liter water).

Experiment 1: female two-way choice

A three-tank set up (see *e.g.* [29,44,58]) was used in order to test female preference based on visual cues alone. Two males were presented, in two outer tanks (40×25×25 cm), to the female, which was placed in a central tank (60×30×30 cm). All tanks were equipped with egg-traps in the form of a plastic grid (eggs would simply fall through the grid so that females could not take them up into their mouth). The outer male tanks contained one shelter (made from a plastic grid) each, whereas three shelters were placed in the central female tank (one on each males' side and one in the center; Figure 2A). Using this set-up, two female two-way choice experiments were conducted. In the first experiment (experiment 1.1) the males' egg-spots differed naturally; we tested males with many (n = 10; egg-spot number mean ± sd = 12.0±2.055, range 9–16) *versus* males with fewer egg-spots (n = 10; egg-spot number mean ± sd = 8.1±0.994, range 6–10). In the second experiment (experiment 1.2) artificial variation was created through experimental manipulations with dry ice to entirely remove all egg-spots ('freeze-branding' method; [23,59]); we tested males with many (n = 11; egg-spot number mean ± sd = 11.7±2.005, range 9–15) *versus* males without egg-spots (n = 11). As a treatment control, both competitors of a trial were freeze-branded, with one male being treated directly on the egg-spots while the other one was treated below the egg-spots. Males of a male pair had similar territorial body coloration and were size-matched (using total length and weight), therefore they did not differ in size nor weight but in the number of anal fin egg-spots (experiment 1.1, Wilcoxon signed-rank test, n = 10: size V = 22, p = 1, weight V = 40, p = 0.221, egg-spot number V = 55, p = 0.005; experiment 1.2, Wilcoxon signed-rank test, n = 11: size V = 35.5, p = 0.859, weight V = 52, p = 0.102, egg-spot number V = 66, p = 0.004; Table S1A). Due to limitations in the number of similar-sized males we used eight male pairs in experiment 1.1 and ten pairs in experiment 1.2 twice. Two pairs in experiment 1.1 and one male pair in experiment 1.2 were tested only once.

To initiate an experimental run, males were given at least 24 hours (in experiment 1.1) or one week (in experiment 1.2 in which recovery from the freeze branding treatment was necessary) to acclimate to the outer tanks and to become territorial (as indicated by nuptial coloration and behavior). Then a gravid female (identifiable through swollen abdomen and enlarged papilla; experiment 1.1: n = 18; experiment 1.2: n = 21; Table S1A) was introduced in the central tank. Female and male behavior was recorded with a video camera (Sony handicam, DCR-HC90E PAL, 3.0 mega pixels). The female was left in the tank until she laid eggs or for a maximum of 7 days and the position of eggs in the egg trap (choice zone next to male 1 (12 cm) *versus* choice zone next to male 2 (12 cm)) was recorded. The percentage of eggs per clutch that a female laid in front of each male was calculated. Due to the fact that often all eggs were laid exclusively next to one male, causing zero inflation in the data, we coded the data as 1 if the male with egg-spots received more than 50% of the eggs and 0 if it received 50% or less of the eggs laid by the female compared to the male with fewer egg-spots (experiment 1.1) or without egg-spots (experiment 1.2). We applied generalized linear mixed models (GLMMs) with a logistic link function (LME4 package [60]), because the response variable was binary (male with egg-spots received more than 50% of the eggs versus male with egg-spots received 50% or less of the eggs). To account for the fact that some pairs of males were used twice, we included male pair as a random factor. We tested whether the probability that a male with egg-spots received more than 50% of eggs was significantly different from 0.5 (i.e. whether the intercept on the logit scale was different from 0). Statistical analyses were performed using the software R, version 2.14.0 [61].

Experiment 2: female four-way choice

This round of experiments made use of the 'partial partition method' (see *e.g.* [16]). A large tank (150×50×50 cm) was divided into five equally sized compartments (30×50×50 cm), which were separated by a plastic grid. The chosen grid-size allowed the smaller females to migrate freely, whereas the larger males were restricted to a single compartment. In each compartment a halved flowerpot served as territory center and hiding place. The middle compartment served as a resting area for the females (Figure 2B).

Four different male phenotypes were produced by freeze branding: (1) no egg-spots; (2) half the amount of egg-spots (remaining at the end of the anal fin); (3) half the amount of egg-spots (remaining close to the genital opening); (4) all egg-spots present (freeze-branding was done at a different area of the fin as treatment control; Figure 4). The males were checked regularly and freeze-branding was repeated if egg-spot pigments reappeared. For this purpose the females were removed from the experimental tank as long as the males needed to recover from the treatment (between two and seven days).

Three replicates with four males each and constantly 12 to 20 females were conducted; males were matched by size and weight (Table S1B) and swapped regularly between compartments to avoid compartment effects. Once a female was mouthbrooding (Figure 1C) she was caught, measured, fin-clipped (for DNA extraction) and the eggs or larvae were removed from her buccal cavity. Fertilization rate was recorded by estimating the number of fertilized eggs or larvae *versus* unfertilized eggs. The fertilized eggs or larvae were incubated in an Erlenmeyer flask for one to six days until they were developed enough for DNA extraction. DNA of ten larvae of each of the total 68 clutches (replicate 1: 23 clutches, replicate 2: 14 clutches, replicate 3: 31 clutches), their corresponding mothers and the putative fathers were used for paternity testing with at least five available un-linked microsatellite markers using a multiplex approach (Qiagen multiplex kit). We used the following markers: Abur82 [62], HchiST68 [63], Osu22d [64], Ppun5, Ppun7, Ppun21 [65], Pzeb3 [66], UNH130 [67], and UNH989 [68]. The amplified DNA samples were genotyped on an Applied Biosystems (ABI) 3130*xl* genetic analyzer and sized in comparison to LIZ 500(-250) (ABI) internal size standard. Genotypes were determined manually using the Genemapper software (version 1.0, ABI). With this procedure the father of each fry could be determined and it became apparent if multiple paternity occurred. Similarly as in experiment 1, we coded the data as 1 if the three males with egg-spots sired 75% or more of the eggs and 0 if the male without egg-spots sired 25% or more of the eggs. The three replicates were analyzed separately using binomial tests with a probability of 0.75 to check if the three egg-spot bearing males had a benefit and therefore received a higher number of clutches.

Experiment 3: male aggression

This set up consisted of a tank (60×30×30 cm) containing a shelter for the focal male (n = 13) and two transparent plastic cylinders (d = 9.5 cm, h = 27 cm), one for each stimulus male (Figure 2C). The focal male was introduced into the aquarium and allowed to acclimate for at least 24 hours. Then two size-matched males (one with intact egg-spots (n = 8; egg-spot number mean ± sd = 7.8±2.123; range 5–11) and the other without egg-spots (n = 8)) were each placed in cylinders to avoid injuries. Three

stimulus male pairs were used once and five stimulus pairs were used twice in alternating positions. Males of a male pair had similar territorial body coloration and were size-matched (by size (total and standard length) and weight), therefore they did not differ in total length, standard length and weight, but they differed in the number of anal fin egg-spots as described above (Wilcoxon signed-rank test, $n = 8$: total length $V = 22$, $p = 0.641$, standard length $V = 22.5$, $p = 0.575$, weight $V = 19$, $p = 0.945$, egg-spot number $V = 36$, $p = 0.014$; Table S1C). We then recorded the behavior of the focal male towards the two intruders by counting the three aggressive behaviors bites, butts and quivers [34]. These measurements were analyzed for a period of ten-minutes (right after the first interaction of the focal male with a stimulus male) from a one-hour video (Sony handicam; see above). The total number of times an aggressive behavior of one of the three categories was performed was used as a total aggression rate for the analysis. Similar to the analysis of experiment 1, the data were coded as 1 if the male with egg-spots had a higher and 0 if it had the same or a smaller aggression rate as the male without egg-spots. Generalized linear mixed models (GLMMs) with binomial error distribution and male pair as a random factor was used to determine if the focal male reacted differently to males with or without egg-spots.

Supporting Information

Table S1 Measurements taken from test animals. (A) Experiment 1.1 and 1.2. (B) Experiment 2. (C) Experiment 3. (PDF)

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Author Contributions

Conceived and designed the experiments: AT WS BE. Performed the experiments: AT BE. Analyzed the data: AT BE. Wrote the paper: AT WS BE.

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Table S1 Measurements taken from test animals. (A) Experiment 1.1 and 1.2. (B) Experiment 2. (C) Experiment 3.
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A

experiment	type of test group	measurement	range of all individuals min - max	mean of all individuals ± sd	range of the difference between individuals of a pair min - max	mean of the difference between individuals of a pair ± sd
experiment 1.1	males (n_{pairs} = 10, n_{ind} = 20)	total length (mm) weight (g) egg-spots (num)	97 - 114 13.24 - 21.16 6 - 16	105.3 ± 4.22 16.83 ± 1.83 10.05 ± 2.54	0 - 3 0.24 - 2.98 2 - 8	1.6 ± 0.97 1.22 ± 0.91 3.90 ± 1.73
	females (n = 18)	total length (mm) weight before testing (g) weight after testing (g) eggs laid (num)	61 - 83 3.73 - 9.08 3.41 - 8.04 7 - 123	7.12 ± 0.65 6.11 ± 1.62 5.38 ± 1.36 61.06 ± 33.89	- - - -	- - - -
experiment 1.2	males (n_{pairs} = 11, n_{ind} = 22)	total length (mm) weight (g) egg-spots artificial (num) egg-spots original (num)	89.33 - 113.90 8.51 - 20.75 0 - 15 6 - 15	105.29 ± 7.81 16.20 ± 3.78 5.86 ± 6.16 10.55 ± 2.48	0.04 - 9.00 0.42 - 4.29 9 - 15 0 - 7	2.37 ± 2.73 1.45 ± 1.10 11.73 ± 2.00 2.36 ± 3.47
	females (n = 21)	total length (mm) weight before testing (g) weight after testing (g) eggs laid (num)	62.23 - 82.19 3.89 - 9.08 3.23 - 7.71 30 - 133	75.00 ± 5.50 6.91 ± 1.34 6.08 ± 1.22 96.33 ± 31.00	- - - -	- - - -

B

experiment	type of test group	measurement	range of all individuals min - max	mean of all individuals ± sd	range of the difference between individuals of a group min - max
experiment 2 - replica 1	males (n_{group} = 1, n_{ind} = 4)	total length (mm) standard length (mm) weight (g) egg-spots artificial (num) egg-spots original (num)	96.24 - 99.58 75.60 - 79.16 11.94 - 14.32 0 - 11 6 - 11	97.38 ± 1.51 78.23 ± 1.75 12.96 ± 1.21 5.25 ± 4.57 8 ± 2.16	0.38 - 3.34 0.05 - 3.56 0.00 - 2.38 2 - 11 1 - 5
	females (n = 23)	total length (mm) standard length (mm) weight after testing (g) eggs laid (num)	4.60 - 6.34 NA 1.50 - 3.32 15 - 79	5.43 ± 0.48 NA 2.30 ± 0.52 44.35 ± 15.92	- - - -
experiment 2 - replica 2	males (n_{group} = 1, n_{ind} = 4)	total length (mm) standard length (mm) weight (g) egg-spots artificial (num) egg-spots original (num)	85.16 - 86.73 67.10 - 68.72 8.20 - 8.55 0 - 7 6 - 11	85.99 ± 0.65 68.19 ± 0.74 8.3 ± 0.17 3.75 ± 2.87 7.5 ± 2.38	0.28 - 1.57 0.07 - 1.62 0.01 - 0.35 3 - 7 0 - 5
	females (n = 14)	total length (mm) standard length (mm) weight after testing (g) eggs laid (num)	47.87 - 64.51 38.97 - 52.68 1.08 - 3.90 18 - 59	53.36 ± 5.86 43.01 ± 4.86 2.08 ± 0.85 32.21 ± 13.40	- - - -
experiment 2 - replica 3	males (n_{group} = 1, n_{ind} = 4)	total length (mm) standard length (mm) weight (g) egg-spots artificial (num) egg-spots original (num)	97.39 - 99.91 77.38 - 79.86 12.00 - 14.54 0 - 5 5 - 6	98.46 ± 1.16 79.12 ± 1.17 13.46 ± 1.18 2.75 ± 2.06 5.25 ± 0.50	0.28 - 2.52 0.12 - 2.48 0.26 - 2.54 2 - 5 0 - 1
	females (n = 31)	total length (mm) standard length (mm) weight after testing (g) eggs laid (num)	46.40 - 61.35 37.09 - 49.49 1.24 - 3.02 14 - 64	53.98 ± 3.81 43.37 ± 3.52 2.14 ± 0.43 35.39 ± 13.88	- - - -

C

experiment	type of test group	measurement	range of all individuals min - max	mean of all individuals ± sd	range of the difference between individuals of a pair min - max	mean of the difference between individuals of a pair ± sd
experiment 3	stimulus males (n_{pairs} = 8, n_{ind} = 16)	total length (mm) standard length (mm) weight (g) egg-spots artificial (num) egg-spots original (num)	85.77 - 103.43 66.60 - 80.41 7.92 - 15.24 0 - 11 5 - 11	94.22 ± 5.93 73.13 ± 4.90 11.33 ± 2.46 3.88 ± 4.26 7.71 ± 1.90	0.01 - 2.18 0.01 - 2.07 0.21 - 1.18 5 - 11 1 - 4	1.02 ± 0.97 1.43 ± 0.80 0.82 ± 0.48 7.75 ± 2.12 2.17 ± 1.17
	focal males (n = 13)	total length (mm) standard length (mm) weight (g) egg-spots (num)	86.02 - 108.85 66.78 - 87.08 8.19 - 18.38 6 - 11	93.90 ± 7.51 73.14 ± 6.67 10.97 ± 2.88 8.31 ± 1.49	- - - -	- - - -

1.3 Egg-spot pattern and body size asymmetries influence male aggression in haplochromine cichlid fishes

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Original Article

Egg-spot pattern and body size asymmetries influence male aggression in haplochromine cichlid fishes

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Assessing an opponent's strength is an important component of attack strategies in territorial combats between males. Body size is often considered to directly influence an individual's strength, but other honest visual signals may also affect the assessment of opponents. Among such visual signals are the so-called egg-spots, a conspicuous ovoid marking on the anal fin of male haplochromine cichlid fishes, made up of carotenoid-containing and other pigment cells. It has long been assumed that egg-spots are mainly relevant in courtship and spawning behavior, and previous work has focused primarily on their function in intersexual selection. Recently, however, both body size and egg-spots have been suggested to play a role in male–male interactions. To test whether egg-spots function in female choice or whether egg-spots and/or body size function as a predictor of strength and the subsequent attack strategy in male–male interactions, we performed a series of behavioral experiments in the haplochromine cichlid *Astatotilapia calliptera*. The trials revealed a limited involvement of egg-spots in female choice, yet a much stronger influence in male interactions. Territorial males combined information from the strength assessment based on body size and egg-spots to adopt their attack strategies. They launched more attacks against the larger intruder with many egg-spots compared with the smaller intruder without or with fewer egg-spots. Our study provides evidence that egg-spots serve as honest visual signal and that the level of asymmetries in egg-spot pattern and body size determines the relative impact of each trait in strength assessment.

Key words: ***Astatotilapia calliptera*, attack strategy, East African cichlid fishes, egg-spots, female choice, Lake Malawi, male aggression.**

INTRODUCTION

Competition over mates constitutes a key mechanism in the process of sexual selection, either through mate choice by the opposite sex or via contests for mates (Darwin 1859, 1871; Andersson 1994). In many territorial species, for example, one of the sexes—most commonly males—competes for a territory in order to gain access to mating partners. Males have thus evolved a variety of strategies to pursue own interests without investing too much energy into fighting or taking the risk of injuries (Maynard Smith and Price 1973). An important component of such male–male interactions is the evaluation of the strength of an opponent, the so-called "resource holding potential" (RHP), which serves to prevent the escalation of fights (Parker 1974). Body size is a direct predictor of the RHP in intraspecific contests because larger males are usually more likely to win combats (e.g., Fryer and Iles 1972; Tokarz 1985; Crespi 1986; Keeley and Grant 1993; Pavey and Fielder 1996; Jenssen et al. 2005; Odreitz and Sefc 2015). In case body size asymmetry is small between opponents or if body size is not a reliable indicator of strength, other factors included into strength assessment either add to or cancel out the effect of body size (Clutton-Brock and Albon 1979; Beaugrand et al. 1996; Sneddon et al. 1997). Other factors that may influence the assessment of an opponent's strength comprise a wide array of male signals, including conspicuous traits such as ornaments (Berglund et al. 1996). The production and display of ornaments that signal male quality involve costs, which in turn prevent dishonest signaling and therefore incorrect strength assessment (Zahavi 1975).

A prime example for honest visual signals are ornaments based on pigment cells containing carotenoids, which cannot be synthesized de novo by animals and, thus, have to be taken up via diet (Goodwin 1986). The costs arising from carotenoid-based visual signals can be manifold and may include competition for carotenoids in environments with carotenoid-poor food (Hill

reallocation of carotenoids from antioxidant activities and/or other physiological processes to the ornament (reviewed in Svensson and Wong 2011). Therefore, only healthy and strong individuals should be able to afford the costs of carotenoid allocation to visual signals (Lozano 1994, 2001). Consistently, reddish signals in general correlate positively with winning combats throughout the animal kingdom (e.g., Evans and Norris 1996; Pryke et al. 2002; Hill and Barton 2005; Hamilton et al. 2013; Sefc et al. 2015). Moreover, there is growing evidence that red coloration may constitute a general signal of intimidation (e.g., Dijkstra et al. 2005; Pryke 2009). The intimidation effect of red coloration and body size seems to be context dependent, though, and can sometimes be defeated by deploying a high-risk strategy. In male three-spined stickleback, for example, red belly coloration has been shown to intimidate opponents outside a settled territory (Bakker and Sevenster 1983; Baube 1997), but to evoke attacks in territorial males toward more reddish intruders (Ter Pelkwijk and Tinbergen 1937; Tinbergen 1948). Additionally, in some species smaller individuals are more aggressive or even prompt a combat (Moretz 2003; Svensson et al. 2012). The initiation and outcome of a combat can therefore not always be predicted based on the contestants' strength alone because an individual's investment often depends on factors such as the subjective value of the contested resource (see "sequential assessment game" and its extension, Enquist and Leimar 1983, 1987). In other words, individuals will fight more, if the subjective value of resource is higher. Therefore, in different contexts, the same visual signal can either inhibit or evoke aggression.

In this study, we focus on a visual signal that is characteristic to the most species-rich group of cichlid fishes and test whether this carotenoid-based ornament and/or body size function as a predictor of strength and subsequent attack strategy in male–male interactions. The visual signal under investigation is the so-called egg-spot pattern of the East African haplochromine cichlids, which are ovoid markings on the anal fins of males (Salzburger et al. 2007; Santos et al. 2014) (Figure 1a) (note that they can also be found in females but are then usually less elaborate). Previous work on the function of egg-spots has primarily focused on their putative role in female choice. Wickler (1962), for example, suggested

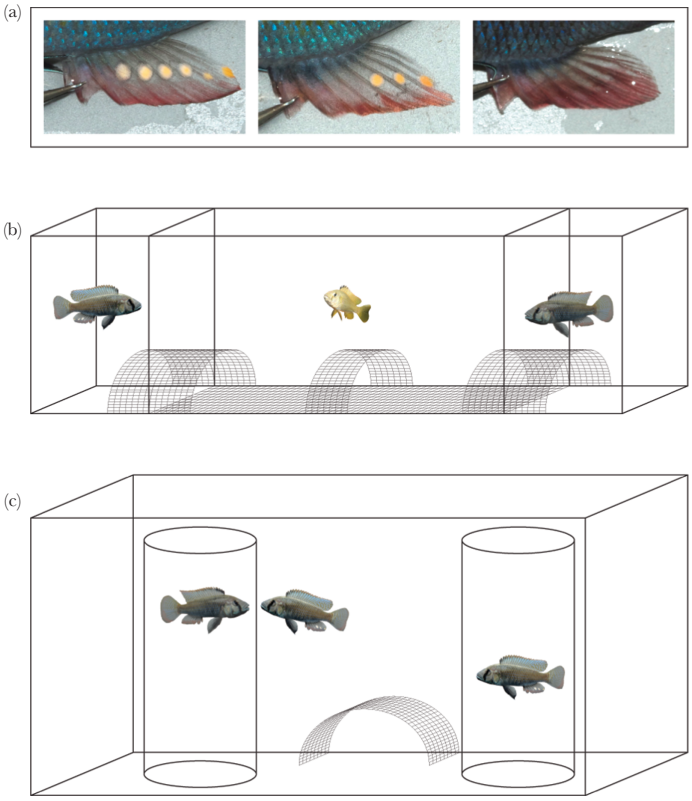

Figure 1
Haplochromine egg-spots and a schematic view of the experimental setups. (a) Egg-spot patterns on male anal fins of *Astatotilapia calliptera* (from left to right: male with many egg-spots; male with few egg-spots; male without egg-spots). (b) The 2-way female choice setup (experiment 1) with the female in the central tank containing an egg-trap and flanked by the stimulus males' tanks (male with many egg-spots vs. male without egg-spots). (c) The setup for the male aggression experiments with the territorial focal male being able to interact with the 2 stimulus intruder males in the plastic cylinders (experiment 2.1: male with many egg-spots vs. male without egg-spots; experiment 2.2: male with egg-spots vs. male with fewer egg-spots).

that egg-spots mimic real eggs, act as releasers for egg-uptake, and maximize fertilization rates. Mate choice experiments in *Astatotilapia elegans* (Hert 1989) and *Pseudotropheus* (*Maylandia*) *aurora* (Hert 1991) revealed that females prefer males with many egg-spots over males with fewer egg-spots. Couldridge (2002), on the other hand, found that *P.* (*M.*) *lombardoi* females preferably choose males with an artificially enlarged egg-spot over males with one natural or many egg-spots. More recently, however, experiments with *Astatotilapia burtoni* demonstrated that females of this species do not show a preference for males with many egg-spots (Henning and Meyer 2012; Theis et al. 2012). Instead, it appears that egg-spots have an intimidating effect in male–male competition in *A. burtoni* (Theis et al. 2012), suggesting that this ornament serves multiple, species-specific functions in haplochromine cichlids. Interestingly, this intimidating effect of egg-spots was not found in the same species during male aggression trials with direct contact between the 2 opponents (Henning and Meyer 2012). The latter study allowed for large asymmetries in body size, though, which was in the end the only trait that determined winning a combat. Taken together, it thus seems that the egg-spot phenotype as well as body size asymmetries of opponents can influence the strength assessment and interact with each other and that the attack strategy is based on the intimidating effect of egg-spots and body size in *A. burtoni*.

Here, we evaluate whether egg-spots function in female choice or in male–male interactions. To this end, we performed a series of behavioral experiments in *Astatotilapia calliptera* (Günther 1893), which represents the Lake Malawi "counterpart" to the previously examined *A. burtoni* from Lake Tanganyika. We first tested, using the same setup as in Theis et al. (2012), whether in *A. calliptera* females also show no preference for males with many egg-spots over males with artificially removed egg-spots (experiment 1). We then examined whether asymmetries in egg-spot pattern alone (experiment 2.1) or in combination with body size asymmetries (experiment 2.2) could be a predictor of strength and subsequent attack strategy, and therefore male aggression, in *A. calliptera*.

METHODS

Study species

Astatotilapia calliptera occurs in shallow, weedy habitats along the shoreline of Lake Malawi, but also inhabits ponds, small lakes, and rivers of its catchment (Konings 2007; Tyers and Turner 2013). With its congener *A. burtoni* from Lake Tanganyika, it shares a generalist lifestyle, the occurrence in lake and stream habitats, and a lek-like breeding system (Theis et al. 2014), in addition to the typical characteristics of haplochromines such as sexual dimorphism, female mouthbrooding, and anal fin egg-spots (Salzburger et al. 2005). The *A. calliptera* test animals used in this study were F1 individuals originating from Chizumulu Island in Lake Malawi, Malawi. Males from this locality display a blue-gray body coloration, which differs from the yellow body coloration of other *A. calliptera* populations (Tyers and Turner 2013).

Females and males were kept in separate tanks ($150 \times 50 \times 50\,cm^3$) providing standardized conditions with constant water temperature (26 °C) and a 12:12-h light:dark cycle. Flake food was fed twice a day, complemented with frozen *Artemia* once a day. The test animals were kept individually in mesh cylinders (d = 16 cm, h = 40 cm) to enable individual identification. All males were photographed (Nikon D5000, Nikon Speedlight SB-900) for later size measurements (Adobe Photoshop CS3 extended, v 10.0.1) and egg-spot counts (a complete egg-spot was counted as 1 and incomplete egg-spots as 0.5; analogous to Albertson et al. 2014). To reduce handling stress, fish were anesthetized during the procedure (3 drops of clove oil per liter water) and were given time to recover before an experimental run (stimulus males at least 2 h, focal males 20 h of acclimation). All experiments were performed at the Zoological Institute of the University of Basel under the permission of the Cantonal Veterinary Office, Basel, Switzerland (permit numbers: 2356, 2403).

Experiment 1: female choice

We used the same experimental setup as in Theis et al. (2012). In each experimental round, we placed a gravid female (n_{female} = 18) in a central tank ($60 \times 30 \times 30\,cm^3$) and allowed visual contact with 2 males with varying egg-spot patterns presented in 2 outer tanks ($40 \times 25 \times 25\,cm^3$) (Figure 1b). The paired males were size matched in standard body length (SL) as precisely as possible ($n_{male\ pairs}$ = 12; $mean_{SL\ difference} \pm$ standard deviation [SD] = 0.97 ± 0.61 mm; $range_{SL\ difference}$ = 0.12–2.12 mm) and introduced at least 20 h before the start of each experimental round to allow for acclimation and territorial behavior to develop. Egg-spots in 1 stimulus male were removed completely ("freeze branding" method; Hert 1986, 1989; see also Theis et al. 2012) but were left unaltered in the other stimulus male ($mean_{egg\text{-}spot\ number\ difference} \pm SD = 4.22 \pm 1.06$; $range_{egg\text{-}spot\ number\ difference}$ = 2.5–6) (Figure 1a). As a treatment control, the unaltered stimulus males were also freeze branded directly above the egg-spots. All manipulations on the anal fins were performed under clove oil anesthesia (3 drops per liter water). In each experimental round, the female was able to see and to interact with both males of the stimulus pair and laid eggs within a period of few hours up to 7 days (the experiment was terminated if the female did not lay eggs within this time period). Because of the grid placed in the aquaria, eggs laid by the female would fall into this "egg-trap" before the female was able to take them up into her mouth for incubation. The egg-trap, which completely covered the floor of the female tank (see Figure 1b), made it possible to assess if the female laid the eggs in front of the male with egg-spots, the male without egg-spots, or in front of both. The position of the eggs laid was used as measure for female preference. Additionally, the interaction time of the female with each of the 2 presented stimulus males was analyzed for the first half an hour (recorded using a Sony handicam HDR-XR550VE, 12.0 mega pixels; analyzed in iMovie, v. 9.0.4) of the experiment to test if females preferably interacted with males with many or without egg-spots and if interaction time correlated with the number of eggs laid.

Because many of the females (9 out of 18) laid their eggs exclusively next to one of the males, the data was coded into 1 and 0 to circumvent the problem of zeroinflation in statistical analyses. The data was coded as 1 if the male with egg-spots received more than or exactly 50% of the eggs and as a 0 if the male with egg-spots received fewer than 50% of the eggs. The binomial data were then analyzed with a generalized linear mixed effects model (GLMM) with a logistic link function using the package lme4 (Bates et al. 2014) in R (version 3.0.3, R Core Team 2014), which was also used for all further statistical analyses. The factor male pair was included as a random effect to account for dependence of the data, that is, the use of 6 male pairs twice. A second model was applied to test if the female spent a different amount of time interacting with one of the 2 stimulus males (note that for models with interaction time sample size is reduced by 1 due to the loss of 1 videotaping). The proportion of time (in seconds) the female interacted in front of the stimulus male with many egg-spots relative to the time in front

of the male without egg-spots was used as response variable in an overdispersed binomial GLMM. An observation level was included as random effect to account for the extravariance in the data. Male pair, as a second random effect, corrected for the dependence of the data due to the repeated usage of the same stimulus male pairs. Using these 2 models, we tested if the intercept on the logit scale was different from 0, which would indicate that the male with egg-spots had a probability significantly higher than 0.5 to receive more eggs (represented as a dashed line in Figure 2a), or more interaction time respectively, than the male without egg-spots. In a third model, we added interaction time as explanatory variable to the above-mentioned first model to test if the choice of egg-laying depended on interaction time.

Experiment 2: male aggression

As described in Theis et al. (2012), the setup to test for male aggression consisted of a tank ($60 \times 30 \times 30\,cm^3$) containing a shelter for the focal male and 2 transparent, perforated plastic cylinders (d = 12 cm, h = 27 cm), one for each of the 2 stimulus males (Figure 1c). The plastic cylinders were used to prevent direct contact between the males, which could lead to injuries. In addition, the plastic cylinders minimize the behavioral response of the opponent through limited space and, hence, prevent asymmetries in the expression of behaviors from opponents, which could influence the aggressive behavior of the focal males (Moore et al. 1997; Wilson et al. 2009). Stimulus males, differing in egg-spot pattern, were always presented in pairs because it is more effective to compare behavioral responses of 1 focal male toward both intruder phenotypes due to among individual differences in aggressiveness reported for many fish species (e.g., Wilson et al. 2011). All males were kept in individual mesh cylinders for at least 3 weeks before the start of the first experiment and a minimum of 24 h between trials if they were used multiple times, to avoid an effect of knowledge about prior fighting success, which might influence the chances of winning (it was previously shown that winner–loser effects persist for no more than 24 h in many fish species, reviewed in Hsu et al. 2006). The focal male was introduced at least 20 h before the experiment to acclimate and to become territorial, which resulted in aggressive attacks as soon as the stimulus males were introduced into the plastic cylinders. The aggressive behaviors as well as the interaction time of the focal males were analyzed for a time period of 30 min. Mouthlocking, bites, butts, circling, displays (frontal and lateral), and quivers (Baerends and Baerends-Van Roon 1950; Fernö 1987) were initially counted separately but added up to the category "attacks" for further analysis due to strong variations in fighting techniques among individuals.

Two different trials were conducted: in a first round (experiment 2.1), the 2 stimulus males were size matched but varied in egg-spot pattern. The egg-spots were completely removed in one of the males and were left unaltered in the other male (n_{focal} = 29; n_{pairs} = 18; $mean_{egg\text{-}spot\ number\ difference} \pm SD = 3.79 \pm 0.97$; $range_{egg\text{-}spot\ number\ difference}$ = 2–5). The stimulus males of a male pair were size matched as precisely as possible among each other ($mean_{SL\ difference\ between\ stimulus\ males} \pm SD = 0.88 \pm 0.74$ mm; $range_{SL\ difference\ between\ stimulus\ males}$ = 0.04–3.27 mm) and with the corresponding focal male ($mean_{SL\ difference\ between\ focal\ and\ stimulus\ males} \pm SD = 1.64 \pm 1.24$; $range_{SL\ difference\ between\ focal\ and\ stimulus\ males}$ = 0.02–5.13). In a second step (experiment 2.2), the presented stimulus male pairs differed in body size, and to a lesser extend as compared with experiment 2.1, in egg-spot number (n_{focal} = 24; n_{pairs} = 17; $mean_{egg\text{-}spot\ number\ difference} \pm SD = 2.52 \pm 0.86$; $range_{egg\text{-}spot\ number\ difference}$ = 0.5–3.5; $mean_{SL\ difference\ between\ stimulus\ males} \pm SD = 6.06 \pm 5.88$ mm; $range_{SL\ difference\ between\ stimulus\ males}$ = 0.39–21.97 mm). The SL of each focal male was in between the 2 corresponding stimulus males ($mean_{SL\ difference\ between\ focal\ and\ average\ of\ the\ corresponding\ stimulus\ males} \pm SD = 1.37 \pm 1.22$ mm; $range_{SL\ difference\ between\ focal\ and\ average\ of\ the\ corresponding\ stimulus\ males}$ = 0.07–4.63 mm).

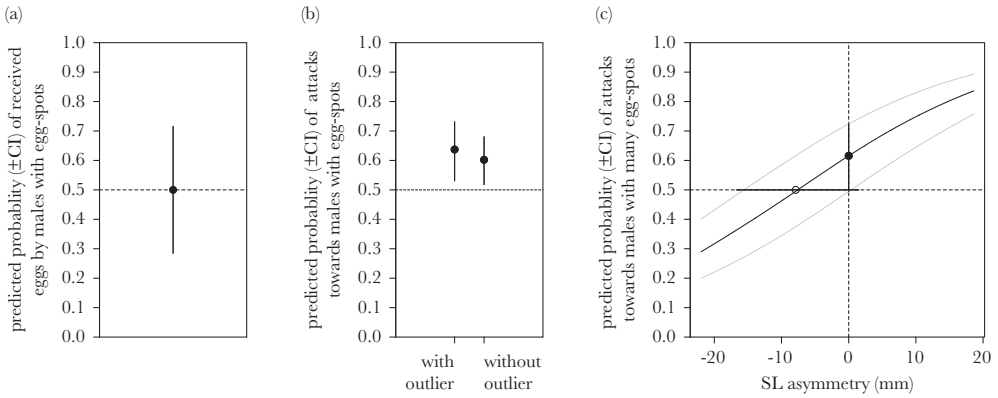

Figure 2
Results of the female choice and male aggression experiments with *Astatotilapia calliptera*. (a) Influence of egg-spot asymmetry on female choice (experiment 1). Predicted probabilities for the stimulus male with egg-spots receiving more of the laid eggs compared with the equally sized male without egg-spots. (b) Influence of egg-spot asymmetry on male aggression (experiment 2.1). Predicted probabilities for the stimulus male with many egg-spots receiving more attacks compared with equally sized male without egg-spots (with and without the outlier). (c) Influence of egg-spots and body size asymmetries (difference between the standard length of the male with many egg-spots and the standard length of the male with fewer egg-spots) on male aggression (experiment 2.2). Predicted probabilities for the stimulus male with many egg-spots compared with the male with fewer egg-spots receiving 1) more attacks at different body size asymmetries (curve), 2) more attacks at equal body size (closed dot), and 3) the same amount of attacks at indicated negative body size asymmetry (open dot).

The recorded number of attacks was grouped into attacks against the male with many egg-spots and attacks against the males with fewer/without egg-spots; the number of attacks against the male with egg-spots relative to the number of attacks against the males without egg-spots was used as a response variable in an overdispersed binomial GLMM. Male pair, as an additional random effect, corrected for the dependence of the data due to the repeated usage of the same stimulus male pairs (experiment 2.1: 11 pairs twice; experiment 2.2: 1 pair twice and 3 pairs 3 times). Although the males were size-matched in the first experiment, body size difference was included as an additional fixed effect in all analyses because even minor size differences are known to influence the outcome of aggressive male–male interactions in cichlid fishes (e.g., Turner and Huntingford 1986). A second model was applied to test if the interaction time of the territorial male differed between the stimulus males. To this end, the same model as described above was adjusted with "interaction time" as response variable instead of "attacks." Using these 2 models, we tested if the intercept on the logit scale was different from 0, which indicates if the male with egg-spots had a probability significantly higher than 0.5 to receive more than 50% of the attacks (represented as a dashed line in Figure 2b,c), or more interaction time, than the male with fewer or without egg-spots. In a third model, we tested if number of attacks correlates with the interaction time. The model as described above was adjusted with interaction time difference as explanatory variable (instead of body size difference).

RESULTS

Experiment 1: female choice

Females showed high variability in total number of eggs laid into the 2 egg-traps (see Supplementary Table 1A for detailed information on egg counts), and 9 out of 18 females laid their eggs exclusively next to one of the males. The male with egg-spots received 50% or more of the eggs in 10 out of 18 trials, resulting in a modeled probability of receiving more eggs in exactly half of the clutches laid (average probability = 0.50, lower confidence interval [CI] = 0.28, upper CI = 0.72) (Figure 2a). Thus, females were not more likely to lay their eggs in front of the male with egg-spots compared with the male without egg-spots (GLMM, $n_{\text{females}} = 18$, $n_{\text{male pairs}} = 12$, $z = 0$, $P = 1$). In the first half an hour of the experiment, females spent more time interacting with the stimulus male without egg-spots compared with the male with egg-spots (GLMM, $n_{\text{females}} = 17$, $n_{\text{male pairs}} = 11$, $z = -2.12$, $P = 0.034$), but this interaction time did not correlate with egg-laying (GLMM, $n_{\text{females}} = 17$, $n_{\text{male pairs}} = 11$, $z = -0.09$, $P = 0.928$).

Experiment 2: male aggression

Focal males were highly active and directed a large amount of attacks toward the stimuli males in all male aggression experiments (see Supplementary Table 1B for detailed information on attack counts). In experiment 2.1, focal *A. calliptera* males allocated significantly more attacks toward the size-matched stimulus male with many egg-spots (experiment 2.1: GLMM, $n_{\text{focal males}} = 29$, $n_{\text{stimulus male pairs}} = 18$, $z_{\text{egg-spots}} = 2.50$, $P_{\text{egg-spots}} = 0.012$, $z_{\text{SL}} = 1.17$, $P_{\text{SL}} = 0.242$). Residual analyses revealed one outlier, in which the focal male directed nearly all attacks against the male with egg-spots (compared with the one without). When the outlier was removed from the analysis, the model revealed additionally that larger males received significantly more attacks (experiment 2.1 without outlier: GLMM, $n_{\text{focal males}} = 28$, $n_{\text{stimulus male pairs}} = 18$, $z_{\text{egg-spots}} = 2.37$, $P_{\text{egg-spots}} = 0.018$, $z_{\text{SL}} = 2.11$, $P_{\text{SL}} = 0.035$). The results of experiment 2.2 support the finding that males with larger body size receive significantly more attacks and indicate a trend that males with many egg-spots receive more attacks by the focal male (GLMM, $n_{\text{focal males}} = 24$, $n_{\text{stimulus male pairs}} = 17$, $z_{\text{egg-spots}} = 1.88$, $P_{\text{egg-spots}} = 0.061$, $z_{\text{SL}} = 2.16$, $P_{\text{SL}} = 0.031$). Thus, the stimulus male with many egg-spots had a modeled average probability of over 0.6 to receive more than 50% of the attacks in all analyses, if the stimulus males were of equal body size (experiment 2.1: average probability = 0.64, lower CI = 0.53, upper CI = 0.73; experiment 2.1 without outlier: average probability = 0.60, lower CI = 0.52, upper CI = 0.68; experiment 2.2: average probability = 0.62, lower CI = 0.49, upper CI = 0.72) (Figure 2b,c). In experiment 2.2, the probability of males with many egg-spots receiving more attacks increased with a larger positive body size asymmetry until these males received all attacks. Complementary, the probability of males with many egg-spots receiving more attacks decreased with a larger negative body size asymmetry. Stimulus males had an equal attack probability if the male with many egg-spots was on average 7.6 mm smaller than the male without or with fewer egg-spots (experiment 2.2: average SL asymmetry = −7.6 mm, lower CI = 1.5 mm, upper CI = −16.7 mm) (indicated by an open dot in Figure 2c).

Focal males also spent more time interacting with the stimulus male with many egg-spots in experiment 2.1. There was a tendency of focal males to interact more with larger males in experiment 2.2 (experiment 2.1: GLMM, $n_{\text{focal males}} = 29$, $n_{\text{stimulus male pairs}} = 18$, $z_{\text{egg-spots}} = 2.60$, $P_{\text{egg-spots}} = 0.009$, $z_{\text{SL}} = 0.74$, $P_{\text{SL}} = 0.460$; experiment 2.1 without outlier: GLMM, $n_{\text{focal males}} = 28$, $n_{\text{stimulus male pairs}} = 18$, $z_{\text{egg-spots}} = 2.06$, $P_{\text{egg-spots}} = 0.042$, $z_{\text{SL}} = 1.019$, $P_{\text{SL}} = 0.306$; experiment 2.2: GLMM, $n_{\text{focal males}} = 24$, $n_{\text{stimulus male pairs}} = 17$, $z_{\text{egg-spots}} = 2.19$, $P_{\text{egg-spots}} = 0.029$, $z_{\text{SL}} = 1.77$, $P_{\text{SL}} = 0.077$). The attacks received by stimulus males correlated with the difference in interaction time of the focal male with the 2 different stimulus males in all experiments (experiment 2.1: GLMM, $n_{\text{focal males}} = 29$, $n_{\text{stimulus male pairs}} = 18$, $z = 8.79$, $P < 0.0001$; experiment 2.1 without outlier: GLMM, $n_{\text{focal males}} = 28$, $n_{\text{stimulus male pairs}} = 18$, $z = 8.65$, $P < 0.0001$; experiment 2.2: GLMM, $n_{\text{focal males}} = 24$, $n_{\text{stimulus male pairs}} = 17$, $z = 16.85$, $P < 0.0001$).

DISCUSSION

The assessment of an opponent's strength is an important mechanism to determine the subsequent attack strategy (Parker 1974). In male–male competitions, the strength of an opponent is often evaluated based on body size, but can also be based on other traits, for example, ornaments (Berglund et al. 1996). In this study, we examined if the carotenoid-based ornament egg-spot and/or body size function as predictors of strength and the subsequent attack strategy in male–male interactions in haplochromine cichlid fish. Additionally, female choice experiments were conducted on the same males because previous work on the function of egg-spots has primarily focused on a role of egg-spots in female choice (e.g., Hert 1989, 1991).

The experiments with the East African cichlid *A. calliptera* presented here revealed limited involvement of egg-spots in female choice when males were size matched, but rather an influence in male interactions, which is in line with our previous results in *A. burtoni* (Theis et al. 2012). However, whereas territorial *A. burtoni* males preferably attacked the presumably weaker stimulus males with fewer egg-spots, *A. calliptera* males adopted an attack strategy spending more time and,

hence, launching more attacks against the male with many egg-spots. In addition, we found that there were more attacks against males with larger body sizes in stimulus pairs with body size asymmetries. The different attack strategies deployed by the 2 *Astatotilapia* species when presented stimulus males with asymmetries in egg-spot pattern, and body size might be explained by different resource values. According to the extension of the "sequential assessment game" theory, weaker or smaller individuals tend to attack stronger or larger competitors if the resource value is higher (Enquist and Leimar 1987)—especially if there are few or no other opportunities to obtain new resources (the "desperado effect," Grafen 1987). Alternatively, the reason leading to the subsequent difference in attack strategy might already be based in the process of strength assessment as such. For example, other color patterns or behaviors could represent additional factors influencing strength assessment in the 2 species, which were not examined in this study. Moreover, the strength assessment based on egg-spots could differ between the species. The seemingly lower intimidation effect of egg-spots in *A. calliptera* compared with *A. burtoni* is most probably connected to the invested costs. *A. calliptera* males have fewer egg-spots (Supplementary Figure A1B in Supplementary Appendix 1), and their egg-spots are less pronounced (Supplementary Figure A1C in Supplementary Appendix 1), suggesting that they might invest less into egg-spots than *A. burtoni* males do. The costliness of egg-spot conspicuousness could also be environmentally induced by being more conspicuous to predators (Goldschmidt 1991) or physiologically, for example, through differences in type and/or density of pigments or different metabolic pathways to produce these pigments (Sefc et al. 2014). However, the possible lower investment costs in *A. calliptera* compared with *A. burtoni* seem to be high enough for egg-spots to constitute a signal of strength evoking attacks. Intruder *A. calliptera* males showing egg-spots always received more attacks if they were larger, similar sized or even slightly smaller than the males with no egg-spots. The effect of egg-spot pattern asymmetry was only overcome by the effect of body size asymmetry if the male with many egg-spots was around 10% smaller than the male with fewer egg-spots (e.g., by approximately 8 mm in experiment 2.2, see Figure 2c). Note, however, that these values should be taken with caution because freeze branding has artificially induced egg-spot variation in our experiment, which might therefore deviate from a setting involving natural variation of egg-spots. Generally, the more similar the contestants are in body size and weight, or the less those traits are used to estimate strength in a species, the more important are asymmetries of other factors (see e.g., Beaugrand et al. 1996). Previous experiments in green swordtail fish (*Xiphophorus helleri*) showed that body size asymmetries of 20–30% are necessary to eliminate other advantages such as prior social experience and prior residency (Beaugrand et al. 1996). Nevertheless, also minor differences, for example, 1 mm in body size (Turner and Huntingford 1986) and few percentages of weight (Barlow et al. 1986; Enquist and Jakobsson 1986) were shown to influence the outcome of combats in cichlids.

Our findings and the above-mentioned examples show that strength assessment and attack strategy can differ greatly between species and, in addition, depend on the experimental setup. The latter could also explain the different outcomes in aggression trials with *A. burtoni* by Henning and Meyer (2012) and Theis et al. (2012). First, the 2 studies differed in the combat setup, with 1 territorial male and 2 intruders (Theis et al. 2012) versus 2 males interacting in a direct combat (Henning and Meyer 2012). Second, one study combined large egg-spot asymmetries with small body size asymmetries (Theis et al. 2012), whereas the other study combined small differences in egg-spot number with larger body size and especially weight differences (see Supplementary Figure S2 in Henning and Meyer 2012). The large body mass asymmetries together with the direct interaction in the study of Henning and Meyer (2012) could possibly explain the fact that body size alone determined the outcome of a combat rather than egg-spot number.

Taken together, these studies suggest that both egg-spot pattern and body size asymmetries influence the strength assessment in *A. burtoni* (Henning and Meyer 2012; Theis et al. 2012) as well as in *A. calliptera* (this study) and that egg-spot asymmetries become more important as the difference in body size between contestants becomes smaller. However, despite the high similarity in lifestyle, the 2 species use different attack strategies. The causes leading to the observed attack strategy in *A. calliptera* could be higher resource value and/or lower intimidating effect of egg-spots compared with *A. burtoni*.

Further support for the hypothesis that the level of intimidation induced by egg-spots could be lower in *A. calliptera* than in *A. burtoni* is provided by the results of the female choice experiments. Females of *A. burtoni* tended to lay eggs in front of males without egg-spots, which could have been due to avoidance of males with egg-spots, which were perceived as more aggressive. In *A. calliptera*, this effect of intimidation seems to be lower because females indeed preferred to interact more with the males without egg-spot at the beginning of the experiment, but showed random mating with respect to the number of eggs laid during the experiment. As several studies have shown, interaction time or time spent does not necessarily predict mate choice (e.g., Kidd et al. 2006), and females may not reveal their mating preferences until the day on which spawning takes place (Kidd et al. 2013). The random mate choice of *A. calliptera* females based on egg-spots might be explained by the lek-like mating system. In this situation, females might choose males indirectly because they either prefer to mate with clustered males ("female preference model"; Bradbury 1981), or with the most superior males in the lek ("hotshot model," Beehler and Foster 1988), or males just formed the lek in areas with high concentration of females ("hot-spot model," Bradbury and Gibson 1983). Alternatively, females might choose directly by assessing males based on other characteristics apart from egg-spots, which were not examined or were excluded in our experimental setup. In our experiments, for example, females were not given the choice between differences of the stimulus males in territory quality, body size, and nonvisual cues. In *A. burtoni*, body size and chemical cues are more likely to affect female choice (Kidd et al. 2013) than egg-spots, for which no preference was found (Henning and Meyer 2012; Theis et al. 2012). In addition, we cannot rule out a putative importance of egg-spots in female choice in our tested species under different conditions. Further tests should be conducted to see if egg-spots could become important in the fertilization process in case of sperm limited males and/or under different environmental conditions, for example, turbid water conditions or strong water current. Under such scenarios, egg-spots could become crucial to ensure close proximity of females with unfertilized eggs next to the male genital papilla during sperm release.

In contrast to the results presented here, the females of some haplochromine species base their mating preference on egg-spot number (Hert 1989, 1991) or egg-spot size (Couldridge 2002). Supposedly, cichlid egg-spots evolved via a female sensory bias (Egger et al. 2011), which suggests an ancestral function in female choice, with a subsequent evolution to multiple functions, for example, species recognition (Axelrod and Burgess 1973) and/or in male interactions (Theis et al. 2012; this study). Until now, no species is known in which egg-spots have a dual function as was shown for other carotenoid-based male ornaments (e.g., Candolin 1999; Griggio et al. 2007). Of course, this

might reflect the situation that, so far, only very few haplochromine species have been subjected to experiments testing for both (and either factor). Nevertheless, our findings together with the previously suggested functions of egg-spots in other cichlid species and the high diversity in egg-spot number, shape, and coloration within and among species (personal observation) show the high flexibility of this trait with respect to function, persistence, and appearance. Furthermore, the observed function of egg-spots in male aggression supports the hypothesis that the process of intrasexual selection on male coloration has played a role in the astonishing radiation of haplochromine cichlids (reviewed in Dijkstra and Groothuis 2011).

In summary, egg-spots constitute an extraordinary example of a color ornament, which evolved in manifold directions with regard to functions in female choice and male–male competition. We have shown that egg-spots are used in quality assessment of competitors, with egg-spots becoming more important as the difference in body size between contestants becomes smaller. Further knowledge on the function of egg-spots over a broader range of haplochromine species could reveal links between their function, pattern, coloration, the species' mating behavior, and their environment.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/

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Table S1 Detailed information of means and ranges of counted eggs in the the female choice experiment (A) and counted attacks in male aggression experiments (B).

A

egg counts	experiment 1	
	mean +-sd	range
eggs total (in both egg traps)	28.17 ± 15.70	4 – 54
eggs next to male with egg-spots	11.78 ± 13.80	0 – 48
eggs next to male without egg-spots	16.39 ± 18.42	0 – 54

B

attack counts	experiment 2.1		experiment 2.1 without outlier		experiment 2.2	
	mean ± sd	range	mean ± sd	range	mean ± sd	range
attacks total (towards both stimuli males)	373.40 ± 93.92	209 – 586	369.96 ± 93.79	209 – 586	284.56 ± 88.19	115 – 449
attacks towards male with egg-spots	214.79 ± 86.96	89 – 468	205.75 ± 73.36	89 – 339	163.84 ± 83.02	41 – 418
attacks towards male without egg-spots	158.59 ± 88.99	1 – 394	164.21 ± 85.21	14 – 394	120.72 ± 85.45	15 – 277

APPENDIX I

Egg-spot differences between *Astatotilapia calliptera* and *Astatotilapia burtoni*

Males of *A. calliptera* and *A. burtoni* (Fig. A1A) were compared with regard to egg-spot number and egg-spot hue. In a first step egg-spot number were counted on photographs, which were taken before the freeze-branding manipulation. The numbers of egg-spots were compared between the species with a generalized linear model (GLM) with the package lme4 (Bates et al. 2014) in the statistical software R (version 3.0.3, R Core Team 2014). The tested males showed an average of four egg-spots in *A. calliptera* (n = 42; average egg-spot number ± sd = 4.21 ± 0.87), whereas *A. burtoni* males possess an average egg-spot number of eight (n = 27; average egg-spot number ± sd = 8.07 ± 1.80). Therefore the tested *A. calliptera* males possess significantly fewer egg-spots compared to males of *A. burtoni* (GLM; $z = -6.426$, $p < 0.0001$) (Fig. A1B).

In a second step, the hue of the first egg-spot on the anal fin was measured for five individuals per species. Reflectance spectra of the colored egg-spot area were taken in the aquaria facilities using a JAZ modular portable spectrometer (Ocean Optics; wavelength range 300-980 nm) with an integrated, pulsed Xenon lamp module (OCOJAZ-PX) and an OCOWS-1 diffuse reflection standard, following the methods described in Gray et al. (2011). Between five to six reflectance spectra were measured for each specimen.

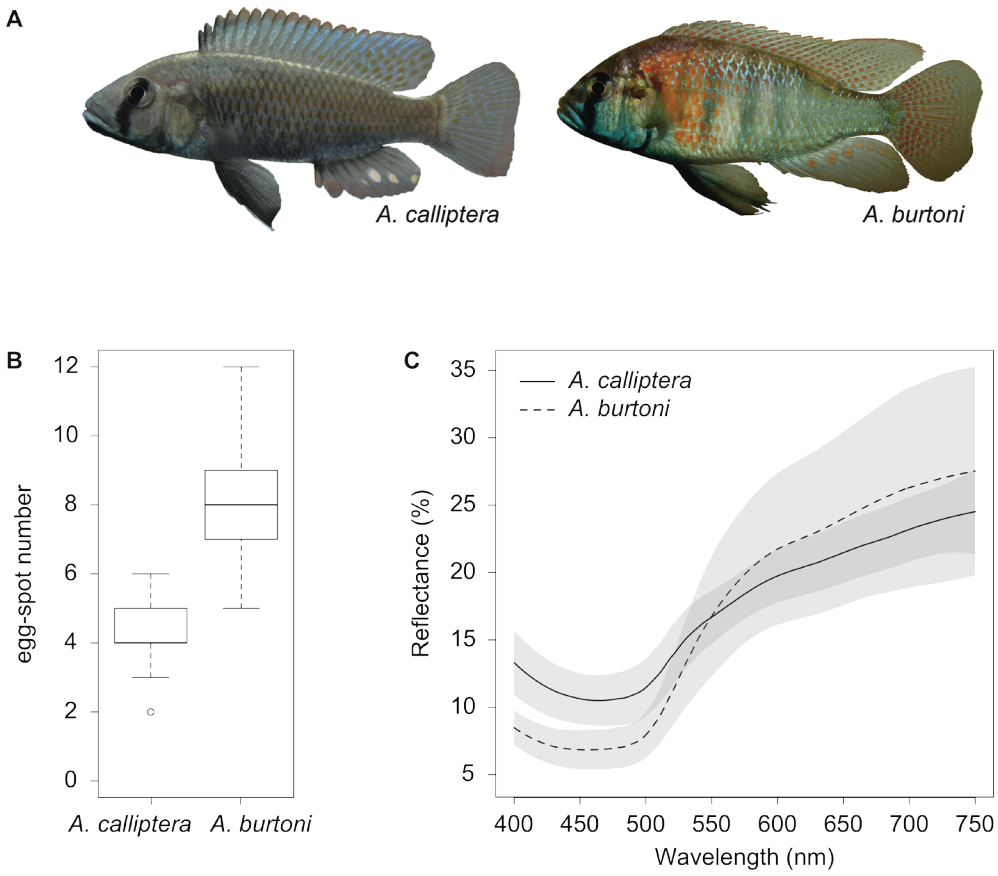

Figure A1 Egg-spot differences between males of *Astatotilapia calliptera* and *Astatotilapia burtoni*. (A) Males of *A. calliptera* and *A. burtoni* showing egg-spots on their anal fins. (B) Difference in number of egg-spots. (C) Difference in hue of egg-spots.

Spectral files were inspected and processed using the R package pavo (Maia et al. 2013). Wavelengths were interpolated in 1-nm bins and considered from 400 to 700 nm. Spectra from individuals belonging to the same species were combined and averaged. Finally, the data was smoothed and spectral curves were plotted for each species. *Astatotilapia calliptera* shows more yellowish egg-spots, whereas the egg-spot of *A. burtoni* seem to be more orange and brighter (cut-on step reflectance occurring at around 500 nm indicate yellow and 550 nm indicate red; Marshall 2000) (Fig. A1C).

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Part 2

divergence in lake-stream systems

2.1 Variation of anal fin egg-spots along an environmental gradient in a haplochromine cichlid fish

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Most authors were involved in fieldwork. I conducted data acquisition on egg-spot characteristics from the photographs, analysed the data thereof, and designed all figures and tables. Further, I wrote the first draft of the manuscript, which was refined together with BE, WS, RO and CF.

Variation of anal fin egg-spots along an environmental gradient in a haplochromine cichlid fish

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Abstract

Male secondary sexual traits are targets of inter- and/or intrasexual selection, but may vary due to a correlation with life-history traits or as by-product of adaptation to distinct environments. Trade-offs contributing to this variation may comprise conspicuousness towards conspecifics *versus* inconspicuousness towards predators, or between allocating resources into coloration *versus* the immune system. Here, we examine variation in expression of a carotenoid-based visual signal, anal-fin egg-spots, along a replicate environmental gradient in the haplochromine cichlid fish *Astatotilapia burtoni*. We quantified egg-spot number, area, and coloration; applied visual models to estimate the trait's conspicuousness when perceived against the surrounding tissue under natural conditions; and measured immune activity. We find that (*i*) males possess larger and more conspicuous egg-spots than females, which is likely explained by their function in sexual selection; (*ii*) riverine fish generally feature fewer but larger and/or more intensively colored egg-spots, which is probably to maintain signal efficiency in intraspecific interactions in long-wavelength shifted riverine light conditions; and (*iii*) egg-spot number and relative area correlate with immune defense, suggesting a trade-off in the allocation of carotenoids. Taken together, haplochromine egg-spots feature the potential to adapt to the respective underwater light environment, and are traded-off with investment into the immune system.

Introduction

Male secondary sexual traits constitute what are amongst the most conspicuous characters in animals and often play a key role in female choice and male-male competition (Darwin 1871; Espmark et al. 2000; Andersson 1994). Signals that aim to attract mating partners and to intimidate rivals are considered 'honest' if comprising a handicap and if being costly to display and/or to produce (Zahavi 1975; Iwasa et al. 1991; Iwasa and Pomianowski 1999; but see, e.g., Számadó 2011 for other models of honest signaling). According to the 'handicap principle', displaying an honest signal should reflect the overall quality of its bearer (Zahavi 1975; Andersson 1994; Rowe and Houle 1996; but see Fisher 1930; Lande 1981; Kirkpatrick and Ryan 1991; Kokko et al. 2006). Importantly, variation in the expression of an honest signal is not expected to be purely under genetic control, but should instead correlate with life-history traits such as age, nutritional status, social status, or parasite load (Kodric-Brown and Brown 1984; van Noordwijk and de Jong 1986; de Jong and van Noordwijk 1992). Further, phenotypic divergence in such signals can emerge as by-product of adaptation to distinct environmental niches (Nosil 2012), since the traits are expected to evolve to a point where viability costs balance out mating advantage (Endler 1978; Jennions et al. 2001). Thus, variation in visual, acoustic and chemical signals can be affected by a wide array of environmental parameters.

A key component in visual signaling is the conspicuousness of the signal as it influences the perceptibility of the visual signal to the potential receivers such as mates and intraspecific rivals, but also interspecific

competitors and, in particular, predators (Endler 1992). High predation pressure is often accompanied by a reduction in conspicuousness of signal expression (Endler 1980; Stuart-Fox and Ord 2004; Schwartz and Hendry 2007), whereas reduced visibility may lead to increased conspicuousness of visual signals, most probably to maintain their function in intraspecific interactions (Marchetti 1993; Zahavi and Zahavi 1997; Kekäläinen et al. 2010; Dugas and Franssen 2011). However, especially in aquatic environments, reduced visibility can also decrease conspicuousness of visual signals, for example when intraspecific receivers reduce their responsiveness to visual signals and/or when investing into this costly trait is maladaptive (e.g. Seehausen et al. 1997, 2008; Wong et al. 2007; Luyten and Liley 1991; Maan et al. 2010; Boughman 2001). Additionally, the size, shape or coloration of visual displays can be influenced by the physical or chemical properties of habitats (e.g. Endler and Houde 1995; Hill and Montgomerie 1994; Moller 1995; Candolin et al. 2007). In case of carotenoid-based visual signals, for example, the expression might be directly influenced by the accessibility to food resources, since carotenoids cannot be synthesized *de novo* by animals and thus have to be obtained through food (Goodwin 1986). The conspicuousness of carotenoid based visual signals should therefore reflect the ability to feed successfully on carotenoid-rich food (Hill 1992) – or even more likely – to be an indicator of the bearer's health, since carotenoids are also used as antioxidants in immune responses (Lozano 1994, 2001; von Schantz et al. 1999; Svensson and Wong 2011; Simons et al. 2012). Consequently, using carotenoids for signaling instead of the immune system is considered to be costly. Under stressful conditions carotenoids may therefore primarily be invested into the immune response or, alternatively, they may be allocated to offspring (Sheldon and Verhulst 1996) or to other life-history traits such as general fitness (Smith et al. 2007) and survival (Pike et al. 2007).

Taken together, visual signals can be shaped by both, sexual selection and a broad range of environmental and physiological factors. Examining the contribution of environmental factors on signal expression in nature is challenging, though, but has been successfully studied with respect to color patterns in some species (Endler 1980). A promising set-up to study the influence of natural selection on color patterns consists of populations of a species displaying secondary male ornaments that occur, in replication, along a marked environmental gradient. Such a setting can be found in the haplochromine cichlid species *Astatotilapia burtoni* (Günther 1894), which occurs both in East African Lake Tanganyika and inflowing rivers. This generalist species displays typical haplochromine features such as sexual dimorphism, female mouthbrooding and egg-spots, i.e. a characteristic carotenoid-containing visual signal and evolutionary innovation (Goldschmidt and de Visser 1990; Salzburger et al. 2005; Santos et al. 2014). Egg-spots are ovoid markings on the anal fin of haplochromines primarily composed of two types of chromatophores (xanthophores and iridophores) (Salzburger et al. 2007; Santos et al. 2014) (Fig. 1A). In male haplochromines egg-spots consist of a conspicuously colored yellow, orange or reddish inner circle and a transparent outer ring (Wickler 1962). The function of anal fin egg-spots has initially been attributed to female choice (Wickler 1962; Hert 1989, 1991; Couldridge 2002) or – more recently in the species examined here – to male-male competition (Theis et al. 2012, 2015). *Astatotilapia burtoni* exhibits a lek-like polygynandrous mating system, with only dominant males gaining access to territories as well as to females (Fernald and Hirata 1977). Moreover, egg-spots appear to play a pivotal role in interactions among males, as they appear to have an intimidating effect in *A. burtoni* (Theis et al. 2012). In both female choice and male-male competition, males are expected to benefit from adapting signal conspicuousness to be effective within their respective environment. Indeed, most haplochromine cichlids from Lake Victoria display fewer but larger and hence, more conspicuous egg-spots in more turbid waters (Goldschmidt 1991). Contrarily, in *Pundamilia pundamilia*, also a haplochromine from Lake Victoria, populations show a trend towards less conspicuous egg-spots with respect to saturation and hue in more turbid waters (Castillo Cajas et al. 2012).

In this study, we focus on the natural variation of egg-spots within and among four lake-stream systems of *A. burtoni*. Previous work has demonstrated that populations from replicate lake-stream systems show similar adaptations to divergent selection regimes with regard to body shape and trophic structures (Theis

et al. 2014). Importantly, the detected trait differences among populations do not reflect pure plastic responses to different environmental conditions, but have a substantial genetic component (Theis et al. 2014). Here, we first explored sex-specific differences in egg-spots by comparing egg-spot number, relative average area, relative total area and coloration inferred from photographs of fish. Due to the proposed function of egg-spots in male-male competition (Theis et al. 2012, 2015), males were expected to display more, larger and more intensely colored egg-spots compared to females. To ascertain habitat-specific differences, the same egg-spot characteristics were then compared among males of the different lake and stream populations. We hypothesized that egg-spot characteristics from replicate lake-stream systems would follow similar trajectories along this environmental gradient. We then examined how the underwater light environment and the status of the immune system affect the conspicuousness of male egg-spots. To this end, we measured immune activity of males and underwater light environments from lake and stream populations and asked whether these factors were associated with divergence in the egg-spot characteristics number, relative average area, relative total area and coloration based on photographs. Finally, reflectance and irradiance spectrophotometry and theoretical fish visual models were used to determine the color contrast between male egg-spots and the surrounding anal fin tissue under natural ambient light conditions. We hypothesized that males from longer wavelength shifted environments, and/or males experiencing less stress to the immune system, would display the most conspicuous egg-spots.

We found sex- and habitat-specific differences in egg-spots of *A. burtoni*. Males had more elaborate egg-spots compared to females, and are likely to use them as honest signals with the potential to adapt their conspicuousness according to underwater light environment and immune defense. This study provides novel insights into the highly complex interactions between sexual and ecological selection that influence the expression of male secondary visual signals.

Methods

Sampling

Astatotilapia burtoni specimens, underwater ambient light measurements and immunological data were obtained between June 2011 and August 2013 from the Southern part of Lake Tanganyika, Zambia. In total, we sampled at 11 locations from four lake-stream systems (Fig. 1B; for detailed description of these localities see Appendix I in Theis et al. 2014), resulting in a dataset comprising 643 individuals (for detailed information on sample sizes see Table S1). Fish were collected using hook and line fishing, minnow traps and/or gill nets under the permission of the Lake Tanganyika Research Unit, Department of Fisheries, Republic of Zambia.

Egg-spot measurements based on photographs

Before taking the photographs, the fish ($n_{females\ per\ population}$ = 6 – 39; $n_{females\ total}$ = 204; $n_{males\ per\ population}$ = 10 – 55; $n_{males\ total}$ = 300; for detailed information on sample sizes see Table S1) were anaesthetized with clove oil (2 - 3 drops per liter water) to reduce stress of handling. Two standardized photographs per individual were taken, one in lateral position to measure body size, and one focusing on the anal fin for subsequent measurements of the egg-spot characteristics (Fig. S1). All images were taken on a grey card to allow for manual white balance. We used digital cameras (Canon EOS 400D, Canon EOS 550D or Nikon D5000) with an external flash (Nikon, Speedlight SB-24).

To assess body size of fish, we recorded 17 homologous landmarks on the full body photographs (for details see Muschick et al. 2012) in the program tpsDIG (v.2.11; Rohlf 2008) followed by a transformation into centroid size in MorphoJ (v.1.05f; Klingenberg 2011). Centroid size was then used as the representative measure for body size. The photographs were further used to assess egg-spot number, relative average egg-spot area, relative total egg-spot area and egg-spot coloration. To this end, egg-spot and anal fin areas were measured using the lasso tool in Photoshop (Adobe Photoshop CS3 extended, v.10.0.1). The

relative total egg-spot area was defined as the proportion of the anal fin area occupied by the pigmented egg-spot area. The relative average egg-spot area was calculated as the relative total egg-spot area divided by the relative number of egg-spots (a complete egg-spot was counted as 1 and incomplete egg-spots as 0.5; following Albertson et al. 2014) to avoid artifacts through smaller - still growing egg-spots - typically at the edge of the anal fin.

In addition, egg-spots were assigned to one of six color categories by AT ranging from a faint, barely pigmented to an intense appearance. The color categories (referred to as coloration from here on) therefore describe the conspicuousness of egg-spots based on a combination of hue, saturation and brightness (representative photographs of the color categories are provided in Fig. S2). Since every specimen displayed more than one egg-spot, an average value was calculated for each individual. Although coloration was defined by a categorical measure, it reflected a continuous variable after calculating the average value across all egg-spots for each specimen.

The differences in egg-spot measurements based on photographs (number, relative average area, relative total area and coloration) were analyzed in two steps: (*i*) sex-specific differences of egg-spots in all populations combined and (*ii*) habitat-specific differences of egg-spots among males of lake and stream populations within each system.

In order to test for differences in egg-spot characteristics between females and males, we conducted sex-specific centering and scaling of the data with respect to centroid size. This was necessary since *A. burtoni* shows pronounced body size dimorphism between males and females (Fernald 1977). Our aim here was to compare average sized females to average sized males (and not same sized females and males). A generalized linear mixed model (GLMM) with Poisson distribution was used in the case of egg-spot number and normal linear mixed models (LME) with ANOVA comparison were used for relative average egg-spot area (square root transformation), total egg-spot area and coloration data. Analyses were conducted using the package LME4 (Bates et al. 2014) in R (version 3.0.3, R Core Team 2014), which was also used for all further statistical analyses. The linear models included population (separately for each sex) as a random effect and were combined with a random slope (the centered body size) in cases where this improved the model (based on ANOVA comparisons). Additionally to the fixed effect sex, the centered body size and/or the interaction thereof was added if necessary (for details on the models see Table S2A).

Before the habitat-specific differences in the egg-spot characteristics were analyzed in detail, we tested for the biggest differences among populations with regard to egg-spot phenotype in males. To this end, we conducted a principal component analysis (PCA) with the function PRCOMP of the R package STATS for the combined egg-spot characteristics (number, relative average area, relative total area and coloration). Due to the large sample size we calculated the mean PC loadings per population for graphical illustration.

Habitat-specific differences of egg-spot characteristics were then analyzed among males of lake populations in comparison to the corresponding stream populations. A generalized linear model (GLM) with Poisson distribution was used in the case of egg-spot number, and normal linear models (LM) were used for relative average area, relative total area and coloration (with square transformation) data. Additionally to the fixed effect population, we included body size as a fixed effect if it improved the model (for details on the models see Table S2B). To correct for multiple comparisons, the function GLHT from the package MULTCOMP (Hothorn et al. 2008) with mcp specification (population comparisons within system) was used, with a correction for variance heterogeneity (vcov argument with sandwich function of the package sandwich; Zeileis 2004, 2006) for the egg-spot characteristics number, relative average area and relative total area, but not for coloration.

Egg-spot reflectance and theoretical fish visual models

Theoretical fish visual models (Vorobyev and Osorio 1998; Vorobyev et al. 2001) from the perspective of *A. burtoni* were used to measure the color contrast (color distance; ΔS) between male egg-spots and the surrounding anal fin tissue under natural ambient light conditions. For this purpose, specimens were

caught in 2013 from each locality ($n_{males\ per\ population}$ = 4 – 9, $n_{males\ total}$ = 45) except for the populations ChL, Ch1 and Lf1 (for detailed information on sample sizes see Table S1). Immediately upon collection, fish were anaesthetized with clove oil (2 - 3 drops per liter water) and reflectance spectra of the second egg-spot and the area above the egg-spots on the anal fin of males (see Fig. 1A) were taken in the field using a JAZ Modular Portable Spectrometer (Ocean Optics; wavelength range 300 – 980 nm) with an integrated, pulsed Xenon lamp module (OCOJAZ-PX) and an OCOWS-1 diffuse reflection standard according to the methods described in Gray et al. (2011). Between four to six reflectance spectra were taken per area and specimen. Spectral files were visually inspected and processed using the R package PAVO (Maia et al. 2013). Wavelengths were interpolated in 1 nm bins over a spectral range from 400 - 750 nm. Spectra from egg-spot and fin measurements were combined and averaged for each individual. To account for the light environment under which egg-spots are viewed, we modeled color discrimination using natural illumination measurements for each population taken from their environment at different water depths (see irradiance measurements below; Fig. S3). Whereby, using natural illumination measurements as part of the model, allows us to recreate what egg-spot colors look like in their environment independent of where (natural environment, laboratory, etc.) the spectral reflectance measurements are taken (see e.g. Cortesi et al. 2015).

A. burtoni photoreceptors are arranged in a classical mosaic pattern with four double cone receptors surrounding a single cone (Fernald and Liebman 1980; Fernald 1981). The single cone expresses a short-wavelength sensitive (SWS) 'blue' pigment with a peak spectral sensitivity (λmax) at 455 nm, the shorter tuned double cone member expresses a middle-wavelength sensitive (MWS) 'green' pigment at 523 nm λmax and the longer tuned double cone member expresses a long-wavelength sensitive (LWS) 'red' pigment at 562 nm λmax (Fernald and Liebman 1980). Members of double cones have previously been shown to contribute separately to color discrimination in some fishes (Pignatelli et al. 2010) and we therefore modeled *A. burtoni* as having a trichromatic visual system with a cone photoreceptor ratio of 1:2:2 (SWS:MWS:LWS) and a 0.05 LWS noise threshold for the Weber fraction (ω) (for similar approaches see Boileau et al. 2015; Cortesi et al. 2015). The visual model calculates ΔS within the visual 'space' of the fish based on an opponent mechanism, which is limited by the noise of different photoreceptor types (Vorobyev and Osorio 1998; Vorobyev et al. 2001). Similar colors will result in low ΔS values, whereas chromatically contrasting colors will result in high ΔS values with ΔS = 1 as the discrimination threshold (just noticeable difference; JND). We would like to note that we currently do not know how *A. burtoni* processes visual stimuli and that behavioral experiments are needed to comprehend what a change in JND beyond the discrimination threshold of 1 signifies. Similarly, behavioral experiments would be needed to assess whether the discrimination threshold varies depending on direction and position in the visual space. Moreover, due to the difficulty of measuring egg-spots in the field we were restricted in sample size, which did not allow for further statistical analyses. However, it is our best estimator in that the larger ΔS is, the more likely it is that the signal can be distinguished, especially when visual information needs to remain reliable over distance in turbid water conditions.

Association tests

Finally, we tested for an association between egg-spot measurements based on photographs and underwater light environments (i.e. orange ratio) as well as immunological parameters. To this end downwelling irradiance was measured for each locality (except Lf1) at the surface and at the following depths: 10, 20, 30, 40, 50, 70 and 100 cm, or to the deepest possible point within the interval. At each depth, we took five measurements using a JAZ modular portable spectrometer (Ocean Optics; wavelength range 300 - 980 nm) with an OFRM25L05 optical fiber and a CC-3-UV-T cosine corrector attached. Before measurements, an OCOWS-1 diffuse reflection standard was used for relative calibration. All measurements were taken in July 2013 on clear days around noon (between 11:30 and 14:00). Spectral data were inspected and processed using the package PAVO (Maia et al. 2013) in R. Wavelengths were interpolated in 1 nm bins from 400 - 700 nm, and five measurements from each depth level were averaged.

As a measure for underwater light environments, irradiance data was transformed into orange ratio values. The orange ratio quantifies the relative transmission of long wavelength light by dividing the integral of 400 - 550 nm absorbance by the integral of 550 - 700 nm absorbance (Endler and Houde 1995). This ratio generally increases with depth and increasing turbidity, as short wavelengths are selectively scattered and absorbed (Levring and Fish 1956). For further statistical comparisons among the localities, the average change in orange ratio for each locality was calculated from the deepest available measurement divided by the number of 10 cm depth levels.

As an immunological measurement, the activity of the immune system that can be found under natural environmental conditions was determined in the field. We measured the lymphocyte ratio in the blood (lymphocyte count / (lymphocyte + monocyte counts)) to estimate the proportion of cells of the adaptive immune system. Measurements were taken during the dry season in July 2013 for all lake-stream localities except for ChL, Ch1 and Lf1. Blood samples were taken from the caudal vein ($n_{\text{males per population}} = 6 – 22$; $n_{\text{males total}} = 94$; for detailed information on sample sizes see Table S1) and directly analyzed with a flow cytometer (BD Accuri C6 Flow Cytometer, Becton and Dickinson, Heidelberg, Germany). Immunological assays were performed according to protocols developed for sticklebacks (Scharsack et al. 2004, 2007a,b) with the modifications reported in Roth et al. (2011) as well as cichlid-specific settings as developed and described in Diepeveen et al. (2013). The distinction of blood cell types (lymphocytes vs. monocytes) was based on differences in their light scatter profiles (FSC - forward scatter, approximation for cell size; SSC - side scatter, approximation for cell complexity).

To test for an association between egg-spot measurements based on photographs, orange ratio and immune response, each egg-spot characteristic (size-corrected, if necessary) was used as response variable in a multiple regression on distance matrices (MRM) with 10'000 permutations using the R package ECODIST (Goslee and Urban 2007). The explanatory variables in the MRMs were pairwise differences in orange ratio, immune response and geographic distance. Note that the MRM excluded the populations ChL, Ch1 and Lf1 due to lack of underwater ambient light and/or immunological data.

Results

Sex-specific differences in egg-spots

Egg-spot number was the only examined egg-spot characteristic that showed no differences between sexes but correlated positively with body size (GLMM: $z_{sex} = -0.52$, $p_{sex} = 0.602$; $z_{CS} = 9.43$, $p_{CS} < 0.0001$) (Fig. 2A). The measurements on egg-spot areas revealed that males tended to have larger average egg-spot areas and a significantly larger total egg-spot area relative to their fin areas compared to females (Fig. 2A). Therefore sex, but not body size improved the model for both egg-spot area characteristics (LME comparison with ANOVA: relative average egg-spot area - $\chi^2_{sex} = 3.4139$, $p_{sex} = 0.0647$; $\chi^2_{CS} = 0.1485$, $p_{CS} = 0.6999$; relative total egg-spot area - $\chi^2_{sex} = 7.5488$, $p_{sex} = 0.0060$; $\chi^2_{CS} = 0.0073$, $p_{CS} = 0.9318$). Male egg-spots showed way more intense coloration, which also increased faster with increasing body size compared to females (LME comparison with ANOVA: $\chi^2_{\text{interaction sex:CS}} = 8.5799$, $p_{\text{interaction sex:CS}} = 0.0034$; $\chi^2_{sex} = 41.691$, $p_{sex} < 0.0001$; $\chi^2_{CS} = 11.757$, $p_{CS} = 0.0006$; Fig. 2A) (for sex-specific mean values with corresponding confidence intervals of each egg-spot characteristic see Table S3A).

Habitat-specific differences in egg-spots

The PCA revealed a clear separation between lacustrine and riverine populations within the lake-stream systems, except for the four populations of the Kalambo system, which clustered together (Fig. 3A). The other three systems - Chitili, Lufubu and Lunzua - were separated into lake and stream populations along principal component 1 (PC1, explaining 46% of the variance) and PC2 (explaining 32% of the variance) (for detailed information on proportions of variance and averaged PC loadings see Table S4). Lake populations generally showed greater egg-spot numbers compared to stream populations. Stream populations had

a larger relative average egg-spot area and more intense coloration, as well as a larger relative total egg-spot area in the case of Lf1.

The more detailed analyses for each egg-spot characteristic separately showed similar overall trends as the PCA results, but revealed lake-stream system-specific differences. The analysis of egg-spot number among populations within systems revealed that more upstream populations had significantly fewer egg-spots in the rivers Lufubu and Lunzua, but not in Kalambo and Chitili (GLM with correction for multiple comparisons: LfL - Lf2: $z = 3.873$, $p = 0.0011$; Lf1 - Lf2: $z = 4.616$, $p < 0.0001$; LzL - Lz1: $z = 5.114$, $p < 0.0001$; only significant values are presented in the text, for all population comparisons within systems see Table S3D and for all population-specific mean values with corresponding confidence intervals for each egg-spot characteristic see Table S3B) (Fig. 2B). The model for egg-spot number also revealed an increase in egg-spot number with increasing body size of the males (GLM: $z = 6.985$, $p < 0.0001$).

The relative average egg-spot area increased with larger distance from the lake within the Lufubu and the Lunzua systems and between two riverine populations of the Kalambo River (LM with correction for multiple comparisons: Ka1 - Ka3: $z = -2.997$, $p = 0.0291$; LfL - Lf2: $z = -4.736$, $p < 0.0001$; LzL - Lz1: $z = -6.470$, $p < 0.0001$). With increasing body size of the males the average egg-spots became smaller in relation to fin area (LM: $t = -9.680$, $p < 0.0001$) (Fig. 2B).

Relative total egg-spot area was the only parameter that showed no divergence along the lake-stream gradient. There was a trend of body size improving the model, indicating a correlation between body size and relative total egg-spot area (LM comparison with ANOVA: $F = 2.8532$, $p = 0.0923$). Note, however, that this result was mainly influenced by the riverine Chitili population (Ch1), as without Ch1, the trend did not persist (LM comparison with ANOVA: $F = 0.4716$, $p = 0.4928$). This most probably reflects the data better and therefore body size was excluded as a fixed effect in this case. However, there were differences among systems with respect to this trait, with the Lufubu populations showing larger relative total egg-spot area compared to the Chitili and Kalambo populations (LM with correction for multiple comparisons: Chitili - Lufubu: $z = 3.378$, $p = 0.0045$; Kalambo - Lufubu: $z = 4.712$, $p < 0.001$; only significant values are presented in the text, for all system comparisons see Table S3E and for system-specific mean values with corresponding confidence intervals see Table S3C) (Fig. 2B).

Based on our color categories, riverine populations showed more intense colored egg-spots than lake populations in the Chitili Creek and in the Lunzua system (LM with correction for multiple comparisons: ChL - Ch1: $z = -3.531$, $p = 0.0050$; LzL - Lz1: $z = -4.889$, $p < 0.0001$). Additionally, egg-spot coloration showed a positive correlation with body size (LM: $t = 12.283$, $p < 0.0001$) (Fig. 2B).

The visual model revealed a higher egg-spot to fin contrast (i.e. larger color distance) in riverine populations compared to lake populations (except for the Ka2 population; Fig. 3B). This pattern was consistent when visual models were calculated with underwater ambient light profiles from different depths (i.e. 10 cm, 30 cm and maximal depth, see Fig. S4).

Association tests

The results of the underwater ambient light and immunological parameters are shown in Figure 4. Within systems, the underwater light environment in stream populations was characterized by higher orange ratio values when compared to lake populations (for detailed information on orange ratio values see Table S5; for underwater ambient light spectral curves see Supplementary Fig. S3). The proportion of lymphocytes showed higher values for stream populations compared to lake populations in the Lufubu and the Lunzua systems, but less variation for the populations from the Kalambo system (Fig. 4).

The MRMs indicated that the examined egg-spot characteristics were influenced to a different extent by the explanatory variables. Relative average egg-spot area and egg-spot number correlated with the proportion of lymphocytes. However, egg-spot coloration correlated with underwater light environment and relative total egg-spot area with geographic distance (Table 1).

Discussion

In this study, we examine natural variation in a putative sexually selected trait, anal fin egg-spots, in lake and stream populations of the haplochromine cichlid *A. burtoni*. Egg-spots constitute a carotenoid based signal that has been suggested to be an evolutionary innovation of haplochromine cichlids (Goldschmidt and de Visser 1990; Salzburger et al. 2005; Santos et al. 2014).

We first show that egg-spot phenotypes differ substantially between sexes, with females showing smaller and less colored egg-spots compared to the larger and more intensely colored egg-spots of males (Fig. 2A). The increased conspicuousness of egg-spots in males is most probably founded in their function. Egg-spots play an important role in strength assessment of a competitor and elicit an intimidating effect in male combats in *A. burtoni* (Theis et al. 2012), as well as in its congener *A. calliptera* (Theis et al. 2015). Interestingly, in some haplochromine species including *A. burtoni* and *A. calliptera*, also female individuals show egg-spots. To the best of our knowledge, no function for female egg-spots has been reported yet, and, additionally to the reduced area and less conspicuous coloration, the female egg-spots also lack the translucent, non-pigmented area around the egg-spots. This translucent ring is likely to enhance contrast of egg-spots in males (Tobler 2006). Reduction or absence of visual signals in females is most probably to decrease energy investment and to reduce conspicuousness towards predators. Alternatively, this might be a corollary of the necessity to invest most of their resources directly into offspring (Trivers 1972). In addition, sexual immune dimorphism could play a role, i.e. whereas males increase fitness through mating success, females need to invest more resources in their immune system as they gain fitness through longevity (Rolff 2002) and should benefit from allocating carotenoids to immune responses instead of a costly trait (Lozano 1994, 2001; Svensson and Wong 2011). The reduced conspicuousness of egg-spots in females due to a reduction in egg-spot area and coloration goes along with a generally more drab body coloration. Interestingly, fin and flank traits seem to be coupled in females of Lake Malawi cichlids, but showed two distinct clusters in males (Brzozowski et al. 2012). This developmental uncoupling might enable males to specifically alter the conspicuousness of the trait in dependence of, e.g., status (Brzozowski et al. 2012). Our finding that egg-spots in *A. burtoni* are only reduced in area and coloration, but not in number between males and females, might be the result of a developmental constraint.

Among males within systems, there is a general trend of increasing conspicuousness of egg-spots from lake towards riverine populations, with the latter generally showing fewer, but larger egg-spots with a more intense coloration and a higher egg-spot to fin contrast (Figs. 3A, B). Within systems, this increase in conspicuousness is either connected with a change to more intense egg-spot coloration (Chitili; no data available for egg-spot to fin color distance), larger relative egg-spot area and higher egg-spot to fin contrast (Lufubu), a combination of all three factors (larger relative average egg-spot area, more intense egg-spot coloration and higher egg-spot to fin contrast; Lunzua) or absent (Kalambo) (Figs. 2B, 3B). Except for the Kalambo system, egg-spots were more conspicuous in areas where predation pressure is presumably lower, i.e. the stream localities. *Astatotilapia burtoni* supposedly experiences predation through piscivorous fishes, other aquatic predators (e.g. otters and snakes), and birds (e.g. kingfisher and cormorants), of which only the latter and some piscivorous fishes also chase regularly in upstream riverine localities. It has been shown that in areas with high predation pressure ornamentation and coloration is reduced or cryptic (Endler 1980; Stuart-Fox and Ord 2004). Because predation pressure most probably correlates negatively with orange ratio in our study system, it is difficult to disentangle their relative influences. Egg-spot conspicuousness could be lower in the lake localities because of higher predation pressure, or – maybe more realistically – increased egg-spot conspicuousness in riverine systems could serve to maintain signal transmission in underwater light environments with higher orange ratios (i.e. long wavelength shifted environments).

Turbidity in aquatic systems can either lead to an increase in the conspicuousness of visual signals, most probably to maintain their function (Dugas and Franssen 2011; Kekäläinen et al. 2010), or a decrease

in conspicuousness, because intraspecific receivers responded less to visual signals (e.g. Seehausen et al. 1997, 2008; Wong et al. 2007; Luyten and Liley 1991). Both scenarios have been discussed in the context of egg-spots (Goldschmidt 1991; Castillo Cajas et al. 2012). These two hypotheses are not mutually exclusive, though, given that the expression of visual signals could be linked to the properties of the ambient light environment or, more general, the overall costs and benefits of carrying and producing the signal. With respect to egg-spot divergence, this would suggest a scenario of increasing egg-spot conspicuousness with increasing turbidity, as long as the benefit outbalances the costs. This corresponds to our finding of more intensely colored egg-spots and higher egg-spot to fin contrast in longer wavelength shifted environments, where also predation is expected to be lower. The reduced expression found in other haplochromine species could be due to the high costs involved in maintenance or due to the absorption of reddish signals in very turbid conditions or deep water (e.g. Seehausen et al. 1997, 2008). This could possibly explain the secondary loss of egg-spots in some deep-water lineages of Lake Malawi haplochromines (Salzburger et al. 2005), and the decrease in egg-spot conspicuousness in more turbid water in *P. pundamilia* (Castillo Cajas et al. 2012), as these examined populations occur in much deeper and more turbid habitats compared to our examined *A. burtoni* populations.

The costs involved in producing and maintaining carotenoid-based ornaments is often linked to immune defense. The relative cost of allocating carotenoid pigments to visual signals is likely to increase upon activation of an immune response, which involves carotenoids (Lozano 1994, 2001; Svensson and Wong 2011). For example, fishes experiencing high levels of stress show reduced immune responses, which may result in a decreased lymphocyte ratio (Ellsaesser and Clem 1986; Witeska 2005). Allocation of carotenoids to the immune response is, in these cases, likely to be beneficial for the immune system. However, if carotenoids are limited or if there is a metabolic constraint for carotenoid conversion, investing in the immune system would likely reduce the conspicuousness of carotenoid based visual signals. In support of this trade-off hypothesis we found that *A. burtoni* populations with a decreased lymphocyte ratio show smaller egg-spots and populations with high lymphocyte ratios possess fewer but larger and more conspicuous egg-spots. (Note, however, that a shift in the lymphocyte ratio could also imply that there are more monocytes present, which are the first line of the immune defense, or fewer lymphocytes, which are indicative of a recovery from a recent infection.)

The relative influence of underwater light environment and immunological parameters seem to vary among egg-spot characteristics. Egg-spot coloration most probably depends on underwater light environment (i.e. orange ratio), whereas egg-spot number and relative average egg-spot area rather correlate with immune defense (Table 1). In systems showing population-specific differences in relative average egg-spot area (Lufubu and Lunzua), the fewer but larger egg-spots of riverine populations result in the same relative total area as the many smaller egg-spots of lacustrine populations. Relative total egg-spot area was therefore the only parameter, which did not differ among populations within systems and, interestingly, did also not correlate with body size. However, there is an among-system variation in relative total egg-spot area, with populations from Lufubu showing a larger relative total egg-spot area compared to the Chitili and Kalambo systems. We would like to note here that the Lufubu populations are, genetically, the most distinct ones (Theis et al. 2014).

Overall, the association between egg-spot characteristics, environmental and immunological parameters suggests that the relative total egg-spot area is rather fixed within systems, whereas egg-spot number, relative average egg-spot area and egg-spot coloration seem to adapt to the respective environment. Likewise, in the guppy *Poecilia reticulata*, the area of the sexually selected orange spots was fixed, but brightness was affected by the environment through scarcity in dietary carotenoids supplied by algae (Grether et al. 1999). However, that carotenoid uptake as such would influence egg-spot conspicuousness is rather unlikely as *A. burtoni* feed mainly on algae, plant material and macroinvertebrates (Theis et al. 2014), which offer plenty of carotenoids. There might be other factors, though, which were not taken into account here, and that might influence egg-spot characteristics as well, e.g. other abiotic environmental factors, special biotic interactions and/or anthropogenic influences. Further, the results on

the association between egg-spots, underwater light environment and immunological parameters should be taken with caution since correlations of data from the field are vulnerable to contain artifacts and are based on a few populations only. Nevertheless, our findings provide a first insight with respect to possible environmental and immunological factors influencing the egg-spot phenotype. The fact that different egg-spot characteristics may be influenced by variable environmental factors illustrates that several replicates need to be examined to elucidate the causes for variation in such a complex trait. To which degree underwater light environment and/or immune response are involved in shaping egg-spot characteristics needs further examinations under controlled laboratory conditions.

In summary, egg-spots show sex- and habitat-specific differences in the haplochromine cichlid *A. burtoni*. Males possess more conspicuous egg-spots compared to females, and, within populations, larger males have more conspicuous egg-spots than smaller ones, both of which could be explained by their function in sexual selection. Further, males of three out of four examined lake-stream systems show similar shifts in egg-spot divergence, with riverine fish possessing fewer but larger and/or more intensely colored egg-spots compared to fish from the corresponding lake habitats. Moreover, the visual model revealed more conspicuous egg-spots in riverine populations as compared to lake populations. Taken together, egg-spots represent an honest trait, which shows the potential to adapt to differences in signal transmittance, and that is traded off with investment into the immune system. Our findings indicate that the expression of a visual signal to maximize both, survival and reproduction is a complex and sensitive equilibrium, which should always be interpreted in the context of several aspects of both, sexual and ecological selection.

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Table 1 Multiple regression on distance matrices (MRM) among pairwise differences in egg-spot characteristics, orange ratio, lymphocyte ratio as well as geographic distance. The egg-spot characteristics number, relative average area and coloration were corrected on centroid size before the analyses. Significance levels: *p < 0.05 and **p < 0.01.

egg-spot characteristic	orange ratio	lymphocyte ratio	geographic distance
number	0.2336	0.0065**	0.0744
relative average area	0.1069	0.0076**	0.1397
relative total area	0.8409	0.7708	0.0247*
coloration	0.0082**	0.1485	0.5281

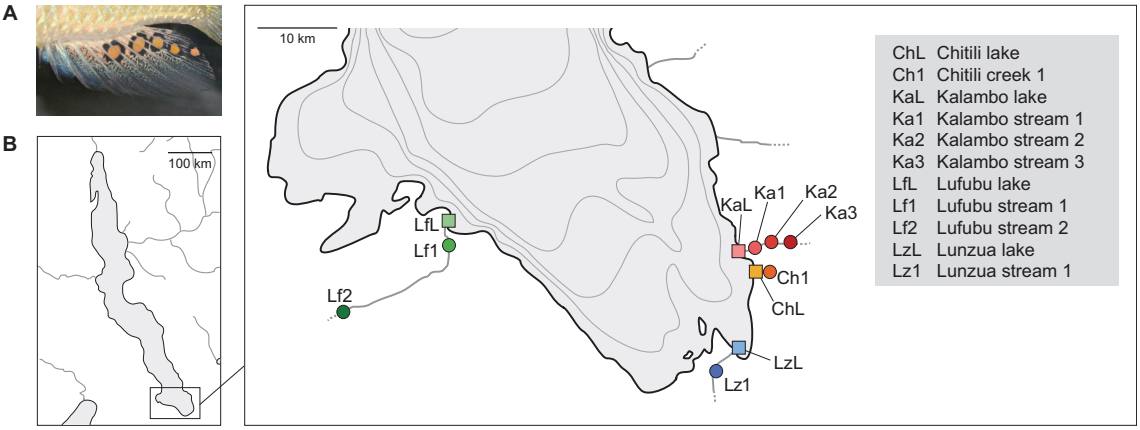

Figure 1 Male secondary sexual trait and populations under investigation. (A) Egg-spots on the anal fin of a male *Astatotilapia burtoni*. (B) Map showing the 11 sampling localities in the southern part of Lake Tanganyika (squares represent lake and circles stream populations; bathymetric lines are placed at every 100 m water depth, after Coulter 1991; full names of populations are listed in the grey box).

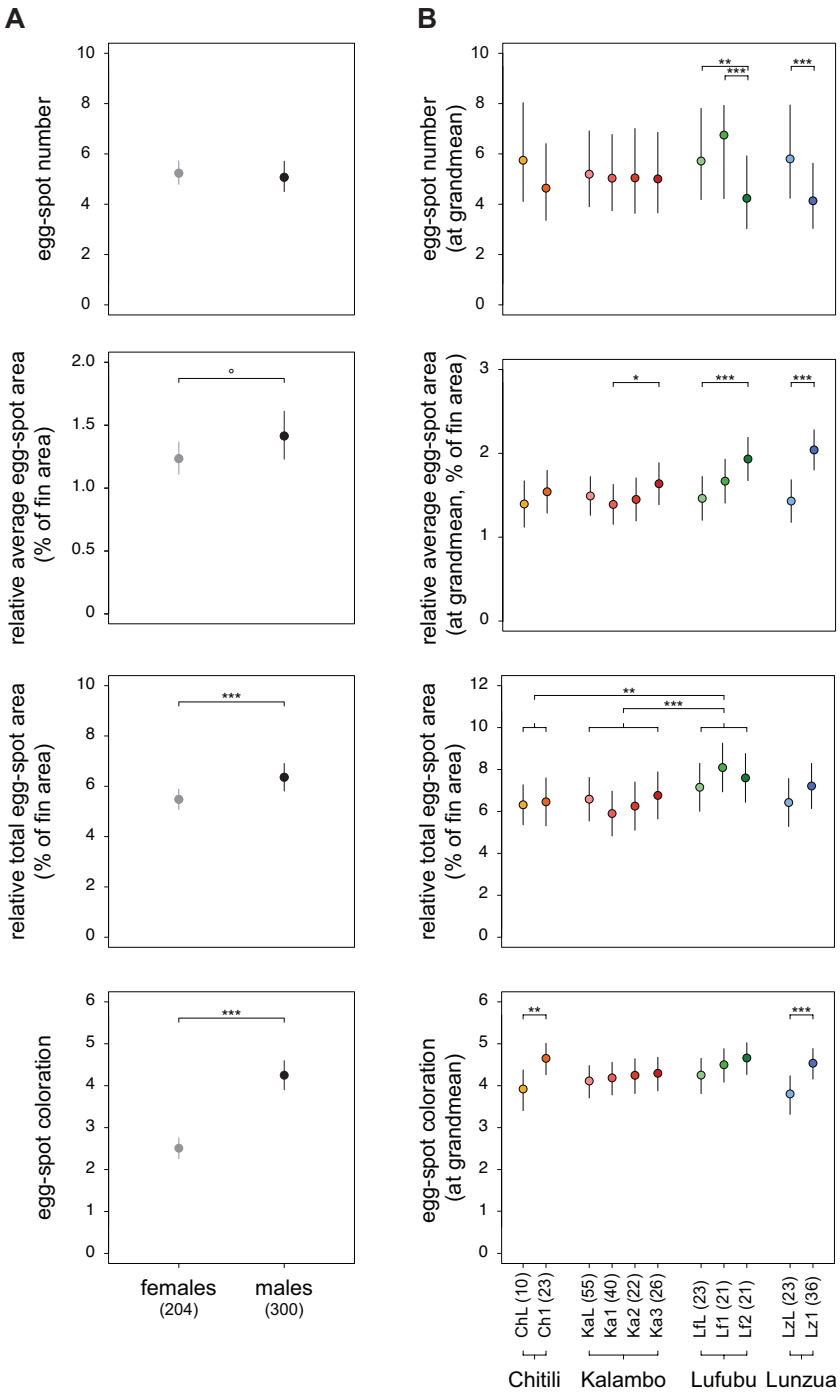

Figure 2 Differences in the four examined egg-spot characteristics measured based on photographs (number, relative average area, relative total area and coloration) between all females and males (A) and among males of the populations within the lake-stream systems (B). Full names of populations are listed in the grey box of Fig. 1. Significance levels: °p < 0. 1, *p < 0.05, **p < 0.01 and ***p < 0.001. Corresponding sample sizes are parenthesized.

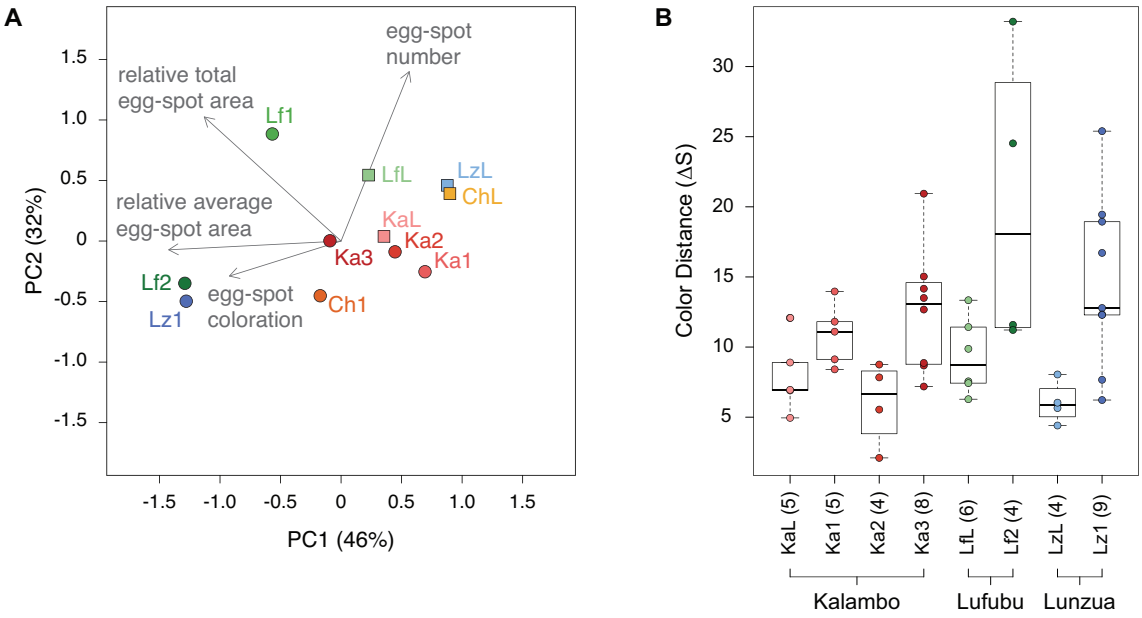

Figure 3 Differences in egg-spot characteristics. (A) PCA-biplot of all populations measured based on the examined egg-spot characteristics measured on the photographs (number, relative average area, relative total area and coloration). The indicated dots represent the mean for all males per population. Sample sizes are the same as reported in Fig. 2B. (B) Color distances resulting from the visual models including the orange ratio for 30 cm below surface. Sample sizes are parenthesized.

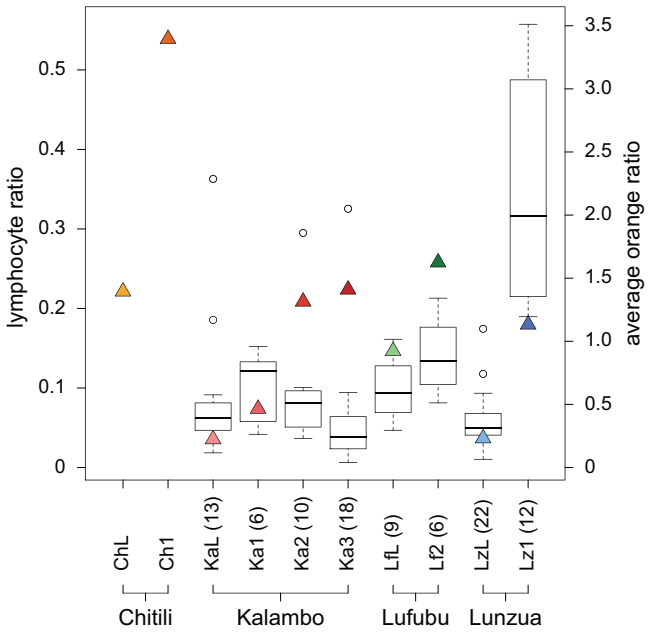

Figure 4 Boxplots of lymphocyte ratios and average orange ratio values (indicated by triangles; average change in orange ratio per 10 cm calculated from the deepest available measurement) per population (note that for the populations ChL and Ch1, no data on lymphocyte ratio was available).

Table S1 Sample size details for analyses on egg-spot characteristics and lymphocyte ratios (blood measurements), with geographic coordinates for each locality.

sampling information				egg-spot characteristic measurements				blood measurements
				taken from the photographs			reflectance spectrometry	lymphocyte ratio
population		latitude	longitude	all	females	males	males	males
ChL	Chitli lake	8°38'18.42"S	31°11'55.34"E	37	27	10	NA	NA
Ch1	Chitli creek 1	8°38'16.91"S	31°12'4.02"E	51	28	23	NA	NA
KaL	Kalambo lake	8°36'6.27"S	31°11'13.24"E	94	39	55	5	13
Ka1	Kalambo stream 1	8°35'35.23"S	31°11'6.18"E	53	13	40	5	6
Ka2	Kalambo stream 2	8°35'6.24"S	31°12'29.32"E	37	15	22	4	10
Ka3	Kalambo stream 3	8°35'41.59"S	31°14'50.32"E	49	23	26	8	18
LfL	Lufubu lake	8°33'36.56"S	30°43'33.79"E	29	6	23	6	9
Lf1	Lufubu stream 1	8°35'49.31"S	30°43'38.96"E	27	6	21	NA	NA
Lf2	Lufubu stream 2	8°41'9.37"S	30°33'51.90"E	36	15	21	4	6
LzL	Lunzua lake	8°44'57.13"S	31°10'21.86"E	39	16	23	4	22
Lz1	Lunzua stream 1	8°47'23.51"S	31° 8'14.33"E	52	16	36	9	10
total sample size per method				**504**	204	300	**45**	**94**

Table S2 Linear models to test for differences in egg-spot measurements based on photographs (number, relative average area, relative total area and coloration) between sexes (A) and among populations (males only) (B).

A

egg-spot characteristic	linear model
number	glmer(number ~ sex + centred_centroid_size + (1|sex_specific_population), data=data, family="poisson")
relative average area	lmer(sqrt(relative_average_area) ~ sex + (centred_centroid_size|sex_specific_population), data=data)
relative total area	lmer(relative_total_area ~ sex + (centred_centroid_size|sex_specific_population), data=data)
coloration	lmer(coloration ~ sex + centred_centroid_size + sex:centred_centroid_size + (centred_centroid_size|sex_specific_population), d ata=data)

B

egg-spot characteristic	linear model
number	glm(number ~ population + centroid_size, data=data, family="poisson")
relative average area	lm(relative_average_area ~ population + centroid_size, data=data)
relative total area	lm(relative_total_area ~ population, data=data)
coloration	lm(coloration^2 ~ population + centroid_size, data=data)

Table S3 Detailed results on sex-, population- and system-specific mean values (with corresponding confidence intervals) of egg-spots and the pairwise comparisons thereof. Mean values with corresponding confidence intervals for each egg-spot measurement based on photographs (number, relative average area, relative total area and coloration) and mean values with corresponding standard deviation and range for body size (standard length and centroid size) for each sex (A) and for the males of each population (B) as well as for relative total area for each system (C). Results of all pairwise comparisons for each egg-spot measurement based on photographs (number, relative average area, relative total area and coloration) between populations within lake-stream systems (D) and for comparisons of total egg-spot area between systems (E). Significance levels: *p < 0.05, **p < 0.01 and ***p < 0.001.

A

sex	egg-spot number			relative average egg-spot area (% of fin area)			relative total egg-spot area (% of fin area)			egg-spot coloration			body size			
													standard length (SL; mm)		centroid size (CS)	
	mean	lower CI	upper CI	mean	lower CI	upper CI	mean	lower CI	upper CI	mean	lower CI	upper CI	mean ± sd	range	mean ± sd	range
females	5.23	4.79	5.72	1.23	1.11	1.37	5.48	5.08	5.88	2.51	2.26	2.76	46.79 ± 7.78	27.80 - 75.40	64.67 ± 10.86	39.88 - 106.20
males	5.07	4.50	5.71	1.41	1.23	1.61	6.36	5.81	6.91	4.25	3.90	4.60	54.81 ± 12.40	33.67 - 106.42	76.14 ± 17.79	45.99 - 148.76

B

population	egg-spot number (at grandmean)			relative average egg-spot area (at grandmean, % of fin area)			relative total egg-spot area (% of fin area)			egg-spot coloration			body size			
													standard length (SL; mm)		centroid size (CS)	
	mean	lower CI	upper CI	mean	lower CI	upper CI	mean	lower CI	upper CI	mean	lower CI	upper CI	mean ± sd	range	mean ± sd	range
ChL	5.75	4.11	8.04	1.40	1.12	1.67	6.32	5.36	7.27	3.92	3.41	4.38	51.98 ± 11.66	40.14 - 75.78	72.22 ± 16.58	55.76 - 107.31
Ch1	4.64	3.36	6.41	1.54	1.29	1.80	6.46	5.31	7.60	4.65	4.26	5.01	54.21 ± 14.26	35.25 - 78.15	75.39 ± 20.04	48.74 - 108.22
KaL	5.19	3.90	6.92	1.49	1.26	1.72	6.58	5.55	7.62	4.11	3.71	4.48	55.13 ± 8.61	42.47 - 78.19	75.87 ± 11.99	58.80 - 108.37
Ka1	5.03	3.74	6.77	1.39	1.15	1.63	5.90	4.83	6.97	4.19	3.78	4.56	53.86 ± 13.59	36.09 - 89.60	74.01 ± 18.99	49.24 - 125.66
Ka2	5.05	3.64	7.01	1.45	1.19	1.71	6.25	5.10	7.41	4.25	3.81	4.64	46.98 ± 5.89	36.53 - 59.20	65.27 ± 8.44	51.08 - 82.71
Ka3	5.01	3.66	6.86	1.64	1.39	1.89	6.76	5.64	7.89	4.30	3.88	4.68	51.59 ± 12.33	33.67 - 89.88	71.22 ± 17.83	45.99 - 128.05
LfL	5.72	4.18	7.81	1.46	1.20	1.72	7.15	6.01	8.30	4.25	3.81	4.65	68.99 ± 14.38	47.17 - 102.20	97.27 ± 21.36	61.41 - 143.66
Lf1	6.75	4.22	7.93	1.67	1.41	1.93	8.09	6.93	9.25	4.50	4.08	4.88	64.32 ± 18.17	44.63 - 106.42	90.40 ± 26.02	61.93 - 148.76
Lf2	4.23	3.03	5.92	1.93	1.67	2.19	7.60	6.44	8.76	4.66	4.27	5.03	52.88 ± 7.57	41.67 - 71.23	74.48 ± 10.99	59.36 - 101.69
LzL	5.80	4.24	7.94	1.43	1.18	1.69	6.43	5.28	7.57	3.80	3.32	4.24	51.56 ± 6.38	41.58 - 67.19	71.56 ± 8.96	57.35 - 93.65
Lz1	4.14	3.04	5.63	2.04	1.80	2.28	7.21	6.13	8.29	4.54	4.16	4.88	52.27 ± 8.44	34.55 - 81.25	72.72 ± 12.18	47.48 - 115.62

C

system	relative total egg-spot area (% of fin area)			body size			
				standard length (SL; mm)		centroid size (CS)	
	mean	lower CI	upper CI	mean ± sd	range	mean ± sd	range
Chitili	6.42	5.88	6.95	53.54 ± 13.39	35.25 - 78.15	74.43 ± 18.86	48.74 - 108.22
Kalambo	6.37	5.78	6.97	52.88 ± 10.90	33.67 - 89.88	72.88 ± 15.28	45.99 - 128.05
Lufubu	7.60	6.95	8.26	62.27 ± 15.45	41.67 - 106.42	87.69 ± 22.33	59.36 - 148.76
Lunzua	6.91	6.24	7.57	52.00 ± 7.65	34.55 - 81.25	72.27 ± 10.97	47.48 - 115.62

D

population comparison	egg-spot number		relative average egg-spot area		relative total egg-spot area		egg-spot coloration	
	z-value	p-value	z-value	p-value	z-value	p-value	z-value	p-value
ChL - Ch1	1.938	0.3793	-1.062	0.9307	-0.235	1.0000	-3.531	0.0050**
KaL - Ka1	0.817	0.9834	1.570	0.6470	2.507	0.1141	-0.633	0.9964
KaL - Ka2	0.759	0.9892	0.509	0.9991	0.963	0.9585	-0.949	0.9616
KaL - Ka3	0.728	0.9916	-1.905	0.4041	-0.624	0.9967	-1.380	0.7787
Ka1 - Ka2	-0.071	1.0000	-0.688	0.9939	-0.974	0.9559	-0.414	0.9998
Ka1 - Ka3	0.111	1.0000	-2.997	0.0291*	-2.781	0.0549	-0.784	0.9869
Ka2 - Ka3	0.161	1.0000	-1.989	0.3492	-1.364	0.7882	-0.299	1.0000
LfL - Lf1	-0.154	1.0000	-2.089	0.2895	-1.677	0.5690	-1.524	0.6818
LfL - Lf2	3.873	0.0011**	-4.736	< 0.001***	-0.832	0.9815	-2.483	0.1214
Lf1 - Lf2	4.616	< 0.001***	-2.274	0.1971	0.820	0.9829	-1.006	0.9479
LzL - Lz1	5.114	< 0.001***	-6.470	< 0.001***	-1.777	0.4961	-4.889	< 0.001***

E

system comparison	relative total egg-spot area	
	z-value	p-value
Chitili - Kalambo	-0.143	0.9989
Chitili - Lufubu	3.378	0.0045**
Chitili - Lunzua	1.457	0.4550
Kalambo - Lufubu	4.712	< 0.001***
Kalambo - Lunzua	2.209	0.1184
Lufubu - Lunzua	-2.195	0.1223

Table S4 Results of the principle component analysis (PCA) testing the differences among males with regard to combined egg-spot characteristics number, relative average area, relative total area and coloration. Indicated are standard deviation, proportion of variance, cumulative variance and the mean of the PC loadings per population.

	PC1	PC2	PC3	PC4
Standard deviation	1.349000	1.123100	0.892800	0.348550
Proportion of Variance	0.455000	0.315300	0.199300	0.030370
Cumulative Proportion	0.455000	0.770300	0.969600	1.000000
ChL	0.898797	0.392387	-0.232222	0.061097
Ch1	-0.172236	-0.451941	0.528927	-0.075866
KaL	0.354268	0.037904	-0.119336	-0.089378
Ka1	0.693357	-0.253529	0.099019	-0.011017
Ka2	0.445778	-0.089104	0.158584	-0.002847
Ka3	-0.092053	0.002139	-0.016620	0.071001
LfL	0.226920	0.544469	0.144157	-0.110003
Lf1	-0.567835	0.884261	0.261697	-0.054627
Lf2	-1.290450	-0.349412	-0.016046	-0.057009
LzL	0.877900	0.459667	-0.414205	0.065597
Lz1	-1.279061	-0.497085	-0.256797	0.224243

Table S5 Orange ratio values for each depth level at the sample locations. The last column describes the average change in orange ratio per 10 cm, which was calculated from the deepest possible measurement (in bold). This average orange ratio was used in the analyses as a representative value for the underwater ambient light at each location.

locality	surface	10 cm	20 cm	30 cm	40 cm	50 cm	70 cm	100 cm	average (per 10 cm)
ChL	3.75	5.86	6.17	6.56	7.17	**7.56**	NA	NA	1.51
Ch1	3.59	6.06	**7.03**	NA	NA	NA	NA	NA	3.51
KaL	2.29	2.46	2.43	2.38	2.51	2.71	3.05	**3.41**	0.34
Ka1	2.82	3.37	3.56	3.77	4.09	4.46	5.30	**8.61**	0.86
Ka2	3.22	3.57	3.86	4.27	4.84	5.43	**10.02**	NA	1.43
Ka3	2.95	3.49	3.94	4.67	5.68	**7.64**	NA	NA	1.53
LfL	3.21	3.45	3.69	4.04	4.44	4.98	6.88	**10.40**	1.04
Lf2	3.02	3.60	4.33	**5.23**	NA	NA	NA	NA	1.74
LzL	2.45	2.57	2.67	2.71	2.78	2.81	2.94	**3.48**	0.35
Lz1	2.90	4.29	4.72	4.99	5.65	**6.25**	NA	NA	1.25

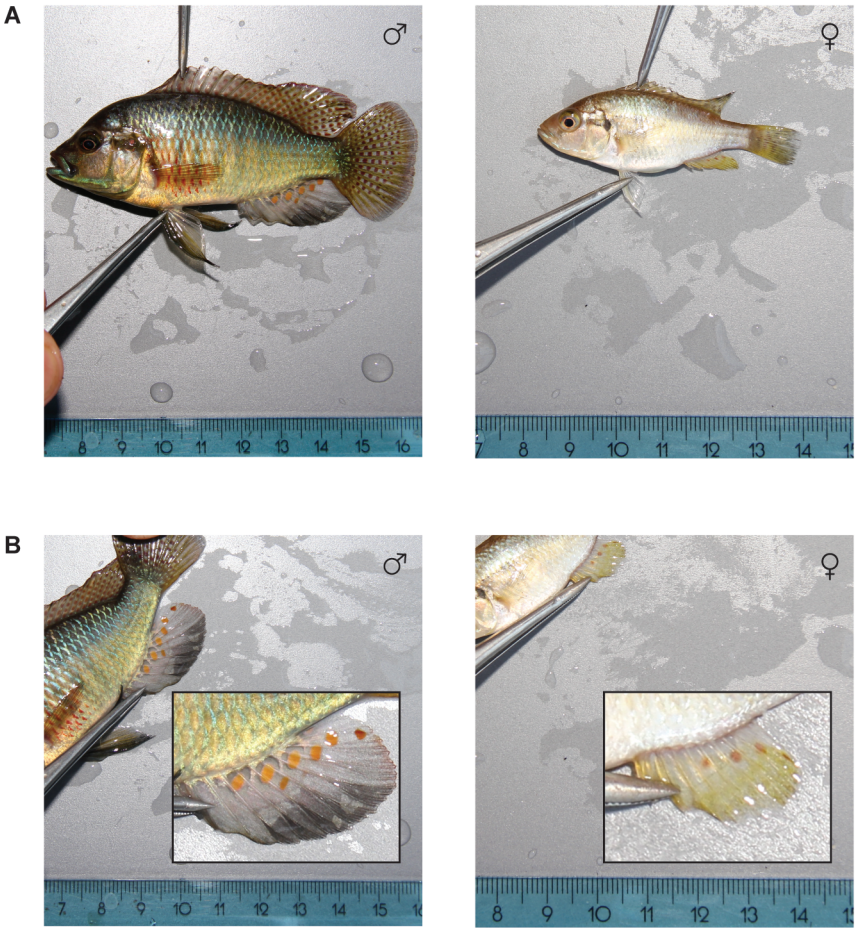

Figure S1 Photographs of a representative male (left side) and female (right side) in lateral position to measure centroid size (A) and focusing on the anal fin for later egg-spot measurements assessing the number, relative average area, relative total area and coloration (B).

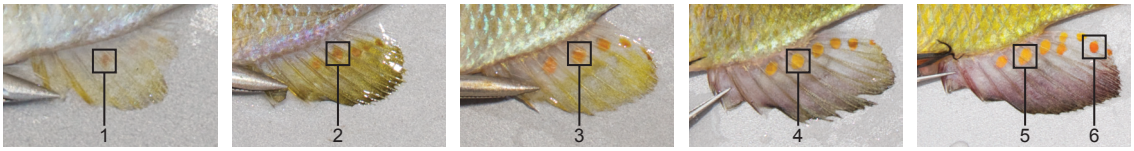

Figure S2 Representative photographs of the six categories used to describe the coloration of egg-spots. The categories ascend with increasing conspicuousness based on a combination of hue, saturation and brightness. 1 dull aggregated pigments; 2 dull egg-spot; 3 intermediate egg-spot; 4 normal egg-spot; 5 bright egg-spot (light orange); 6 bright and more saturated egg-spot (dark orange).

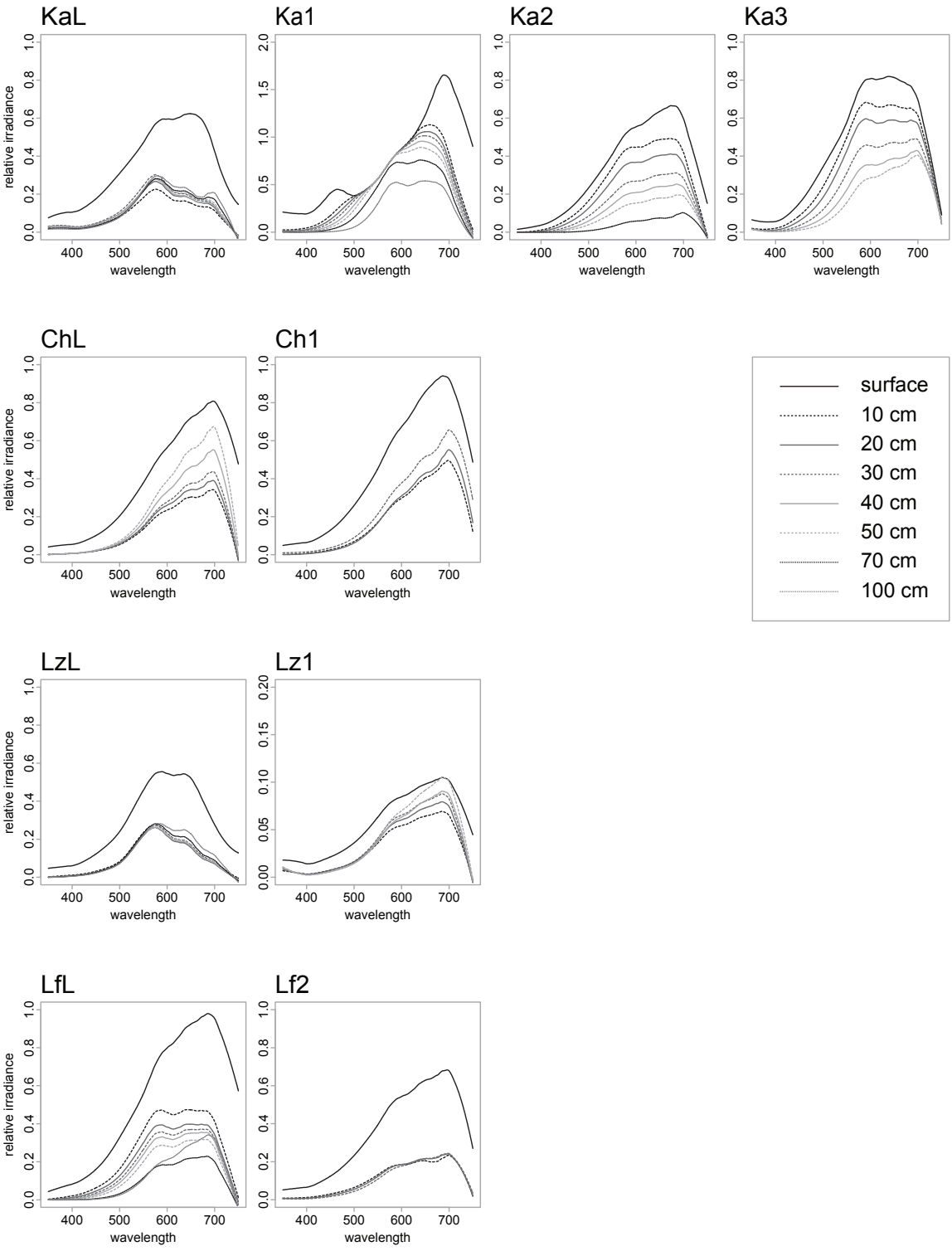

Figure S3 Underwater light environments. In each panel, the curves show underwater ambient light spectra at different depths.

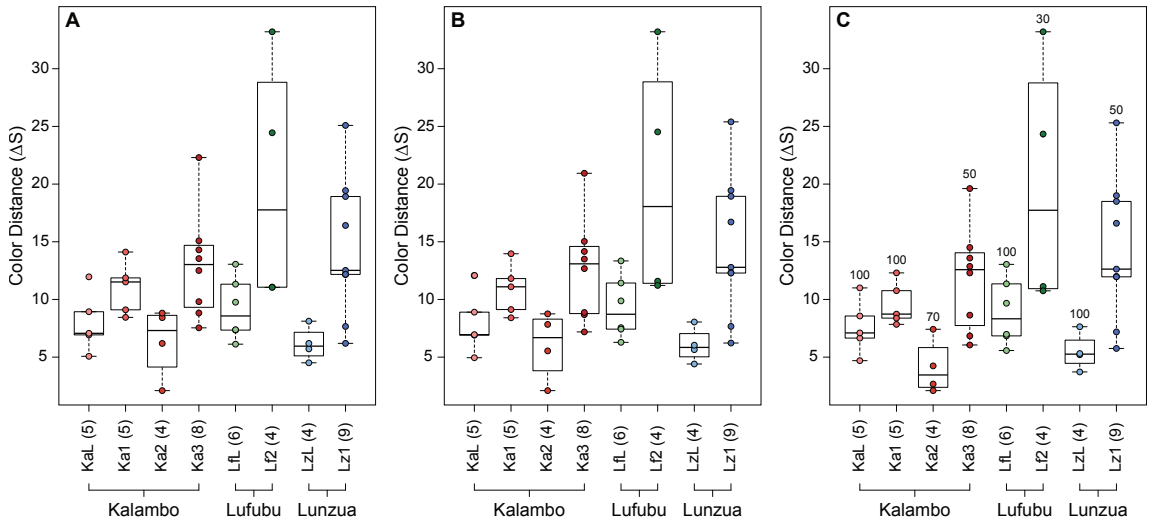

Figure S4 Color distances resulting from the visual models generated for 10 cm below water surface (A), 30 cm below surface (B) and for the deepest measurable depth for each locality (C; the corresponding depth is specified above the boxes). Corresponding sample sizes per population are parenthesized.

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

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2.2.1 Manuscript: p. 95 - 113
2.2.2 Supporting information: p. 114 - 134

I was involved in planning the study, together with BE and WS. All authors were involved in fieldwork. I generated and analyzed body shape data and conducted isolation-by-distance tests as well as the analyses to test for an association between genetic differentiation, morphometric traits and environment. Further I designed all figures and tables and was involved in writing and discussing of the manuscript.

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

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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.

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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%).

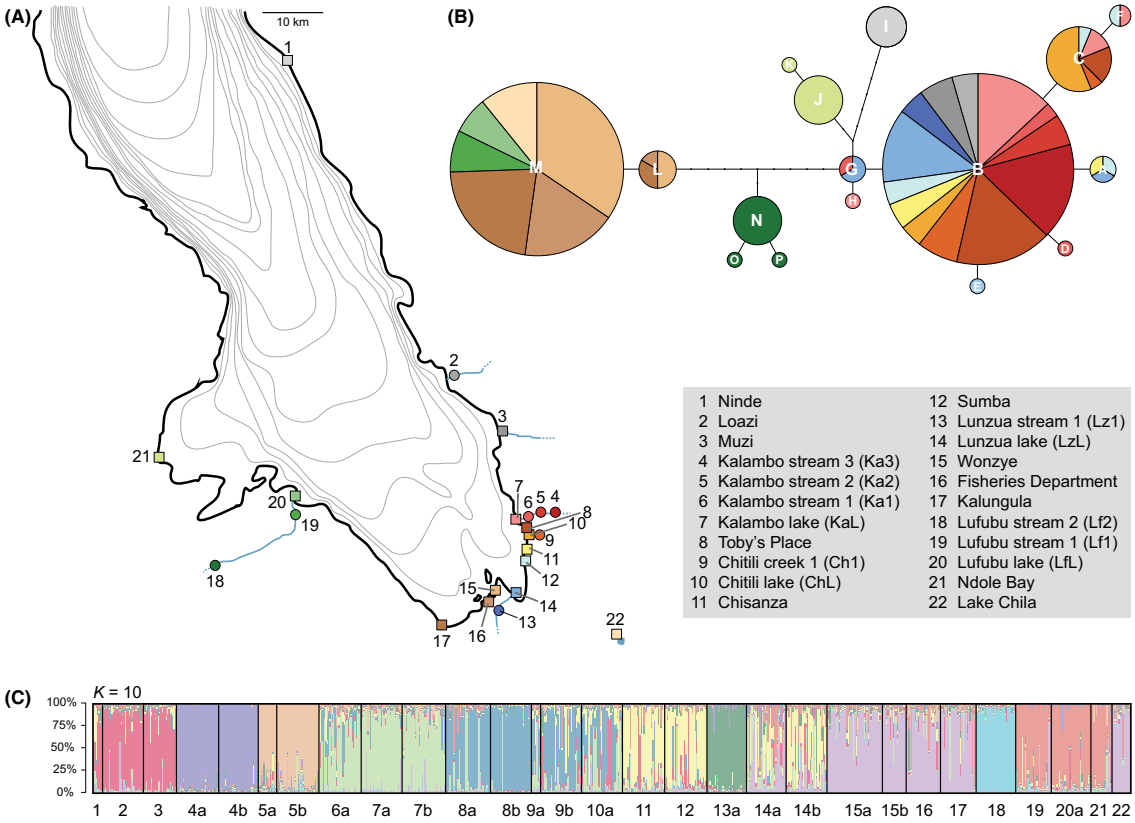

Fig. 1 Sampling locations and genetic differentiation among all populations revealed by microsatellite and mtDNA analyses. (A) The 22 sampling localities indicated by numbers on the southern part of LT (squares represent lake and circles stream populations; bathymetric lines are placed at every 100 m 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.

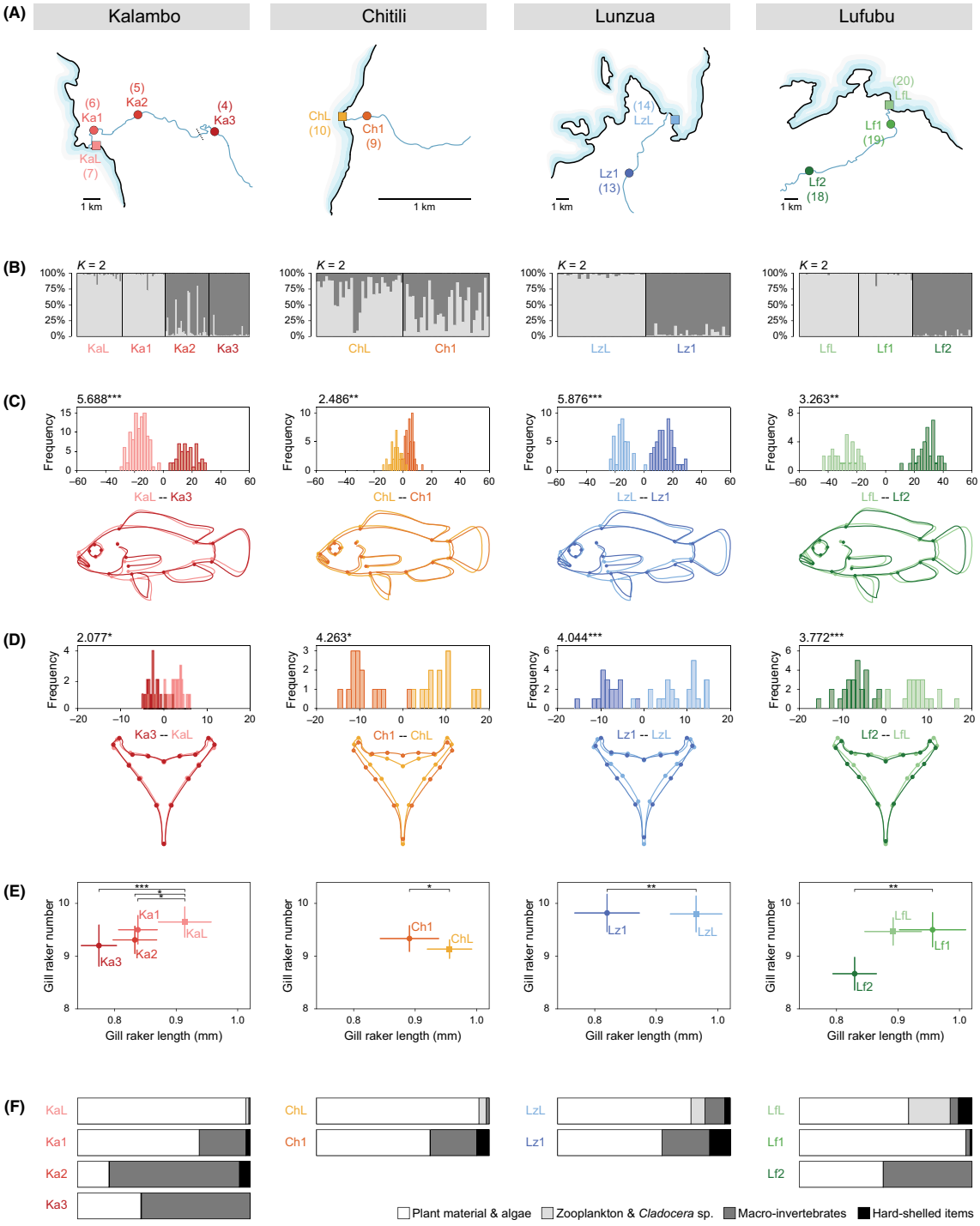

Fig. 2 Divergence between lake and stream habitats in four systems. (A) Maps showing sampling localities for each lake–stream system (see grey box in Fig. 1 for full names of localities). (B) Structure plots for each lake–stream system (shades of grey represent different genetic clusters; K = number 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

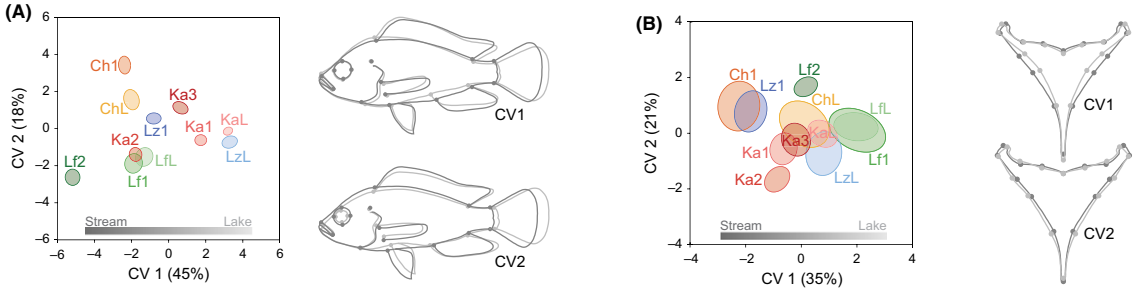

Fig. 3 Body shape and lower pharyngeal jaw (LPJ) shape differentiations of all populations from the lake–stream systems. Canonical variate analyses (CVA) plots illustrate the distribution of the populations on CV1 and CV2 (ellipses represent the 95% confidence intervals of the means) and the shifts are 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 **$P < 0.01$.

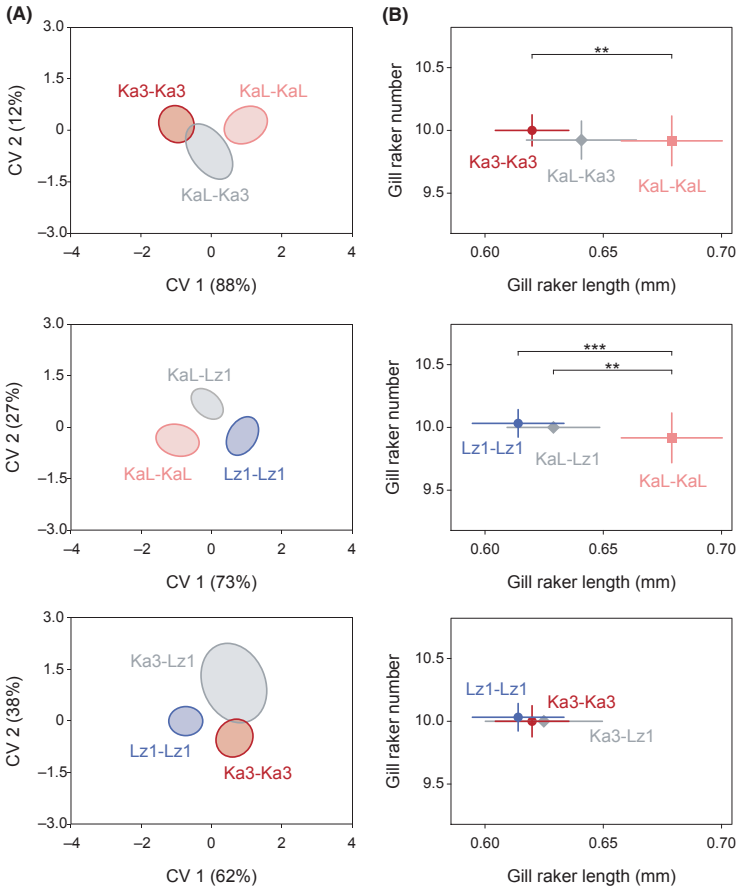

Fig. 4 Body shape (A) and gill raker comparisons (B) of each interpopulation cross with the corresponding within-population crosses from the pond experiment (Fig. S6, Supporting information for corresponding CV outlines and Table S7, Supporting information for distance and significance values).

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.

Table S1 Sample size details for each analysis with information about sampling year and geographic coordinates for each locality (note that some individuals were used for more than one analysis).

	sampling information						body shape analysis				LPJ analysis					gill raker analysis				microsatellite analysis				mtDNA analysis				stomach and gut content analysis			
	population	locality	specification	latitude	longitude	year	total	males	females	juveniles	total	males	females	juveniles	undef. sex	total	males	females	juveniles	total	males	females	juveniles	total	males	females	juveniles	total	males	females	juveniles
Kalambo system	1	Ninde	lake population adjacent to stream	7°40'51.10"S	30°43'20.67"E	2011	7	2	5	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7	2	5	0	7	2	5	0	n/a	n/a	n/a	n/a
	2	Loazi	lake population adjacent to stream	8°18'49.55"S	31° 2'57.54"E	2011	32	17	15	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	31	16	15	0	7	7	0	0	n/a	n/a	n/a	n/a
	3	Muzi	lake population adjacent to stream	8°23'1.84"S	31° 7'47.15"E	2011	24	12	12	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	25	14	11	0	9	7	2	0	n/a	n/a	n/a	n/a
	4a Ka3	Kalambo stream 3	stream population	8°35'41.59"S	31°14'50.32"E	2010	n/a	n/a	n/a	n/a	20	10	9	1	0	n/a	n/a	n/a	n/a	32	4	10	18	27	4	9	14	n/a	n/a	n/a	n/a
	4b Ka3					2011	52	29	23	0	n/a	n/a	n/a	n/a	n/a	29	15	14	0	30	17	13	0	n/a	n/a	n/a	n/a	10	10	0	0
	5a Ka2	Kalambo stream 2	stream population	8°35'6.24"S	31°12'29.32"E	2011	n/a	n/a	n/a	n/a	14	2	12	0	0	14	2	12	0	14	1	13	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	5b Ka2					2012	38	23	15	0	14	10	4	0	0	16	11	5	0	32	19	12	1	8	6	2	0	10	10	0	0
	6a Ka1	Kalambo stream 1	stream population	8°35'35.23"S	31°11'6.18"E	2011	59	41	18	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	32	17	15	0	6	4	2	0	n/a	n/a	n/a	n/a
	6b Ka1					2012	n/a	n/a	n/a	n/a	25	13	11	0	1	28	14	14	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	10	10	0	0
	7a KaL	Kalambo lake	lake population adjacent to stream	8°36'6.27"S	31°11'13.24"E	2010	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	31	27	4	0	29	25	4	0	n/a	n/a	n/a	n/a
	7b KaL					2011	102	59	43	0	23	11	8	0	4	25	17	8	0	33	19	14	0	n/a	n/a	n/a	n/a	10	10	0	0
	7c KaL					2012	n/a	n/a	n/a	n/a	3	1	2	0	0	3	0	3	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	8a	Toby's place	pure lake population	8°37'25.99"S	31°12'2.86"E	2010	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	34	16	11	7	30	15	10	5	n/a	n/a	n/a	n/a
	8b					2012	33	19	14	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	31	19	12	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Chitili system	9a Ch1	Chitili creek 1	stream population	8°38'16.91"S	31°12'4.02"E	2010	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7	1	4	2	7	1	4	2	n/a	n/a	n/a	n/a
	9b Ch1					2011	53	24	29	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	31	14	17	0	10	6	4	0	n/a	n/a	n/a	n/a
	9c Ch1					2012	n/a	n/a	n/a	n/a	14	13	1	0	0	24	15	9	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	10	10	0	0
	10a ChL	Chitili lake	lake population adjacent to stream	8°38'18.42"S	31°11'55.34"E	2011	38	10	28	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	31	11	20	0	10	6	4	0	n/a	n/a	n/a	n/a
	10b ChL					2012	n/a	n/a	n/a	n/a	12	8	3	0	1	23	15	8	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	5	5	0	0
	11	Chisanza	lake population adjacent to stream	8°39'57.39"S	31°11'41.26"E	2011	41	22	19	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	32	19	12	1	9	6	3	0	n/a	n/a	n/a	n/a
	12	Sumba	lake population adjacent to stream	8°40'18.58"S	31°11'33.94"E	2011	33	22	11	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	32	21	11	0	9	6	3	0	n/a	n/a	n/a	n/a
Lunzua system	13a Lz1	Lunzua stream 1	stream population	8°47'23.51"S	31° 8'14.33"E	2011	53	36	17	0	18	11	7	0	0	n/a	n/a	n/a	n/a	30	17	12	1	7	5	2	0	10	10	0	0
	13b Lz1					2012	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	18	11	7	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	14a LzL	Lunzua lake	lake population adjacent to stream	8°44'57.13"S	31°10'21.86"E	2010	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	30	21	9	0	24	20	4	0	n/a	n/a	n/a	n/a
	14b LzL					2011	40	23	17	0	11	3	8	0	0	16	5	11	0	31	16	15	0	n/a	n/a	n/a	n/a	5	5	0	0
	14c LzL					2012	n/a	n/a	n/a	n/a	11	7	4	0	0	15	10	5	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	5	5	0	0
	15a	Wonzye	pure lake population	8°45'1.76"S	31° 7'49.02"E	2010	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	42	24	8	10	39	21	8	10	n/a	n/a	n/a	n/a
	15b					2012	36	17	19	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	18	10	8	0	10	6	4	0	n/a	n/a	n/a	n/a
	16	Fisheries Department	pure lake population	8°45'58.52"S	31° 6'23.99"E	2010	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	26	15	5	6	24	19	5	0	n/a	n/a	n/a	n/a
	17	Kalungula	lake population adjacent to stream	8°48'33.39"S	31° 7'49.02"E	2010	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	27	17	10	0	28	17	10	1	n/a	n/a	n/a	n/a
Lufubu system	18 Lf2	Lufubu stream 2	stream population	8°41'9.37"S	30°33'51.90"E	2012	36	21	15	0	26	14	12	0	0	29	15	14	0	30	15	15	0	13	4	9	0	10	10	0	0
	19 Lf1	Lufubu stream 1	stream population	8°35'49.31"S	30°43'38.96"E	2011	27	21	6	0	12	8	4	0	0	15	10	5	0	27	21	6	0	10	8	2	0	7	7	0	0
	20a LfL	Lufubu lake	lake population adjacent to stream	8°33'36.56"S	30°43'33.79"E	2011	30	24	6	0	14	10	4	0	0	16	11	5	0	30	24	6	0	9	8	1	0	6	6	0	0
	20b LfL					2012	n/a	n/a	n/a	n/a	7	3	4	0	0	10	4	6	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	4	4	0	0
	21	Ndole bay	pure lake population	8°28'34.61"S	30°26'57.48"E	2012	45	24	21	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	16	8	8	0	13	5	8	0	n/a	n/a	n/a	n/a
	22	Lake Chila	pure lake population	8°50'8.68"S	31°22'49.44"E	2012	12	4	8	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	14	4	8	2	14	4	8	2	n/a	n/a	n/a	n/a
	total sample size per analysis						**791**	**450**	**341**	**0**	**224**	**124**	**93**	**1**	**6**	**281**	**155**	**126**	**0**	**786**	**429**	**309**	**48**	**359**	**212**	**113**	**34**	**102**	**102**	**0**	**0**

Table S2 Sample size details and result summary of the pond experiment. (A) Number of stocked adult fish per population and information about survival and reproduction. (B) Number of F1 individuals used for body shape and gill raker analyses.

A

pond	original stock males			original stock females			surviving males			surviving females			non-surviving reproducing males			non-surviving reproducing females			reproducing males	reproducing females	genotyped offspring	mating events
	KaL	Ka3	Lz1	KaL	Ka3	Lz1	KaL	Ka3	Lz1	KaL	Ka3	Lz1	KaL	Ka3	Lz1	KaL	Ka3	Lz1				
1	4	4	4	7	7	7	1	1	2	1	3	3	0	0	1	0	0	1	3	8	148	24
2	4	4	4	7	7	7	2	1	1	1	2	5	1	2	0	0	2	1	5	11	160	26
3	4	4	4	7	7	7	1	1	2	1	3	2	0	0	1	0	0	0	2	8	95	15
4	4	4	4	7	7	7	1	0	0	1	4	4	0	1	0	0	0	0	2	9	111	18
5	4	4	4	7	7	7	2	2	0	3	3	2	0	0	0	0	0	0	2	6	79	15
total	**20**	**20**	**20**	**35**	**35**	**35**	**7**	**5**	**5**	**7**	**15**	**16**	**1**	**3**	**2**	**0**	**2**	**2**	**14**	**42**	**593**	**98**

B

F1 juveniles	body shape	gill raker
KaL-KaL	25	25
Ka3-Ka3	24	24
Lz1-Lz1	31	32
KaL-Ka3	13	13
KaL-Lz1	26	26
Ka3-Lz1	11	12
total	130	132

Table S3 Pairwise genetic and morphometric (body size) distances between populations (full names of localities are given in the grey box in Fig. 1 and Table S1). (A) Pairwise genetic differentiation: DEST-values (upper triangular matrix) and F_{ST}-values (lower triangular matrix). Both indices indicate significant differentiation (P values < 0.05) between most populations (non-significant values are underlined). (B) Pairwise body shape differentiation among populations: Procrustes (upper triangular matrix) and Mahalanobis (lower triangular matrix) distances from the CVA (Fig. S4A). All comparisons showed significant body shape differences (P values < 0.05).

A

population	1	2	3	4a	4b	5a	5b	6a	7a	7b	8a	8b	9a	9b	10a	11	12	13a	14a	14b	15a	15b	16	17	18	19	20a	21	22
1		0.5450	0.6476	0.7410	0.7528	0.7166	0.7754	0.5761	0.5899	0.6407	0.6578	0.6486	0.6970	0.6573	0.6161	0.6554	0.6300	0.6773	0.6453	0.6003	0.6412	0.6333	0.6351	0.6578	0.7470	0.6216	0.6655	0.6624	0.6907
2	0.0925		0.3848	0.6392	0.6646	0.6361	0.7401	0.4525	0.4374	0.4860	0.5584	0.5472	0.5172	0.4678	0.3930	0.4036	0.4120	0.5416	0.4667	0.3968	0.4887	0.4827	0.5121	0.4972	0.6498	0.4745	0.4926	0.5976	0.5610
3	0.1118	0.0513		0.6317	0.6486	0.5918	0.6942	0.3512	0.4235	0.4443	0.5425	0.4995	0.4250	0.3380	0.3183	0.4006	0.3507	0.4906	0.3661	0.3507	0.4920	0.4609	0.5078	0.5254	0.6776	0.5332	0.5121	0.6015	0.5262
4a	0.1430	0.1134	0.1126		-0.0087	0.6502	0.5503	0.6848	0.5581	0.5785	0.5677	0.6405	0.5553	0.6398	0.6050	0.5682	0.5981	0.4527	0.4345	0.5110	0.5681	0.6076	0.5490	0.5943	0.6727	0.4306	0.4634	0.6278	0.5313
4b	0.1577	0.1256	0.1236	-0.0018		0.6382	0.5497	0.6898	0.6248	0.6422	0.6422	0.6841	0.6098	0.6267	0.6209	0.5875	0.5832	0.4352	0.4716	0.4965	0.5914	0.6429	0.5820	0.6397	0.6602	0.4656	0.5044	0.6579	0.5555
5a	0.2053	0.1379	0.1350	0.1719	0.1773		0.1128	0.3582	0.4219	0.3503	0.5523	0.5373	0.6257	0.5373	0.4357	0.5269	0.4961	0.6008	0.6377	0.5163	0.6777	0.6678	0.6311	0.5992	0.7017	0.6676	0.6408	0.5476	0.6259
5b	0.2154	0.1707	0.1659	0.1560	0.1613	0.0422		0.5291	0.4378	0.3579	0.5354	0.6415	0.5772	0.6369	0.5614	0.6299	0.6157	0.6668	0.5818	0.6264	0.6384	0.6557	0.5710	0.5932	0.7059	0.6950	0.6743	0.5443	0.5409
6a	0.1039	0.0671	0.0436	0.1309	0.1391	0.0818	0.1398		0.5291	0.1467	0.3445	0.3142	0.3983	0.3149	0.1728	0.3078	0.3022	0.5222	0.4162	0.3323	0.5202	0.5074	0.4909	0.5093	0.6767	0.5084	0.4730	0.4701	0.4930
7a	0.1052	0.0741	0.0727	0.0922	0.1110	0.1045	0.1030	0.0615		-0.0053	0.2640	0.4185	0.2659	0.4136	0.2243	0.3483	0.3616	0.5369	0.2220	0.3715	0.3418	0.3094	0.3311	0.3085	0.6529	0.4233	0.4280	0.3903	0.3756
7b	0.1240	0.0888	0.0821	0.1026	0.1220	0.0993	0.0887	0.0740	0.0006		0.2424	0.4437	0.2318	0.4171	0.2517	0.3967	0.3987	0.6047	0.2631	0.3982	0.3872	0.4222	0.3590	0.3717	0.6343	0.4850	0.4727	0.4230	0.3898
8a	0.1334	0.1011	0.1009	0.1135	0.1357	0.1437	0.1376	0.0915	0.0370	0.0346		0.1788	0.2516	0.3862	0.3416	0.5063	0.5220	0.6175	0.4398	0.5535	0.5639	0.5682	0.5506	0.5464	0.7040	0.4957	0.5201	0.4737	0.5773
8b	0.1496	0.1037	0.0865	0.1542	0.1717	0.1594	0.1958	0.0526	0.1042	0.1176	0.0789		0.4689	0.2878	0.2595	0.4627	0.4957	0.5815	0.5763	0.5227	0.6696	0.6482	0.6495	0.6972	0.7306	0.6498	0.6589	0.6168	0.6790
9a	0.1282	0.0623	0.0373	0.1267	0.1376	0.1088	0.1511	0.0227	0.0714	0.0816	0.0905	0.0450		0.2260	0.2549	0.2184	0.2473	0.4618	0.3573	0.2845	0.4853	0.4388	0.4555	0.5103	0.6709	0.5284	0.5416	0.5376	0.5607
9b	0.1372	0.0788	0.0703	0.0977	0.1154	0.1493	0.1473	0.0819	0.0333	0.0347	0.0442	0.0577	0.0789		0.0952	0.4628	0.3521	0.5596	0.3127	0.4332	0.4381	0.3847	0.3680	0.4434	0.6248	0.4096	0.4208	0.5379	0.3992
10a	0.1408	0.0874	0.0520	0.1480	0.1545	0.1422	0.1878	0.0451	0.1042	0.1151	0.0919	0.0572	0.0674	0.0142		0.3266	0.3269	0.5092	0.4391	0.3709	0.5790	0.5637	0.5762	0.5892	0.6841	0.5251	0.5744	0.6248	0.6055
11	0.1052	0.0538	0.0418	0.0929	0.1003	0.1138	0.1469	0.0379	0.0680	0.0792	0.0938	0.0752	0.0735	0.0463	0.0278		0.0892	0.4358	0.2670	0.1940	0.4398	0.4005	0.4366	0.4242	0.6550	0.4286	0.3965	0.5390	0.4644
12	0.0797	0.0474	0.0340	0.0929	0.0987	0.0987	0.1362	0.0322	0.0586	0.0703	0.0872	0.0765	0.0496	0.0470	0.0289	0.0095		0.3829	0.2465	0.1214	0.3546	0.3484	0.3775	0.3983	0.6399	0.4277	0.3935	0.5134	0.4150
13a	0.1286	0.0957	0.0786	0.1058	0.1097	0.1534	0.1869	0.0818	0.1074	0.1246	0.1375	0.1247	0.1144	0.1004	0.0810	0.0630	0.0520		0.4966	0.3472	0.5844	0.6242	0.5866	0.5359	0.6777	0.5717	0.6258	0.7185	0.5394
14a	0.1013	0.0728	0.0608	0.0618	0.0776	0.1400	0.1254	0.0868	0.0207	0.0228	0.0537	0.1200	0.0330	0.1027	0.0790	0.0513	0.0460	0.0943		0.1211	0.2074	0.1525	0.2331	0.3151	0.6680	0.3023	0.2637	0.4265	0.3091
14b	0.0800	0.0455	0.0313	0.0796	0.0845	0.1014	0.1384	0.0330	0.0630	0.0730	0.0920	0.0751	0.0587	0.0469	0.0289	0.0119	0.0069	0.0453	0.0410		0.3132	0.2815	0.3157	0.3570	0.6836	0.3839	0.3665	0.5661	0.3695
15a	0.1004	0.0633	0.0654	0.0823	0.0913	0.1293	0.1302	0.0857	0.0362	0.0444	0.0764	0.1247	0.0480	0.1050	0.0791	0.0560	0.0474	0.0959	0.0198	0.0432		-0.0202	0.0808	0.1652	0.6550	0.4034	0.3584	0.4657	0.1277
15b	0.0960	0.0566	0.0603	0.0878	0.0998	0.1330	0.1390	0.0822	0.0350	0.0477	0.0769	0.1247	0.0425	0.1022	0.0740	0.0538	0.0426	0.0983	0.0215	0.0396	-0.0021		0.0648	0.2240	0.6696	0.3461	0.3325	0.4295	0.2123
16	0.0943	0.0607	0.0644	0.0843	0.0938	0.1262	0.1218	0.0812	0.0379	0.0480	0.0784	0.1235	0.0398	0.1034	0.0761	0.0576	0.0454	0.0960	0.0250	0.0420	0.0065	0.0020		0.0748	0.6558	0.3653	0.3424	0.4662	0.0575
17	0.1135	0.0689	0.0703	0.0950	0.1074	0.1238	0.1287	0.0876	0.0378	0.0487	0.0771	0.1384	0.0510	0.1095	0.0833	0.0605	0.0503	0.0473	0.0987	0.0318	0.0143	0.0178	0.0054		0.6973	0.3978	0.3745	0.4929	0.1040
18	0.1978	0.1483	0.1541	0.1644	0.1690	0.2100	0.2161	0.1686	0.1471	0.1575	0.1709	0.2147	0.1493	0.1894	0.1664	0.1491	0.1383	0.1394	0.1771	0.1413	0.1270	0.1286	0.1272	0.1364		0.5617	0.6571	0.6803	0.6186
19	0.0853	0.0528	0.0581	0.0713	0.0799	0.1208	0.1345	0.0735	0.0500	0.0606	0.0691	0.1175	0.0415	0.0965	0.0748	0.0491	0.0422	0.0419	0.0966	0.0345	0.0360	0.0292	0.0289	0.0348	0.1021		0.0676	0.3984	0.4382
20a	0.0838	0.0661	0.0766	0.0693	0.0795	0.1396	0.1526	0.0900	0.0583	0.0716	0.0767	0.1278	0.0548	0.1045	0.0910	0.0652	0.0583	0.0556	0.1043	0.0405	0.0527	0.0449	0.0452	0.0508	0.1318	0.0115		0.4544	0.3790
21	0.1505	0.0956	0.0956	0.1143	0.1255	0.1459	0.1379	0.0772	0.0418	0.0603	0.0828	0.1481	0.0809	0.1343	0.1040	0.0822	0.0616	0.0739	0.1364	0.0555	0.0463	0.0504	0.0509	0.0611	0.1485	0.0404	0.0550		0.5562
22	0.1214	0.0745	0.0720	0.0898	0.0982	0.1330	0.1253	0.0862	0.0471	0.0573	0.0894	0.1371	0.0528	0.1137	0.0884	0.0633	0.0506	0.0994	0.0395	0.0502	0.0170	0.0168	0.0047	0.0183	0.1344	0.0620	0.0392	0.0751	

B

population	1	2	3	4b	5b	6a	7b	8b	9b	10a	11	12	13a	14b	15b	18	19	20a	21	22
1		0.0295	0.0404	0.0484	0.0233	0.0511	0.0327	0.0228	0.0420	0.0379	0.0369	0.0457	0.0438	0.0276	0.0316	0.0395	0.0401	0.0319	0.0324	0.0337
2	4.9489		0.0220	0.0320	0.0279	0.0346	0.0225	0.0190	0.0273	0.0188	0.0194	0.0258	0.0269	0.0236	0.0205	0.0290	0.0231	0.0219	0.0223	0.0235
3	5.5377	3.5329		0.0309	0.0398	0.0303	0.0224	0.0337	0.0370	0.0269	0.0275	0.0268	0.0380	0.0331	0.0257	0.0399	0.0332	0.0340	0.0164	0.0252
4b	6.8230	5.1413	5.0948		0.0458	0.0220	0.0252	0.0382	0.0314	0.0300	0.0290	0.0198	0.0238	0.0330	0.0430	0.0473	0.0338	0.0381	0.0326	0.0364
5b	6.0672	4.7985	5.2939	5.3491		0.0537	0.0371	0.0169	0.0350	0.0307	0.0322	0.0426	0.0397	0.0297	0.0304	0.0289	0.0313	0.0258	0.0309	0.0322
6a	5.8194	4.2485	4.5856	3.9140	5.8216		0.0250	0.0433	0.0456	0.0399	0.0379	0.0276	0.0342	0.0373	0.0418	0.0518	0.0402	0.0435	0.0339	0.0349
7b	4.6544	3.9309	4.7515	4.4821	5.9213	3.6119		0.0271	0.0362	0.0290	0.0259	0.0266	0.0319	0.0202	0.0262	0.0423	0.0322	0.0300	0.0178	0.0219
8b	5.0837	3.6133	4.9001	4.8953	3.2746	4.5877	4.1048		0.0323	0.0262	0.0267	0.0343	0.0311	0.0225	0.0214	0.0292	0.0260	0.0210	0.0259	0.0227
9b	7.4268	5.4561	5.8027	5.2976	5.6321	6.4922	6.4594	5.7587		0.0157	0.0207	0.0267	0.0232	0.0344	0.0426	0.0369	0.0295	0.0302	0.0383	0.0405
10a	6.4120	4.3150	4.5920	4.9249	4.8566	5.5860	5.4444	4.8670	2.7773		0.0122	0.0208	0.0246	0.0301	0.0313	0.0275	0.0191	0.0199	0.0275	0.0309
11	5.3302	3.9713	4.4396	5.4390	5.5530	5.2339	4.0371	4.7344	4.6413	3.2506		0.0175	0.0226	0.0237	0.0307	0.0297	0.0185	0.0201	0.0266	0.0330
12	5.3235	4.1567	3.9157	5.0405	5.4377	4.7458	4.4480	4.7718	4.3730	3.1550	2.6681		0.0195	0.0302	0.0370	0.0382	0.0253	0.0309	0.0307	0.0355
13a	5.5699	3.9608	4.9743	4.2981	4.7427	4.7551	5.0274	4.2964	4.2820	4.1919	4.4350	3.7757		0.0279	0.0411	0.0370	0.0266	0.0302	0.0390	0.0384
14b	4.7761	4.7438	5.9381	6.0316	6.3602	5.3310	3.1124	4.8900	7.2722	6.4282	4.6146	5.0731	5.0915		0.0280	0.0366	0.0313	0.0276	0.0266	0.0304
15b	5.9430	4.0590	4.9601	5.8978	4.9754	5.3011	4.3202	3.1856	7.2228	5.9421	5.1375	5.6335	5.6291	5.0148		0.0324	0.0308	0.0271	0.0189	0.0211
18	8.6069	6.8003	7.1742	7.4456	5.6354	7.6059	8.6780	6.9380	7.0098	6.1314	7.5099	7.0728	5.8962	8.8590	7.8515		0.0236	0.0221	0.0355	0.0380
19	7.2622	5.0887	5.7369	5.7504	5.0762	5.5812	6.1696	5.2621	6.2073	4.7727	4.8053	5.2103	5.2406	6.9723	5.7678	5.6799		0.0119	0.0298	0.0312
20a	6.1171	4.7340	5.5192	5.5934	4.9598	5.3450	5.4051	4.8552	5.8256	4.1782	4.1792	4.7464	4.7929	6.1352	5.5719	5.7718	1.9520		0.0277	0.0276
21	5.4945	3.6410	3.4655	4.2158	3.8495	4.1331	3.5529	3.3760	5.7653	4.4868	4.2807	4.3444	4.9261	5.0558	3.5793	6.8204	5.0064	4.6630		0.0200
22	6.1168	5.0319	5.0086	5.6808	4.4431	5.0999	5.0892	3.7617	6.9245	5.5506	5.8239	5.5646	5.7433	6.4322	4.3525	7.5547	5.6727	5.1649	3.7673	

Table S4 Pairwise morphometric (body shape and LPJ) distances within systems with more than two populations. Procrustes (upper triangular matrix) and Mahalanobis (lower triangular matrix) distances from the CVA (Fig. S5A & B) (non-significant values are underlined). (A) Pairwise body shape differentiation among the four Kalambo populations. (B) Pairwise body shape differentiation among the three Lufubu populations. (C) Pairwise LPJ shape differentiation among the four Kalambo populations. (D) Pairwise LPJ shape differentiation among the three Lufubu populations.

A

population	KaL	Ka1	Ka2	Ka3
KaL		0.0251	0.0368	0.0253
Ka1	3.8781		0.0535	0.0220
Ka2	6.3056	6.0287		0.0456
Ka3	5.3659	4.2863	6.3437	

B

population	LfL	Lf1	Lf2
LfL		0.0122	0.0182
Lf1	2.1637		0.0208
Lf2	4.0045	4.2414	

C

population	KaL	Ka1	Ka2	Ka3
KaL		0.0175	0.0217	0.0158
Ka1	1.9260		0.0122	0.0192
Ka2	3.3438	2.4847		0.0257
Ka3	1.9681	1.8445	3.2216	

D

population	LfL	Lf1	Lf2
LfL		0.0064	0.0266
Lf1	0.6663		0.0258
Lf2	2.1046	2.1603	

Table S5 Pairwise morphometric (body shape and LPJ) distances of all populations from the lake-stream systems. Procrustes (upper triangular matrix) and Mahalanobis (lower triangular matrix) distances from the CVA (Fig. 3) (non-significant values are underlined). (A) Pairwise body shape differentiation. (B) Pairwise LPJ shape differentiation.

A

population	Ka3	Ka2	Ka1	KaL	Ch1	ChL	Lz1	LzL	Lf2	Lf1	LfL
Ka3		0.0457	0.0220	0.0253	0.0314	0.0295	0.0238	0.0331	0.0474	0.0341	0.0386
Ka2	5.6679		0.0535	0.0369	0.0349	0.0312	0.0396	0.0296	0.0288	0.0301	0.0243
Ka1	3.9344	5.9029		0.0251	0.0455	0.0393	0.0341	0.0373	0.0518	0.0406	0.0442
KaL	4.5737	6.1411	3.5549		0.0361	0.0288	0.0320	0.0202	0.0423	0.0321	0.0301
Ch1	5.4110	5.8736	6.5003	6.6979		0.0158	0.0231	0.0344	0.0370	0.0291	0.0300
ChL	5.0602	4.9994	5.5704	5.7198	2.7821		0.0242	0.0302	0.0278	0.0182	0.0196
Lz1	4.3098	5.0077	4.6585	5.0996	4.3658	4.2179		0.0279	0.0371	0.0268	0.0307
LzL	6.0366	6.6764	5.2110	3.0927	7.4857	6.6939	5.1698		0.0366	0.0309	0.0273
Lf2	7.7497	5.8296	7.8774	9.0269	7.0925	6.1512	6.0970	9.2435		0.0229	0.0214
Lf1	5.6788	4.9360	5.4026	6.1579	6.0796	4.5449	5.0511	6.9778	5.6664		0.0121
LfL	5.4917	4.7787	5.1340	5.3243	5.6660	3.8408	4.5813	6.0876	5.7561	2.0123	

B

population	Ka3	Ka2	Ka1	KaL	Ch1	ChL	Lz1	LzL	Lf2	Lf1	LfL
Ka3		0.0273	0.0214	0.0160	0.0414	0.0086	0.0277	0.0257	0.0317	0.0287	0.0247
Ka2	2.7659		0.0122	0.0226	0.0260	0.0310	0.0254	0.0202	0.0193	0.0188	0.0212
Ka1	1.8301	2.1889		0.0188	0.0269	0.0256	0.0191	0.0171	0.0193	0.0234	0.0232
KaL	2.0079	2.9577	2.0749		0.0421	0.0162	0.0278	0.0158	0.0255	0.0238	0.0216
Ch1	3.5801	3.4244	3.1368	4.1603		0.0445	0.0280	0.0419	0.0291	0.0377	0.0392
ChL	1.7643	3.2636	2.5246	1.9657	3.6404		0.0296	0.0291	0.0347	0.0318	0.0270
Lz1	2.5159	3.3232	2.2841	2.9991	2.8786	2.8934		0.0313	0.0200	0.0392	0.0388
LzL	2.8324	3.2146	1.9479	2.1601	4.4232	3.1740	3.7602		0.0252	0.0233	0.0239
Lf2	3.0152	3.6780	2.7341	2.4114	3.3427	2.9693	2.9560	3.0189		0.0310	0.0339
Lf1	3.1319	3.5420	3.3800	2.6641	4.5602	3.1829	4.4215	3.2175	3.0839		0.0074
LfL	2.8893	3.6144	3.2140	2.3889	4.4794	2.8310	4.2185	3.0297	3.0012	0.6559	

Table S6 P values for within system gill raker length comparisons for males and females. P values were obtained with an ANOVA and adjusted with a TukeyHSD in systems with more than two populations to correct for multiple testing (Fig. 2E, Fig. S5C).

sex	Kalambo						Chitili	Lunzua	Lufubu		
	KaL-Ka1	KaL-Ka2	KaL-Ka3	Ka1-Ka2	Ka1-Ka3	Ka2-Ka3	ChL-Ch1	LzL-Lz1	LfL-Lf1	LfL-Lf2	Lf1-Lf2
males	0.0211*	0.0149*	< 0.0001***	0.9979	0.0864	0.1407	0.0419*	0.0003**	0.1544	0.1107	0.0017**
females	0.3340	< 0.0001***	< 0.0001***	0.0001**	0.0001**	0.9967	0.1531	0.0001**	0.0840	< 0.0001***	< 0.0001***

Table S7 Pairwise morphometric (body shape and LPJ) distances between F1 crosses. (A) Pairwise morphometric distances described by Procrustes (upper triangular matrix) and Mahalanobis (lower triangular matrix) distances from the CVAs comparing each inter-population cross with the corresponding within population crosses (non-significant values are underlined, for CVA plots see Fig. 4A). (B) P values for pairwise comparisons of gill raker length among all within and inter-population crosses (Fig. 4B).

A

F1 juveniles	KaL-KaL	KaL-Ka3	Ka3-Ka3
KaL-KaL		0.0086	0.0097
KaL-Ka3	1.2961		0.0048
Ka3-Ka3	1.9713	1.2240	

F1 juveniles	KaL-KaL	KaL-Lz1	Lz1-Lz1
KaL-KaL		0.0081	0.0110
KaL-Lz1	1.3536		0.0078
Lz1-Lz1	1.8514	1.3714	

F1 juveniles	Ka3-Ka3	Ka3-Lz1	Lz2-Lz1
Ka3-Ka3		0.0081	0.0079
Ka3-Lz1	1.6021		0.0090
Lz1-Lz1	1.4724	1.7618	

B

F1 juveniles	Ka3-Ka3	Lz1-Lz1	KaL-Ka3	KaL-Lz1	Ka3-Lz1
KaL-KaL	0.00078	0.00004	0.22130	0.00588	0.02763
Ka3-Ka3		0.99788	0.82486	0.98741	0.99975
Lz1-Lz1			0.57282	0.86382	0.98707
KaL-Ka3				0.98122	0.96682
KaL-Lz1					0.99990

Table S8 Pairwise morphometric (body shape) distances and P values of gill raker comparisons among different groups of the pond experiment. Procrustes (upper triangular matrix) and Mahalanobis (lower triangular matrix) distances of the CVA comparing body shape among the within population F1 offspring and their corresponding wild populations (A) and among population of surviving adults at the beginning and at the end of the experimental period (B). (C) Comparison of gill raker length among the within population F1 offspring and their corresponding wild populations. (Fig. S7)

A

F1 and wild populations	KaL-KaL	Ka3-Ka3	Lz1-Lz1	KaL-wild	Ka3-wild	Lz1-wild
KaL-KaL		0.0094	0.0108	0.0119	0.0266	0.0241
Ka3-Ka3	1.5840		0.0080	0.0145	0.0261	0.0225
Lz1-Lz1	1.3126	1.3175		0.0154	0.0309	0.0284
KaL-wild	2.1099	2.0466	1.7501		0.0235	0.0242
Ka3-wild	3.4504	3.2127	3.3877	3.6574		0.0103
Lz1-wild	2.8738	2.2854	2.9527	3.2975	1.9800	

B

parental populations	KaL-before	Ka3-before	Lz1-before	KaL-after	Ka3-after	Lz1-after
KaL-before		0.0215	0.0218	0.0079	0.0134	0.0106
Ka3-before	2.6663		0.0131	0.0196	0.0132	0.0225
Lz1-before	2.4212	1.9504		0.0211	0.0109	0.0184
KaL-after	1.1066	2.5476	2.4792		0.0127	0.0138
Ka3-after	1.9615	1.7624	1.0353	2.0022		0.0119
Lz1-after	1.8311	2.5275	1.7273	1.7073	1.2464	

C

F1 and wild populations	Ka3-Ka3	Lz1-Lz1	KaL-wild	Ka3-wild	Lz1-wild
KaL-KaL	0.00214	0.00401	0.69902	< 0.00001	0.00005
Ka3-Ka3		0.99760	0.30149	< 0.00001	0.42067
Lz1-Lz1			0.47098	< 0.00001	0.20750
KaL-wild				< 0.00001	0.01044
Ka3-wild					0.09142

sampling information			locus										
population	year		Ppun7	Ppun21	UNH130	Abur82	Ppun5	HchiST46	HchiST68	UNH989	Pzeb3	average
Ninde	2011	N_Z	7	7	6	3	7	1	7	6	7	5.67
		N_A	6	4	6	3	8	1	3	7	4	4.67
		H_O	0.85714	0.42857	0.83333	0.33333	0.85714	na	0.00000	0.66667	0.42857	0.55
		H_E	0.8022	0.73626	0.87879	0.60000	0.91209	na	0.48352	0.83333	0.57143	0.73
Loazi	2011	N_Z	31	31	30	31	27	31	28	28	31	29.78
		N_A	27	20	21	15	24	2	18	22	6	17.22
		H_O	0.93548	0.87097	**0.70000**	0.74194	0.88889	0.12903	0.71429	0.92857	0.70968	0.74
		H_E	0.9413	0.92491	0.92147	0.86409	0.94689	0.12269	0.85390	0.92208	0.71232	0.80
Muzi	2011	N_Z	25	25	24	25	25	24	24	24	24	24.44
		N_A	15	14	15	15	15	2	12	18	6	12.44
		H_O	0.96000	0.84000	0.79167	0.76000	0.72000	0.04167	0.79167	**0.66667**	0.58333	0.68
		H_E	0.90531	0.90531	0.91135	0.90776	0.90367	0.04167	0.86968	0.94681	0.64539	0.78
Kalambo stream 3	2010	N_Z	32	31	32	32	32	32	32	32	32	31.89
		N_A	9	11	15	7	16	2	6	8	7	9.00
		H_O	0.90625	0.87097	0.87500	0.84375	0.84375	0.46875	0.65625	0.71875	0.75000	0.77
		H_E	0.82192	0.83131	0.88790	0.76290	0.91915	0.44792	0.75694	0.84226	0.80655	0.79
	2011	N_Z	30	30	30	30	30	30	30	30	30	30.00
		N_A	11	10	10	7	15	2	4	10	6	8.33
		H_O	0.93333	0.76667	0.76667	0.73333	0.63333	0.43333	0.63333	**0.80000**	0.60000	0.70
		H_E	0.87627	0.79887	0.87175	0.78588	0.89492	0.48079	0.75763	0.82316	0.73446	0.78
Kalambo stream 2	2011	N_Z	14	14	13	14	14	1	13	14	14	12.33
		N_A	7	12	10	6	13	1	6	6	6	7.44
		H_O	0.64286	0.71429	0.69231	0.42857	0.71429	na	0.69231	0.64286	0.78571	0.66
		H_E	0.69312	0.79630	0.84308	0.43915	0.92593	na	0.71385	0.72751	0.76720	0.74
	2012	N_Z	32	32	32	32	31	32	32	32	32	31.89
		N_A	9	13	9	10	14	2	9	12	6	9.33
		H_O	0.50000	0.56250	0.84375	0.59375	0.74194	0.09375	0.81250	0.78125	0.71875	0.63
		H_E	0.57391	0.62351	0.74504	0.68006	0.84294	0.09077	0.82440	0.76885	0.75198	0.66
Kalambo stream 1	2011	N_Z	32	32	32	32	31	32	32	31	32	31.78
		N_A	21	23	23	20	19	3	12	20	6	16.33
		H_O	0.96875	0.90625	0.93750	0.93750	0.83871	0.15625	**0.37500**	0.93548	0.40625	0.72
		H_E	0.91419	0.94643	0.93800	0.94444	0.94289	0.17708	0.76935	0.91962	0.49603	0.78
Kalambo lake	2010	N_Z	31	31	30	31	30	30	31	31	31	30.67
		N_A	18	25	22	19	20	3	14	19	5	16.11
		H_O	0.96774	0.93548	0.86667	0.96774	**0.76667**	0.26667	0.54839	1.00000	0.58065	0.77
		H_E	0.91645	0.94342	0.94407	0.93971	0.94068	0.24350	0.80539	0.93178	0.59598	0.81
	2011	N_Z	33	33	32	33	33	33	33	33	33	32.89
		N_A	18	21	20	18	22	3	17	20	5	16.00
		H_O	0.87879	0.93939	0.84375	0.87879	0.81818	0.24242	0.81818	0.9697	0.42424	0.76
		H_E	0.87832	0.93706	0.93204	0.91422	0.95058	0.29324	0.86993	0.93986	0.43357	0.79
Toby's place	2010	N_Z	34	34	31	33	34	34	34	34	33	33.44
		N_A	13	14	20	14	15	2	11	16	7	12.44
		H_O	0.91176	0.85294	0.93548	0.93939	0.88235	0.05882	0.73529	0.91176	0.54545	0.75
		H_E	0.80114	0.84372	0.88525	0.87925	0.90386	0.11238	0.83055	0.89245	0.52214	0.74
	2012	N_Z	31	31	28	31	31	31	31	27	31	30.22
		N_A	11	10	12	13	14	2	4	9	5	8.89
		H_O	0.96774	0.83871	0.82143	0.90323	0.96774	0.03226	0.61290	0.74074	0.48387	0.71
		H_E	0.85405	0.77737	0.80779	0.83765	0.85616	0.03226	0.68324	0.83718	0.45267	0.68
Chitili creek 1	2010	N_Z	7	7	7	7	7	1	7	7	6	6.22
		N_A	9	7	9	7	9	1	7	5	4	6.44
		H_O	1.00000	0.71429	0.85714	**0.42857**	1.00000	na	0.85714	1.00000	1.00000	0.86
		H_E	0.94505	0.89011	0.91209	0.85714	0.93407	na	0.85714	0.83516	0.72727	0.87
	2011	N_Z	31	31	31	31	31	31	30	29	31	30.67
		N_A	15	17	14	13	16	2	12	12	5	11.78
		H_O	0.90323	0.80645	0.64516	0.80645	0.87097	0.06452	0.70000	0.89655	0.32258	0.67
		H_E	0.87996	0.87943	0.86039	0.85087	0.90164	0.06346	0.85424	0.87719	0.34320	0.72
Chitili lake	2011	N_Z	31	31	29	31	31	1	28	30	31	27.00
		N_A	16	13	18	19	19	1	14	14	6	13.33
		H_O	0.87097	0.90323	0.96552	0.83333	0.86667	na	**0.57143**	0.73333	0.41935	0.77
		H_E	0.90375	0.91698	0.92257	0.91808	0.91751	na	0.88636	0.91751	0.46007	0.86
Chisanza	2011	N_Z	32	30	30	32	31	32	31	32	32	31.33
		N_A	25	24	19	23	20	2	13	21	10	17.44
		H_O	0.96875	1.00000	**0.63333**	0.81250	0.83871	0.37500	**0.61290**	**0.75000**	0.59375	0.73
		H_E	0.94792	0.95593	0.92712	0.94990	0.91539	0.30952	0.85669	0.92808	0.67560	0.83
Sumba	2011	N_Z	32	31	32	32	32	32	32	32	31	31.78
		N_A	27	25	22	23	20	2	14	20	9	18.00
		H_O	0.93750	0.96774	**0.75000**	**0.81250**	0.78125	0.21875	**0.53125**	**0.62500**	0.70968	0.70
		H_E	0.96081	0.95346	0.93204	0.94891	0.93056	0.19792	0.90228	0.92808	0.72343	0.83
		p	0.10437	0.41513	**0.00142**	**0.00379**	**0.01741**	1.00000	**0.00000**	**0.00000**	0.37605	0.21
Lunzua stream 1	2011	N_Z	30	30	30	30	30	30	30	30	30	30.00
		N_A	11	13	12	12	13	2	10	9	6	9.78
		H_O	0.83333	0.90000	0.66667	0.66667	**0.60714**	0.23333	**0.60000**	0.70000	0.60000	0.65
		H_E	0.87458	0.85480	0.83446	0.75819	0.86104	0.20960	0.85706	0.82542	0.60791	0.74
Lunzua lake	2010	N_Z	30	30	29	30	28	28	30	29	30	29.33
		N_A	24	29	31	26	20	3	18	22	8	20.11
		H_O	0.93333	0.96667	0.89655	0.93333	0.89286	0.42857	**0.63333**	0.93103	0.66667	0.81
		H_E	0.95876	0.96667	0.97217	0.96271	0.94675	0.38247	0.92825	0.95523	0.5887	0.85
	2011	N_Z	31	31	31	31	31	31	31	31	31	31.00
		N_A	25	30	27	25	20	3	23	23	8	20.44
		H_O	0.87097	1.00000	**0.74194**	0.80645	0.80645	0.32258	**0.70968**	0.87097	0.67742	0.76
		H_E	0.94553	0.96616	0.96140	0.95928	0.94236	0.32311	0.94342	0.94553	0.68852	0.85
Wonzye	2010	N_Z	41	42	42	42	37	42	42	42	42	41.33
		N_A	29	33	30	24	23	3	15	24	10	21.22
		H_O	**0.95122**	**0.95238**	**0.88095**	**0.95238**	**0.86486**	0.38095	**0.61905**	**0.90476**	0.73810	0.80
		H_E	0.95574	0.96644	0.95668	0.95726	0.95002	0.32014	0.82760	0.94894	0.79891	0.85
	2012	N_Z	18	18	17	18	15	18	18	18	18	17.56
		N_A	23	23	22	20	12	2	13	19	9	15.89
		H_O	0.94444	0.83333	0.88235	1.00000	0.66667	0.16667	0.83333	0.94444	0.88889	0.80
		H_E	0.96349	0.96508	0.96791	0.96825	0.91264	0.15714	0.82698	0.95714	0.85556	0.84
Fisheries Department	2010	N_Z	25	26	24	25.00000	26	26	26	26	26	25.56
		N_A	27	20	24	23	17	3	15	23	11	18.11
		H_O	1.00000	1.00000	0.95833	0.80000	0.80769	0.15385	**0.61538**	**0.76923**	0.84615	0.77
		H_E	0.94694	0.93288	0.96188	0.95020	0.93363	0.14706	0.88612	0.94872	0.84238	0.84
Kalungula	2010	N_Z	26	27	26	26	20	27	27	27	27	25.89
		N_A	23	20	18	19	15	3	15	17	10	15.56
		H_O	0.88462	0.92593	0.92308	0.84615	**0.70000**	0.11111	0.62963	**0.70370**	0.70370	0.71
		H_E	0.94344	0.94340	0.92609	0.94118	0.92821	0.10832	0.84696	0.90566	0.82250	0.82
Lufubu stream 2	2012	N_Z	30	30	29	30	30	30	30	29	30	29.78
		N_A	9	20	14	17	15	2	6	10	5	10.89
		H_O	0.80000	0.96667	0.79310	0.93333	0.75862	0.03333	0.60000	**0.96552**	**0.30000**	0.68
		H_E	0.71469	0.94407	0.76830	0.90904	0.91712	0.03333	0.60904	0.69752	0.58136	0.69
Lufubu stream 1	2011	N_Z	27	27	27	27	24	27	27	27	27	26.67
		N_A	19	28	21	25	21	3	16	21	12	18.44
		H_O	0.88889	0.88889	**0.66667**	0.85185	**0.62500**	0.25926	**0.74074**	0.92593	0.85185	0.74
		H_E	0.95318	0.96995	0.95038	0.94200	0.94592	0.23410	0.92872	0.94829	0.85325	0.86
Lufubu lake	2011	N_Z	30	30	30	30	30	30	29	30	30	29.89
		N_A	18	28	22	22	24	4	14	19	13	18.22
		H_O	0.9	0.86667	**0.46667**	0.93333	**0.73333**	0.46667	**0.55172**	**0.83333**	0.86667	0.74
		H_E	0.93616	0.9661	0.86723	0.93164	0.95028	0.45989	0.79492	0.94746	0.83051	0.85
		p	0.08827	0.12133	**0.00008**	0.77188	**0.00048**	0.61206	**0.00000**	**0.00672**	0.33487	0.22
Ndole bay	2012	N_Z	16	16	8	16	13	16	16	15	16	14.67
		N_A	15	9	12	7	12	2	7	10	7	9.00
		H_O	1.00000	0.93750	0.87500	0.68750	**0.61538**	0.18750	0.68750	0.53333	0.68750	0.69
		H_E	0.93347	0.88105	0.96667	0.74194	0.93846	0.17540	0.71371	0.85057	0.74395	0.77
Lake Chila	2012	N_Z	13	14	13	14	12	1	14	14	14	12.11
		N_A	13	19	15	16	14	1	12	12	8	12.22
		H_O	1.00000	1.00000	0.76923	1.00000	0.75000	na	0.78571	0.71429	0.85714	0.86
		H_E	0.92000	0.96825	0.94154	0.94974	0.94203	na	0.84656	0.88624	0.84656	0.91

Table S9 Microsatellite diversity in populations of *Astatotilapia burtoni*. NG, number of genotypes per locus; NA, number of alleles per locus; HO, obsevered heterozygosity; HE, expected heterozygosity. Deviations from Hardy-Weinberg expectations at a 0.05 significance level after sequential Bonferroni correction are indicated in bold print.

Table S10 Genetic diversity of mtDNA sequences. N, number of sequences per population; H, number of haplotypes; He, gene diversity; π, nucleotide diversity.

population	*N*	*H*	*He*	*π*
Ninde	7	1	0.00000	0.00000
Loazi	7	1	0.00000	0.00000
Muzi	9	1	0.00000	0.00000
Kalambo stream 3	27	1	0.00000	0.00000
Kalambo stream 2	8	1	0.00000	0.00000
Kalambo stream 1	6	3	0.60000	0.00182
Kalambo lake	29	3	0.25400	0.00071
Toby's place	30	2	0.18600	0.00051
Chitili creek 1	17	2	0.44100	0.00120
Chitili lake	10	2	0.55600	0.00151
Chisanza	9	3	0.41700	0.00182
Sumba	9	3	0.58300	0.00227
Lunzua stream 1	7	1	0.00000	0.00000
Lunzua lake	24	4	0.30800	0.00098
Wonzye	49	2	0.08000	0.00022
Fisheries Department	24	1	0.00000	0.00000
Kalungula	28	1	0.00000	0.00000
Lufubu stream 2	13	3	0.41000	0.00119
Lufubu stream 1	10	1	0.00000	0.00000
Lufubu lake	9	1	0.00000	0.00000
Ndole	13	2	0.15400	0.00042
Lake Chila	14	1	0.00000	0.00000

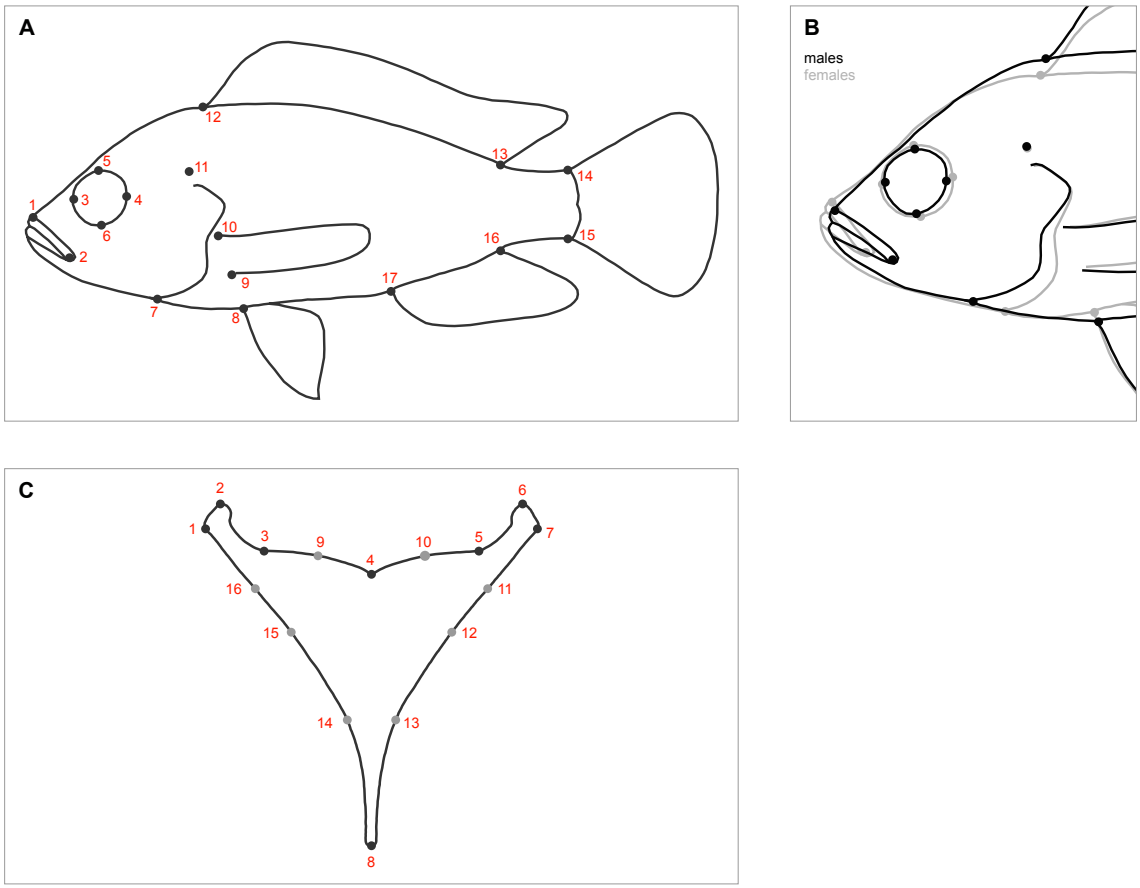


Figure S1 Landmark positions for body shape and LPJ analyses and sex differences in head shape. (A) All 17 landmarks were used for body shape analyses comparing the wild populations, whereas only the 6 landmarks 1, 2, 8, 12, 14 and 15 were used for comparisons of the body shape of adults and F1 offspring of the pond experiment and only the four landmarks 1, 2, 7 and 12 were included in the mouth position analysis. (B) Only the landmarks describing head shape (1-8, 11 and 12) were used to compare head morphology of males (black outline) and females (grey outline). A DFA showed that females generally have more slender, but longer heads (DF differences are increased tenfold in the outlines). (C) True (black) and semi-landmarks (grey), which were included in the comparisons of the LPJ shape.

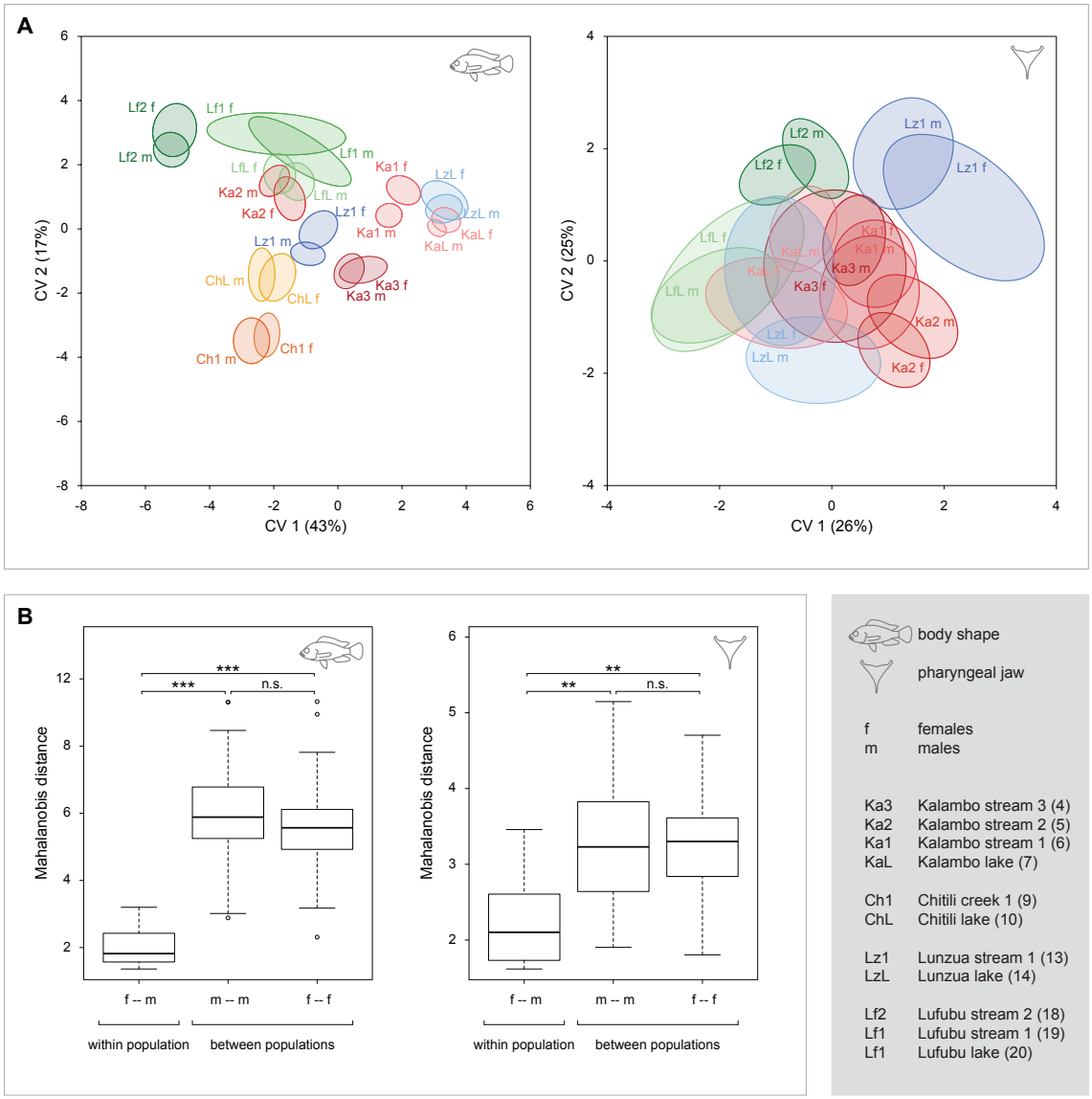


Figure S2 Comparison of inter-sexual within population differences and intra-sexual differences among populations in morphometric traits (body shape and LPJ). (A) CVA plots show strong population specific overlap of male and female body, as well as in LPJ shape (ellipses represent the 95% confidence intervals of the means). The Chitili system was excluded for LPJ shape since sample size was low in females (Table S1). (B) ANOVAs with additional TukeyHSD show significantly smaller Mahalanobis distances in inter-sexual comparisons within populations, compared to intra-sexual comparisons among populations for body shape as well as for LPJ shape. Significance levels: *P < 0.05, **P < 0.01 and ***P < 0.0001.

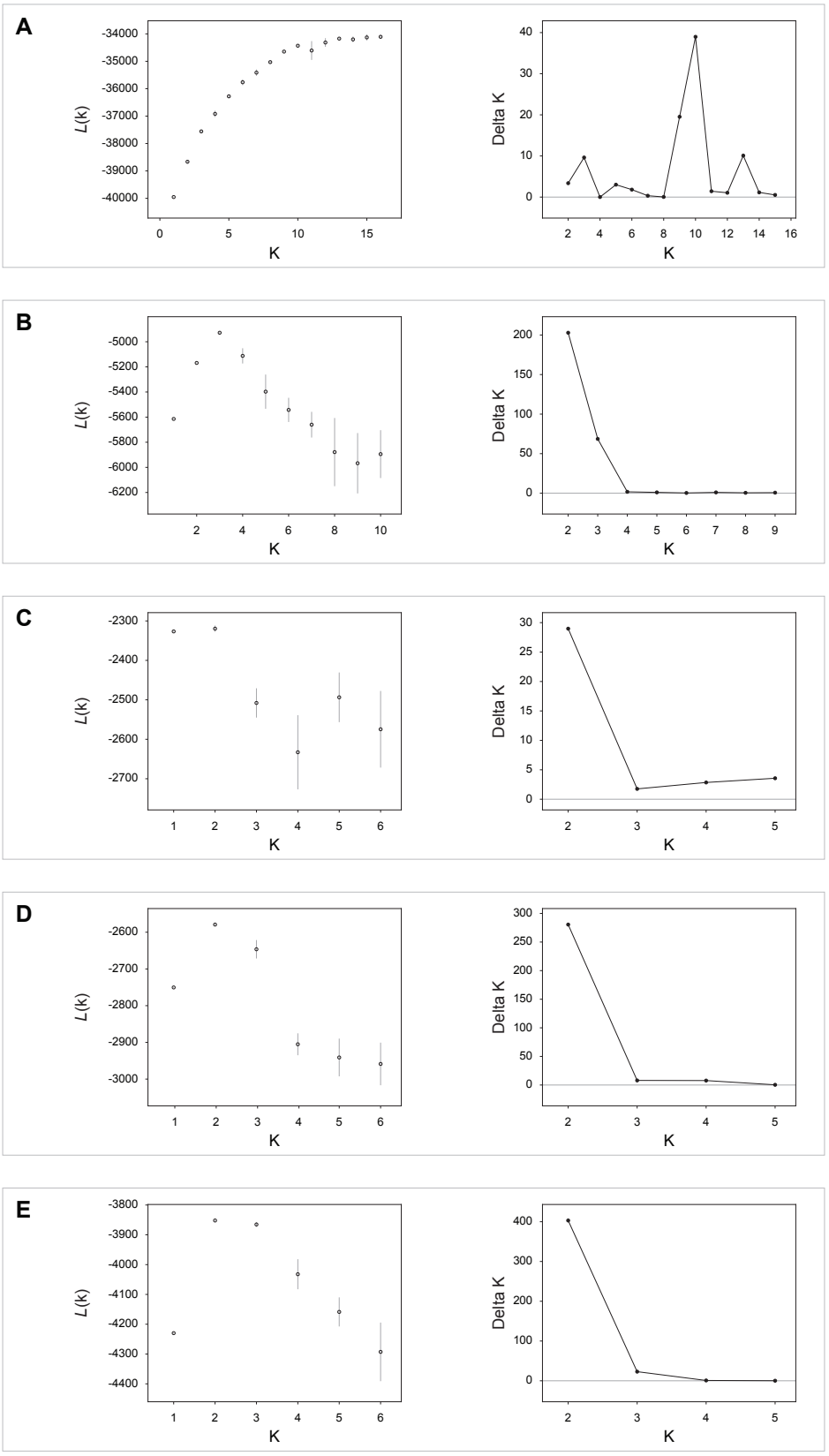
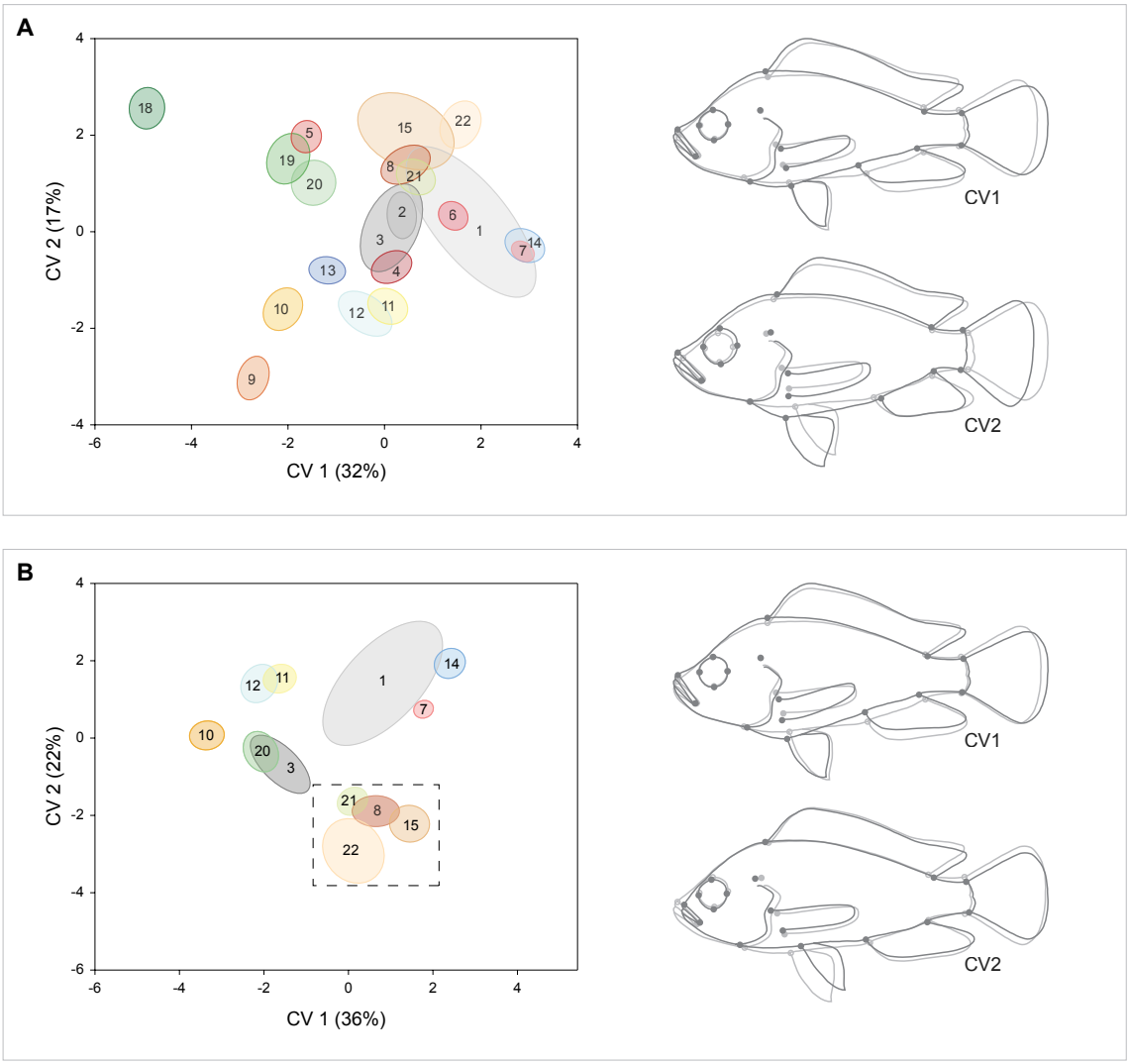


Figure S3 Mean likelihood (L(K) ± SD) over 10 Structure runs assuming K clusters (left); ΔK statistic (right); (A) full data, (B) samples from the Kalambo river, (C) samples from the Chitili creek, (D) samples from the Lunzua river, (E) samples from the Lufubu river.



1 Ninde	6 Kalambo stream 1 (Ka1)	11 Chisanza	18 Lufubu stream 2 (Lf2)
2 Loazi	7 Kalambo lake (KaL)	12 Sumba	19 Lufubu stream 1 (Lf1)
3 Muzi	8 Toby's Place	13 Lunzua stream 1 (Lz1)	20 Lufubu lake (LfL)
4 Kalambo stream 3 (Ka3)	9 Chitili creek 1 (Ch1)	14 Lunzua lake (LzL)	21 Ndole Bay
5 Kalambo stream 2 (Ka2)	10 Chitili lake (ChL)	15 Wonzye	22 Lake Chila

Figure S4 Body shape differentiation among the 20 sampled populations and among the 11 shoreline populations only (ellipses represent the 95% confidence intervals of the means). (A) Overall body shape differentiation among 20 populations (numbers and colors of the populations correspond with Fig. 1). The most extreme shape changes of the first two CVs are illustrated by landmark shifts (from grey to black with increasing values) (Table S3B). (B) CVA plot for the first two CVs and corresponding landmark shifts for the shoreline populations only. The clustering of populations in the morpho-space indicates stronger clustering of pure lacustrine populations (framed with a dashed line) compared to the other, more scattered shoreline populations, which are adjacent to streams.

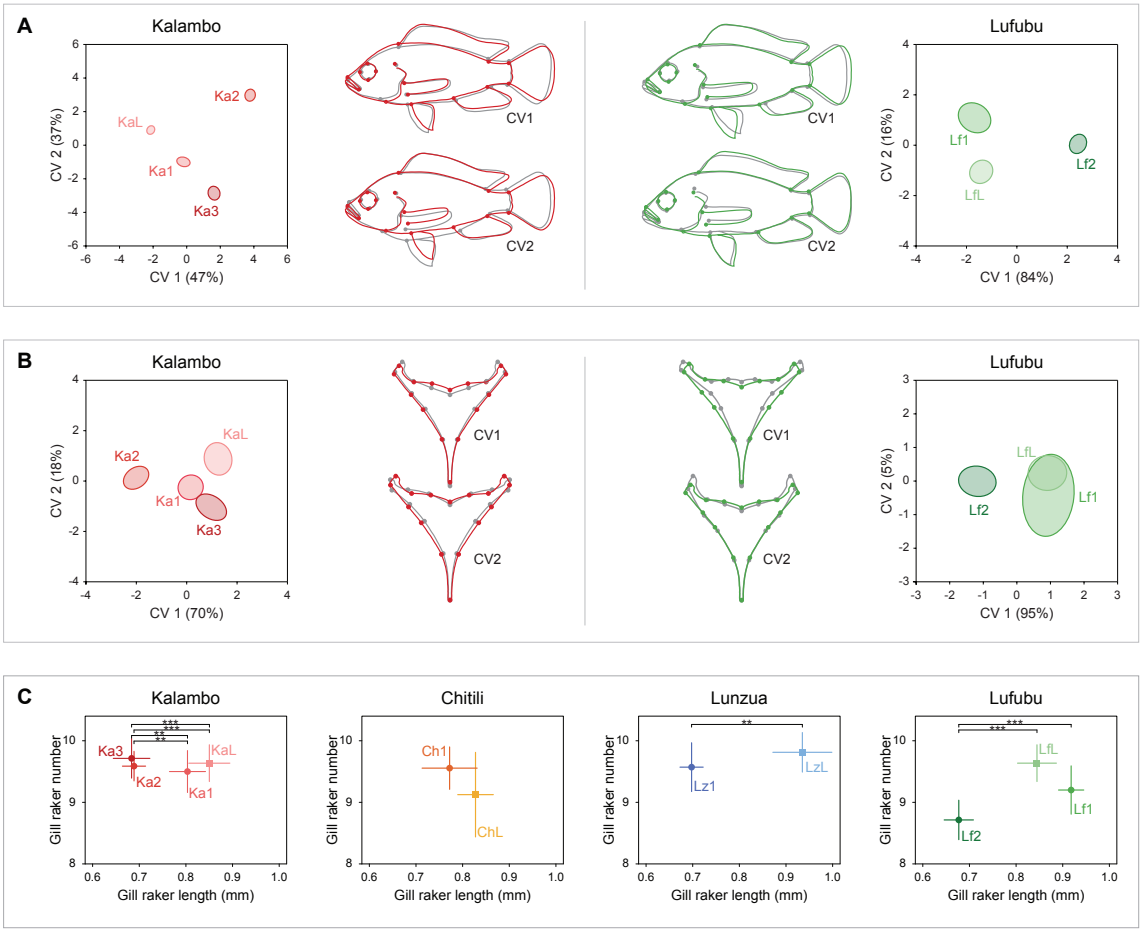


Figure S5 Body shape and LPJ shape differentiation within systems with more than two populations and gill raker length and number in females. (A) Body shape differentiation separately for the four Kalambo populations (ellipses represent the 95% confidence intervals of the means, outlines from colored to grey with increasing CV-values, Table S4A) as well for the three Lufubu populations (Table S4B). (B) LPJ shape differentiation for the four Kalambo populations separately (Table S4C) as well for the three Lufubu populations (Table S4D). (C) Differences in size corrected female gill raker lengths and number between populations within each lake-stream system (error bars represent 95% confidence intervals of the means) (Table S6). Significance levels: *P < 0.05, **P < 0.01 and ***P < 0.0001.

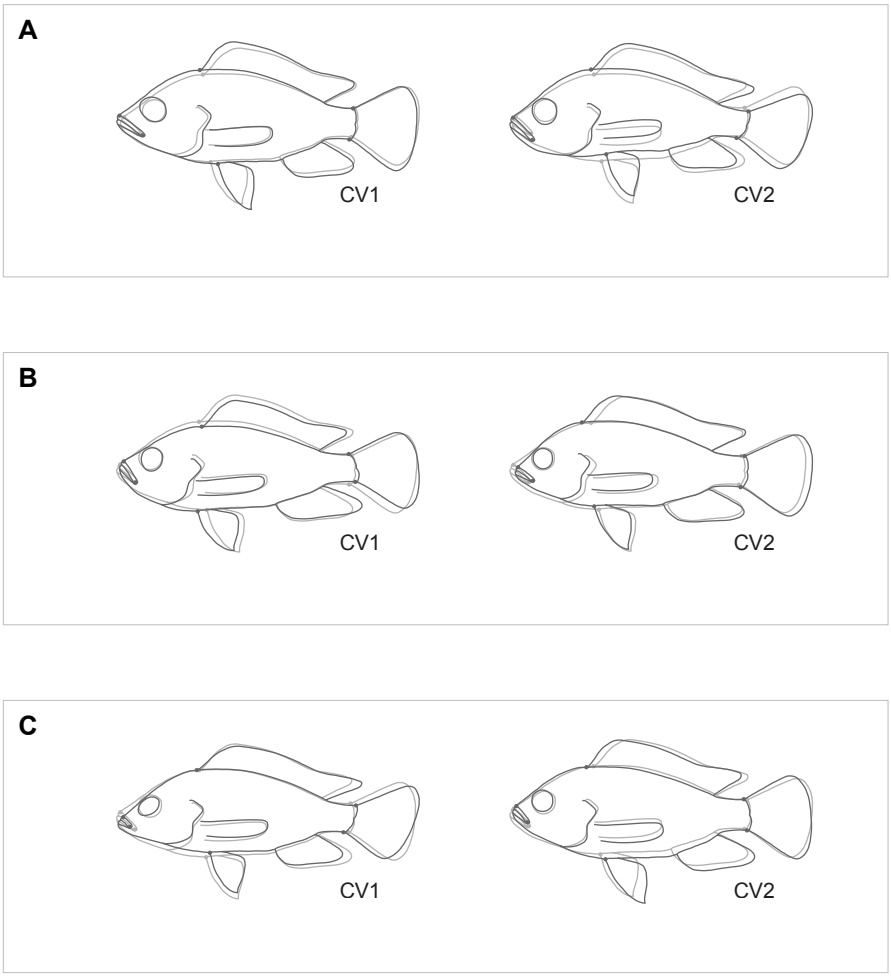
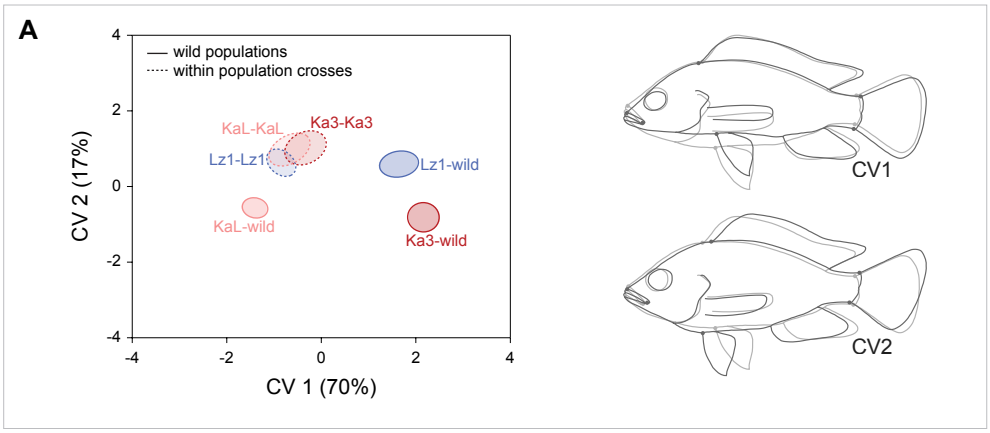
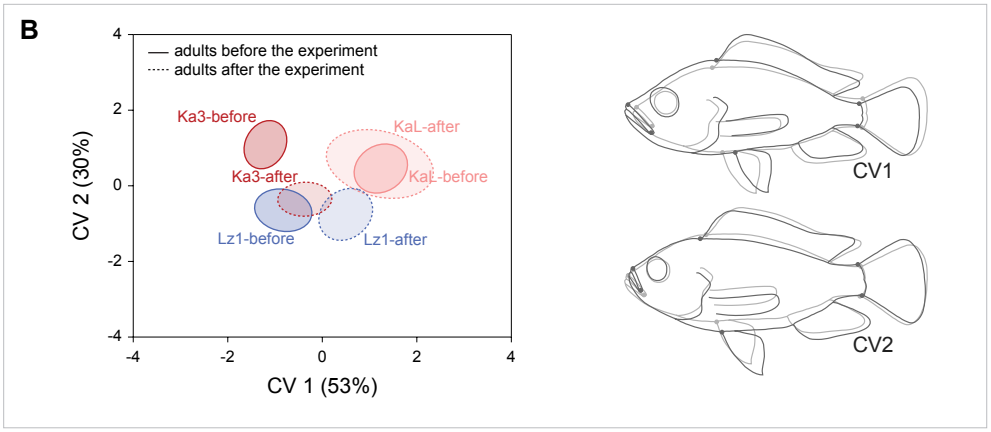


Figure S6 Outlines to illustrate the body shape changes in F1 individuals of the pond experiment (CVA plots in Fig. 4A; distance values Table S7). From light grey to dark outlines with increasing values, scaling factor ten by default.
(A) KaL-KaL/KaL-Ka3/Ka3-Ka3, (B) KaL-KaL/KaL-Lz1/Lz1-Lz1 and (C) Ka3-Ka3/Ka3-Lz1/Lz1-Lz1.





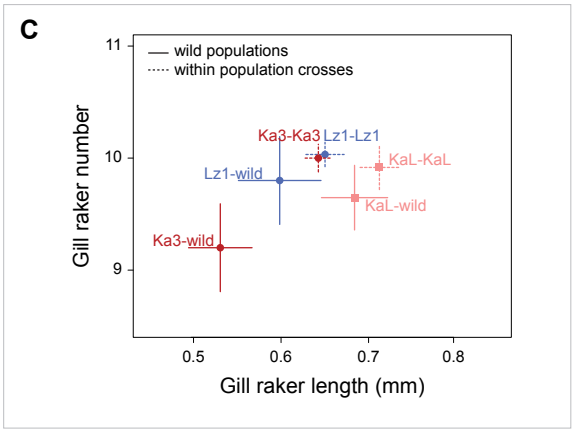


Figure S7 Plasticity in body shape and gill raker length. (A) CVA of body shape among the within population F1 offspring and their corresponding wild populations. Outlines for illustration purposes only, from light grey to dark outlines with increasing values, scaling factor ten by default. (B) CVA comparing the body shape of surviving adults at the beginning and at the end of the experimental period. (C) Comparison of gill raker length among the within population F1 offspring and their corresponding wild populations. (Table S8)

Appendix S1: Description of river systems

Kalambo

The catchment of the Kalambo River is located mainly in Tanzania, with a small portion in Zambia. The lake population of the Kalambo system (KaL) was collected at Chipwa village, close to the Kalambo River mouth at the border between Zambia and Tanzania (Fig. A1A, Fig. 1A and Fig. 2A). The habitat at Chipwa is characterized by mainly sandy bottom with bulrush (*Typha* spp.) vegetation and a maximum depth of 1.5 m. The first riverine population (Ka1) was sampled 1500 m upstream from KaL, within a slowly flowing, maximally 3 m deep water and vegetation comprising mainly hippo grass (*Vossia cuspidata*). The second upstream population (Ka2) originates from predominantly rocky habitat with a maximum depth of 1 m. The third upstream population (Ka3) is separated from downstream populations by the Kalambo Falls – with a drop of more than 200 m the second-tallest waterfall in Africa. Compared to Ka2 there is less water current at Ka3, fewer rocks but more vegetation (predominantly reeds and hippo grass).

Chitili

The Chitili Creek is a very small yet permanent stream flowing through Chitili village, and is therefore greatly affected by human activities including agriculture (Fig. A1B). The corresponding lake population (ChL) dwells in a heterogeneous shallow (max. 0.6 m) habitat with rock and sand bottom covered with aquatic plants and hippo grass belts. At the relatively close upstream sampling site, the creek is narrow, shallow (max. 0.3 m deep) and densely vegetated.

Lunzua

Although the Lunzua catchment is almost three times smaller in area than that of the Kalambo, both catchments are comparable with regard to slope angles, water discharge rates and drainage densities (Sichingabula 1999; Kakogonzo *et al.* 2000). The habitat of the Lunzua lake population (LzL) is similar to KaL, with mostly sandy bottom, bulrush vegetation and relatively shallow waters (max. 0.6 m depth) (Fig. A1C). A 3 m tall waterfall close to the river mouth and several rapids separate the lake population from the upstream riverine population (Lz1). The habitat at Lz1 consists mainly of sand and mud bottom, the water depth was around 0.5 m.

Lufubu

The Lufubu River is the largest tributary of southern LT (Langenberg *et al*. 2003). The sampling site at the river mouth (LfL) is shallow (0.3 – 2 m), densely vegetated with papyrus (*Cyperus papyrus*), hippo grass and balsa wood trees (*Aeshynomene elaphroxylon*) (Fig. A1D). The first upstream population (Lf1) was sampled at a location with very similar habitat conditions to LfL with very slowly flowing water. The upstream population (Lf2) was collected more than 30 km upstream the estuary, with habitat comprising pebbles and submerged vegetation and fast flowing waters (max. depth 0.5 m).

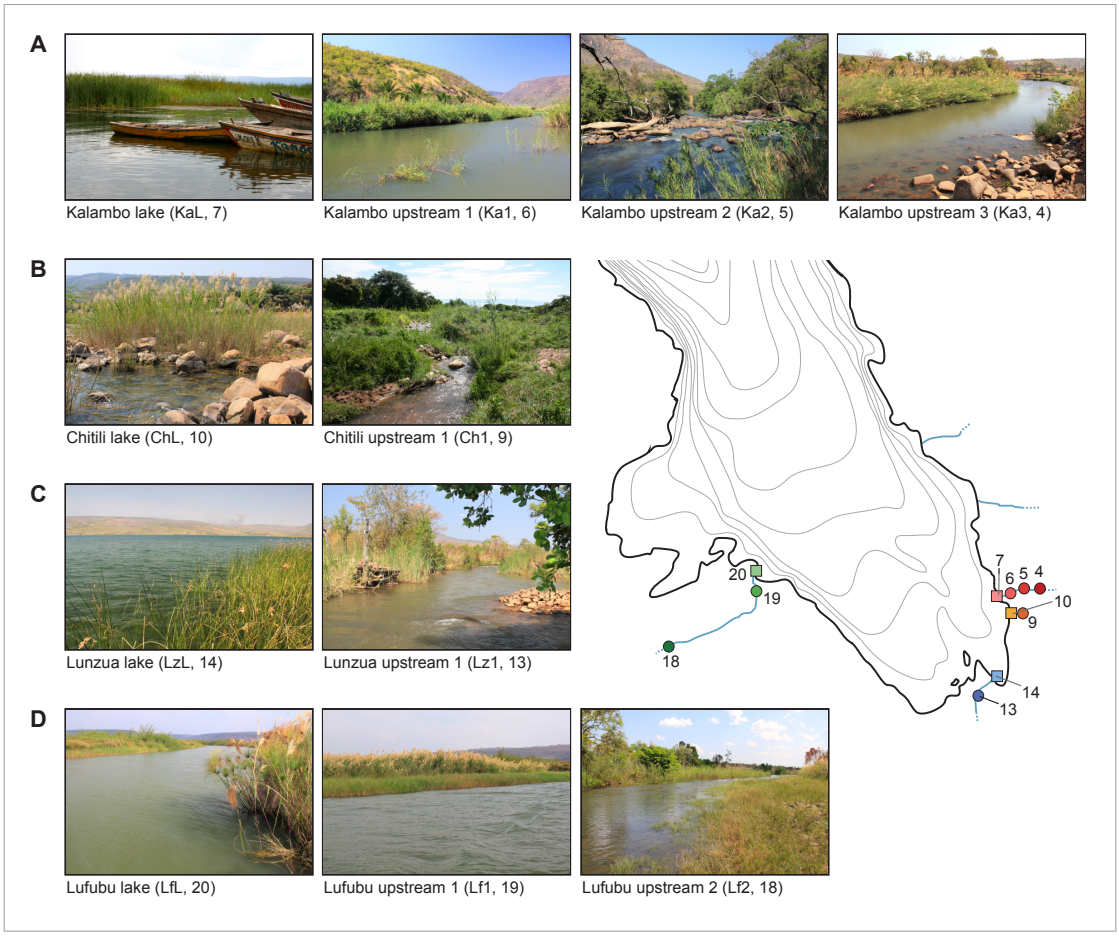


Fig. A1 Map of the southern part of LT (altered from Fig. 1A) showing the populations of the four lake-stream systems with corresponding habitat photographs. (A) The four Kalambo populations, (B) the two populations from the Chitili Creek, (C) the two Lunzua populations and (D) the three populations from the large Lufubu River.

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Sichingabula H (1999) Analysis and results of discharge and sediment monitoring activities in the Southern Lake Tanganyika basin, Zambia. *Pollution Control and Other Measures to Protect Biodiversity in Lake Tanganyika*, UNDP/GEF, RAF/92/G32, Technical Report No: 4.

Appendix S2: Pond experiment– Simulation

To test whether biased mating with respect to body size might explain the observed pattern, we simulated the experiment under the conditions of random mating with an increased mating probability, however, for large males. The simulations were conducted for each pond separately, with the observed number of male and female survivors per pond and reproductively active individuals (based on the paternity analyses, Table S2A). The frequencies for all 9 possible mating combinations were simulated for the observed number of mating events per pond (Table S2A) with 1'000 iterations. We tested 43'910 models with different mating probabilities for four dominant males per pond: for the two largest males at the starting point of the experiment (accounting for dominance in the early phase) and at the end point of the experiment (accounting for dominance in the late phase). We assigned dominance for two males per phase (early and late) to include possible dynamics in dominance ranks. The models covered a range from 1- to 20-fold mating probabilities for the four dominant males. Females were sampled randomly with equal probabilities in each model. To find the best fitted model we calculated the absolute deviation of the observed data form each of the iterations per model (Δ_{SIM}). Then the sum of the mean Δ_{SIM} (SUM_{Δ}) over all ponds was calculated. Therefore the model with the smallest SUM_{Δ} represents the model, which fits the observed data best. The macro for the simulations was written in R.

Comparing the SUM_{Δ} of the 43'910 models revealed that the model assuming random mating (without dominance) shows the highest SUM_{Δ} whereas several models accounting for biased mating with respect to size fit the observed data very well (Fig. A2). Generally, the model improves with increasing probability for the largest male to mate at the end point of the experiment. Further, SUM_{Δ} decreases with increasing mating probability for the largest male at the starting point of the experiment, achieving an optimum when the probability to mate is 10- to 12-fold higher for the largest, i.e. dominant male(s). If the mating probabilities for the two largest males (starting point and end point) increase, SUM_{Δ} decreases asymptotically resulting in several well fitting models. Thereby an increasing mating probability for the second largest male in the late phase does not substantially contribute to an improvement of the model. However the model improves with 4- to 6-fold higher mating probability for the second largest male in the early phase.

Comparing the best-fitting models with the observed data revealed that the observed frequencies of all mating combinations overlap with the 95% confidence limits of the simulated model (1'000 iterations) in all 5 ponds (Fig. A3). This suggests that the model assumptions of an increased mating probability for the largest males (10- to 12-fold higher for males in the early phase and 15- to 20-fold higher in the late phase of the experiment), plus a 4- to 6-fold higher probability for the second largest males in the early phase, explain best the observed frequencies of mating combinations. The lower mating probability for the dominant male in the early phase in combination with an increased probability for the second largest males might reflect an unstable dominance status and relatively early changes in dominance ranks. The observed aggressive territorial fights within the first two weeks (which led to high mortality in the early phase of the experiment) also support this.

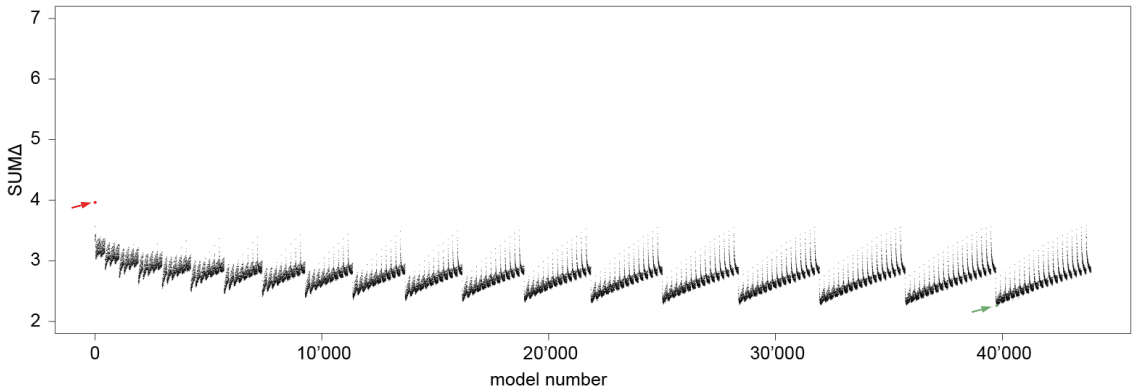

Fig. A2 SUM_{Δ} of the 43'910 models tested. The different combinations of mating probabilities (from 1- to 20-fold) for the four dominant males sorted by increasing mating probabilities for (*i*) the largest male at the end point of the experiment, (*ii*) the largest male at the starting point of the experiment, (iii) the second largest males at the end point and (*iiii*) the second largest males at the starting point of the experiment. The model without assigning any dominance to the males is marked in red and the best fitting model (lowest SUM_{Δ}) in green.

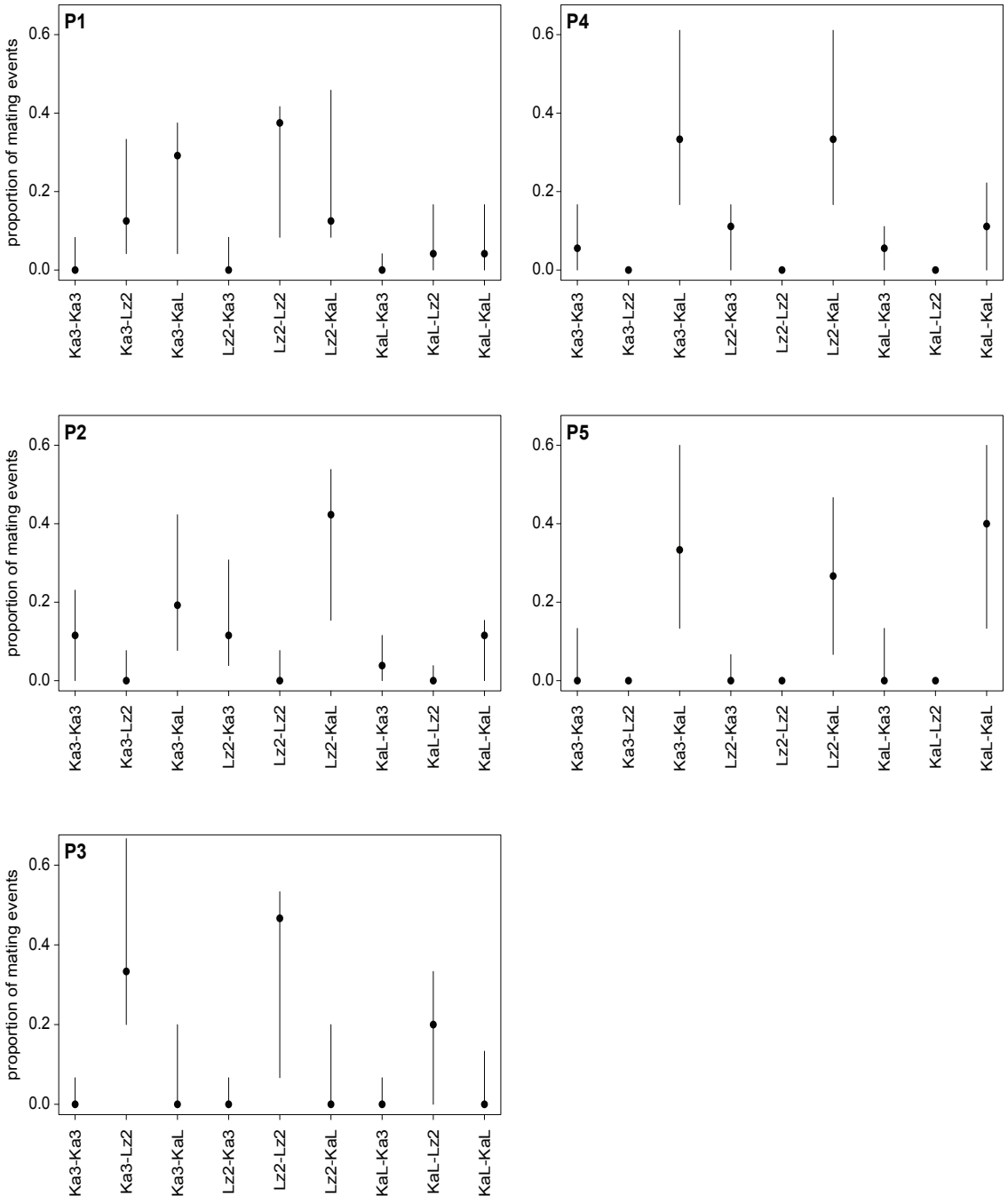

Fig. A3 Observed frequencies of mating combinations per replicate (filled circles) and simulated mating combinations with 1‘000 iterations (bars show the 95% confidence limits) using the best fitting model (green arrow in Fig. A2) with following mating probabilities: 10-folded and 5-folded mating probabilities for the largest and the second largest males at the starting point of the experiment and 20- and 1-folded probabilities for the largest and the second largest males at the end point of the experiment.

Part 3

side projects

3.1 Phylogeographic and phenotypic assessment of a basal haplochromine cichlid fish from Lake Chila, Zambia

Egger B, Klaefiger Y, Indermaur A, Koblmüller S, Theis A, Egger S, Näf T,
Van Steenberge M, Sturmbauer C, Katongo C and Salzburger W
Hydrobiologia (2014)
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3.1.1 Manuscript: p. 141 - 154
3.1.2 Supporting information: p. 155 - 162

I was involved in sampling, conducted the statistical analyses on male body shape and body colouration data, designed some of the figures and helped with proofreading of the manuscript.

Hydrobiologia
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ADVANCES IN CICHLID RESEARCH

Phylogeographic and phenotypic assessment of a basal haplochromine cichlid fish from Lake Chila, Zambia

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Abstract The basal haplochromine genus *Pseudocrenilabrus* comprises three valid species, although the current taxonomy most probably underestimates species richness. Previous phylogeographic studies on the *P. philander* species complex revealed a clear structuring of populations, shaped by river capture events. Here we report the discovery of *P.* cf. *philander* in Lake Chila, a small lake south of Lake Tanganyika. We were interested whether discrete morphs, similar to what has been found in Lake Mweru and the Lunzua River, were present in Lake Chila. We evaluated the phenotypic variability of the population in relation to other lacustrine and riverine populations by quantifying colouration and body shape. To place the specimens in a phylogeographic framework, we inferred a phylogeny based on the most variable part of the mitochondrial control region. We found two divergent mtDNA lineages in Lake Chila and tested for population structure and admixture between the lineages using microsatellite data. Our study reveals a complex phylogeographic pattern and demonstrates admixture of distant mtDNA lineages in Lake Chila, producing a hybrid swarm with substantial phenotypic variability. Unlike in Lake Mweru, *Pseudocrenilabrus* has not diversified further into discrete morphs in Lake Chila, probably because

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of the long-term instability of the lake and the presumed recency of the admixture event.

Keywords Phylogeography · Nuptial colouration · *Pseudocrenilabrus* · Hybridization

Introduction

Cichlid fishes from the East African Great Lakes, Tanganyika (LT), Malawi (LM) and Victoria (LV), are well-known model systems for studying the mechanisms underlying adaptive radiation and explosive speciation (see e.g. Kocher, 2004; Salzburger, 2009; Santos & Salzburger, 2012). Within African cichlids, the Haplochromini stand out as the most species-rich lineage, comprising the species flocks of LM and LV, the Tropheini from LT, as well as riverine and lacustrine species from northern, eastern, southern and central Africa and the levant (Turner et al., 2001; Verheyen et al., 2003; Joyce et al., 2005; Salzburger et al., 2005; Koblmüller et al., 2008a). The majority of haplochromine cichlids belongs to the derived 'modern' clade (as defined in Salzburger et al., 2005), the members of which are mostly lacustrine, 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 cichlid fauna of many rivers and smaller lakes, especially in central and southern Africa, is typically dominated by more basal haplochromine lineages. These lineages are considered comparably species poor, which has been explained by the lack of ecological opportunity in temporally unstable riverine ecosystems (Joyce et al., 2005). One of these basal riverine haplochromine lineages is represented by the genus *Pseudocrenilabrus,* which is distributed across many river systems and ichthyogeographic regions in northern, eastern, central and southern Africa (Skelton, 1991). The genus currently comprises three valid species, *P. multicolour* (two subspecies: *P. m. multicolour* and *P. m. victoriae*), *P. nicholsi* and *P. philander* (three subspecies: *P. p. dispersus*, *P. p. luebberti* and *P. p. philander*), although the current taxonomy likely underestimates species richness (Twentyman-Jones et al., 1997; Katongo et al., 2005; Stelkens & Seehausen, 2009). *Pseudocrenilabrus* are all considered generalist species, typically inhabiting calm parts of rivers, swamps and flooded areas (Greenwood, 1989). Males of the genus *Pseudocrenilabrus* generally show less elaborate nuptial colouration compared to 'modern' haplochromines and lack egg-spots, but most populations feature a red to orange blotch on the posterior margin of their anal fin.

The phylogeographic relationships within the genus *Pseudocrenilabrus* have so far mainly addressed the *P. philander* species complex in southern Zambian rivers. Two previous studies revealed a clear structuring of populations, possibly shaped by tectonic movements that allowed for past temporal connections between watersheds (Katongo et al., 2005; Koblmüller et al., 2012). Based on sequences of the most variable part of the mitochondrial control region (d-loop), Katongo et al. (2005) identified four distinct clades: the Chambeshi-Bangweulu clade, the Lake Mweru clade, the Lunzua clade and the Kafue–Zambezi clade. In more recent studies, Koblmüller et al. (2008a, 2012) included a previously undescribed haplochromine species from the Lufubu River (*P.* sp. 'Lufubu A'), which turned out as the most basal lineage in the genus. *P.* sp. 'Lufubu A' is found in sympatry with another *Pseudocrenilabrus* that represents a fifth lineage within the *P. philander* species complex (*P.* sp. 'Lufubu B'; Koblmüller et al., 2012). Despite the existence of several subspecies and many geographically separated, often morphologically distinct populations (Greenwood, 1989; Katongo et al., 2005), the genus was considered species poor in comparison to other riverine taxa (Skelton, 1994). However, Koblmüller et al. (2008b) described a population from the upper Lunzua River that contains two (blue and yellow) colour morphs sharing a single mitochondrial haplotype, but showing weak differentiation at nuclear markers suggesting that they might be undergoing incipient speciation. In addition, Stelkens and Seehausen (2009) reported the occurrence of at least 13 distinct morphs of *Pseudocrenilabrus* cf. *philander* in Lake Mweru. The morphs were assigned to two divergent mitochondrial lineages, of which the more frequent one diversified with respect to eco-morphology and nuptial colouration. In mate choice experiments, it was shown that the degree of divergence between morphological traits, but not genetic distance, was associated with the level of reproductive isolation between morphs (Stelkens & Seehausen, 2009). The existence of a small adaptive radiation in Lake Mweru

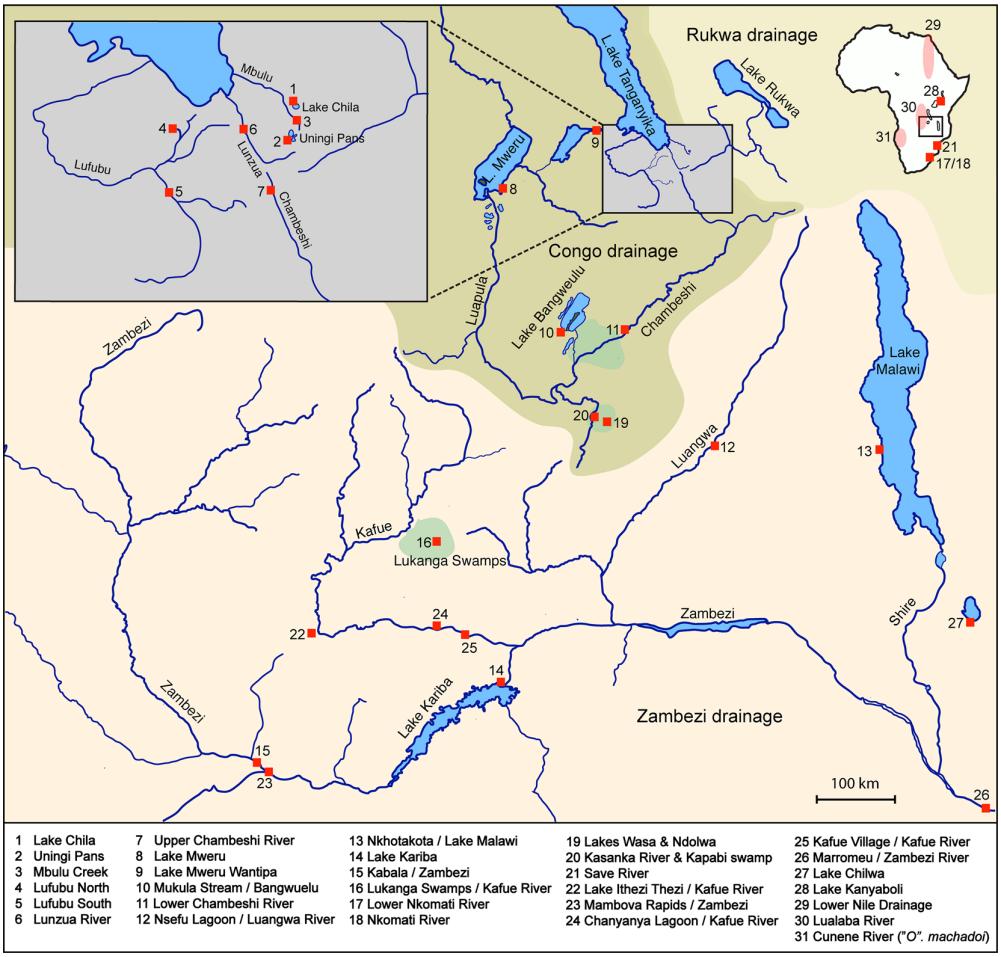

Fig. 1 Simplified map of the major water bodies in our study area showing the 28 sampling sites (*red squares*). Locations 29–31 roughly indicate the natural range of specimens acquired from the aquaria trade or where the exact location was unknown (translucent *red areas*). *Dark green* patches indicate swampy areas. *Different background colours* designate the major drainages indicated in the figure, namely Zambezi, Congo and Rukwa (including eastward draining rivers). Sampling site 19 and 20 each designate two sites that are very close together and belong to the same system

suggests that *Pseudocrenilabrus* are more likely to diversify in a stable heterogeneous (lake) environment, providing more ecological opportunity as compared to rivers (see e.g. Schluter, 2000; Wagner et al., 2012).

During a field trip in February 2012, we discovered a population of *P.* cf. *philander* in Lake Chila, a small (approximately 1,200 m long and 900 m wide) and shallow (maximum depth = 4 m) but permanent lake 20 km south of Lake Tanganyika (Fig. 1). Apart from *P.* cf. *philander*, the lake harbours a cichlid fauna typical for the Chambeshi, Zambezi and the Zambian/Congo watersheds (*Serranochromis angusticeps*, *S. robustus*, *S. thumbergi*, *Tilapia sparmanii*, *Oreochromis macrochir*) and *Astatotilapia burtoni* from the LT basin (see also Skelton, 1993). *Pseudocrenilabrus* from this population showed phenotypes distinct from other populations belonging to the *P. philander* species complex, with deeper bodies compared to nearby riverine populations and very elaborate colour patterns in males. We evaluated the phenotypic variability of the Lake Chila population in relation to other lacustrine (Mweru-Wantipa) and riverine (Lunzua and Chambeshi) populations by quantifying male nuptial colouration and body shape based on

standardized photographs. To place the Lake Chila specimens in a phylogeographic context, we reconstructed a phylogeny based on the most variable part of the mitochondrial control region using available *Pseudocrenilabrus* sequences from GenBank and additional samples recently collected from the area (Fig. 1). Interestingly, we found that two divergent mtDNA lineages were present in the small lake and further tested for population structure (in relation to neighbouring riverine populations) and admixture between the two mtDNA lineages using microsatellite data.

Materials and methods

Sampling

Sampling of *Pseudocrenilabrus* spp. was carried out during several field trips to Zambia between September 2003 and February 2012 (see Fig. 1; Tables S1, S2 and S3 for details on sample size and locations). Specimens were collected using gill nets and hook and line fishing under the permission of the Lake Tanganyika Research Unit, Department of Fisheries, Ministry of Agriculture and Livestock, Republic of Zambia. Fish were anaesthetized using clove oil (2–3 drops clove oil per litre water) and photographed in a standardized manner for later colour pattern and geometric morphometric analyses. Fin clips were taken from the specimens directly in the field and subsequently preserved in 96 % ethanol for further whole genomic DNA extraction. From each sampling location, at least one whole specimen was preserved in 96 % ethanol.

Male body colouration

To evaluate differences in nuptial colouration within and between populations, we used standardized photographs of males from Lake Chila ($n = 49$), Lunzua River ($n = 7$), Mbulu Creek ($n = 2$), Lufubu River ($n = 3$), Chambeshi River ($n = 2$), Lake Mweru-Wantipa ($n = 15$) and the Uningi Pans ($n = 3$) (Table S3) to extract nine features related to colouration (see Salzburger et al., 2006): anal fin colour (red/yellow/red–yellow/none); anal fin blotch colour and presence (orange/red/none); dorsal fin colouration (black–red/red–grey/none); pelvic fin colouration (intensity of black stripe); caudal fin pattern (spotted/half spotted); dorsal body colouration (bluish/yellowish/blue–yellowish/none); central body colouration (bluish/yellowish/blue-yellowish/none); ventral body colouration (bluish/yellowish/blue-yellowish/none) and eye bar presence. Characters were translated into a categorical data matrix and analysed in a Multiple Correspondence Analysis (MCA) in R (v.3.0.3, R Development Core Team, 2014; package FactoMineR, Husson et al., 2014).

Body shape

The photographs of males from Lake Chila ($n = 49$), Lunzua River ($n = 18$), Lufubu River ($n = 5$), Chambeshi River ($n = 2$) and Lake Mweru-Wantipa ($n = 14$) (Table S3) were used to obtain data for the geometric morphometric analyses by recording the coordinates of 17 homologous landmarks (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 non-shape variation (Rohlf & Slice, 1990). Additionally, the data were corrected for allometric size effects by using the residuals of the regression of shape on centroid size for further analyses. A canonical variate analysis (CVA; Mardia et al., 1979) was used to assess shape variation among the populations. The mean shape distances of the CV analysis were obtained using permutation tests (10,000 replications). Additionally, a PCA was conducted to assess within-population variance in body shape for Lake Chila only.

Molecular methods

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 Magna Pure extraction using a robotic device (Magna Pure LC, Roche Diagnostics) and following the manufacturer's protocol (Roche, Switzerland).

We genotyped a total of 249 *Pseudocrenilabrus* specimens from the Lunzua River ($n = 167$; 73 specimens sampled in 2004 partly used in Koblmüller et al., 2008b; 94 specimens sampled in 2010), Mbulu Creek ($n = 13$, sampled in 2010) and Lake Chila ($n = 69$, sampled in 2012) (see Table S2 for details) at

5 microsatellite loci (HchiST46, HchiST94 (Maeda et al., 2008), UNH002 (Kellogg et al., 1995), Pmv3 and Pmv4 (Crispo et al., 2007)).

Fragment size calling was carried out on an ABI 3130*xl* genetic analyser (Applied Biosystems) in comparison to the LIZ 500(−250) (Applied Biosystems) size standard. Genotypes were determined manually using Peak Scanner (v.1.0; Applied Biosystems), controlled and rounded to integers with the software TANDEM (v.1.09; Matschiner & Salzburger, 2009). STRUCTURE (v.2.3.3; Pritchard et al., 2000) was then used to infer population structure (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 $K = 1$–8 and the most likely number of genetic clusters was inferred using the ΔK method (Evanno et al., 2005) implemented in the software STRUCTURE HARVESTER (Earl & von Holdt, 2012). Initially, we intended to genotype all 249 *Pseudocrenilabrus* spp. specimens with a larger set of microsatellite loci, but only 5 loci (see above) could be amplified in both the Lake Chila and the Lunzua River/Mbulu Creek samples. We, therefore, tested additional loci and selected, based on amplification success and the level of polymorphism, 7 loci for the Lake Chila subset (HchiST46, HchiST94 (Maeda et al., 2008), UNH002 (Kellogg et al., 1995), Pmv3, Pmv4 (Crispo et al., 2007), Ppun21 (Taylor et al., 2002), Pzeb3 (Van Oppen et al., 1997) and 6 loci for the Lunzua River/Mbulu Creek subset: (Pmv1, Pmv3, Pmv4, Pmv15 (Crispo et al., 2007), UNH989 and UNH002 (Kellogg et al. 1995)). We then performed STRUCTURE analyses for the Lake Chila set and the Lunzua River/Mbulu Creek set separately to test for substructure within the two datasets. Conditions were the same as for the combined dataset, except the ten replicated simulations were performed for $K = 1$–5 for Lake Chila and $K = 1$–10 for Lunzua River/Mbulu Creek. Genetic differentiation among all populations and between morphs within the Lunzua River samples, as well as between yellow morphs sampled in 2004 and 2010 (the low sample size of blue males from the same location did not allow for a contrast between different sample years) was estimated as θ_{ST} (Weir & Cockerham, 1984) in ARLEQUIN (v.3.5; Excoffier & Lischer, 2010) for both the dataset containing 5 loci and the Lunzua River/Mbulu Creek dataset with 6 loci.

We also determined the DNA sequence of the most variable part of the mitochondrial control region (359 bp in total) for 82 samples (see Table S1 for details) using published primers (L-ProF or L-Pro-F_Tropheus and TDK-D; Meyer et al., 1994; Lee et al., 1995; Koblmüller et al., 2011). Amplification and sequencing were performed as described elsewhere (Duftner et al., 2005; Koblmüller et al., 2011). 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 analyser (Applied Biosystems). Additionally, sequences of the most variable part of the mitochondrial control region for *Pseudocrenilabrus* spp. were obtained from GenBank (from Joyce et al., 2005; Katongo et al., 2005; Koblmüller et al., 2008a, 2012; Wagner et al., 2012; see Table S1 for details). Note that we also included '*Orthochromis' machadoi* (Poll, 1967), since previous studies demonstrated the placement of this species within the genus *Pseudocrenilabrus* (see e.g. Koblmüller et al., 2008a). Together with the sequences from GenBank (total $n = 155$), the mitochondrial DNA sequences were aligned in MAFFT v.6 (Katoh et al., 2002) under the FFT-NS-i option, i.e. with fast construction of an initial alignment followed by iterative refinement until convergence, with default gap penalties. Identical sequences were collapsed into haplotypes using DNA collapser implemented in the online tool FaBox (Villesen, 2007). Bayesian inference (BI) was carried out in MrBayes v.3.2.2 (Ronquist et al., 2012). Posterior probabilities were obtained from MCMC simulations in two independent runs (10 chains with 10 million generations each, chain temperature: 0.25, trees sampled every 1,000 generations) using the best-fit model of molecular evolution as suggested by JMODELTEST (Posada, 2008). A 50 % majority-rule consensus tree was constructed after a one million generation burn-in (chain stationarity and run parameter convergence were checked with Tracer v.1.6 (Rambaut et al., 2013), using posterior probability as a measure of clade support).

Results

Nuptial colouration

Results from the MCA on the colour matrix including all populations are shown in Fig. 2A. Dimension 1

Table 1 Pairwise population differentiation between *Pseudocrenilabrus* populations and morphs

Dataset 1 (5 loci)	Lunzua loc. 1 (blue '04)	Lunzua loc. 1 (yellow '04)	Lunzua loc. 1 (yellow '10)	Lunzua loc. 2 (blue '10)	Lunzua loc. 2 (yellow '10)	Mbulu yellow ('10)
Lunzua loc. 1 (yellow '04)	−0.01474					
Lunzua loc. 1 (yellow '10)	0.16427***	0.17607***				
Lunzua loc. 2 (blue '10)	0.41273***	0.37886***	0.16337**			
Lunzua loc. 2 (yellow '10)	0.18593***	0.19913***	0.03094*	0.05452		
Mbulu (yellow '10)	0.16224**	0.17008***	0.08917**	0.19097**	0.11138**	
Chila ('12)	0.32118***	0.38302***	0.29676***	0.30133***	0.35078***	0.19948***
Dataset 2 (6 loci)	Lunzua loc. 1 (blue '04)	Lunzua loc. 1 (yellow '04)	Lunzua loc. 1 (yellow '10)	Lunzua loc. 2 (blue '10)	Lunzua loc. 2 (yellow '10)	
Lunzua loc. 1 (yellow '04)	−0.01156					
Lunzua loc. 1 (yellow '10)	0.08357***	0.08048***				
Lunzua loc. 2 (blue '10)	0.24296***	0.24822***	0.15457***			
Lunzua loc. 2 (yellow '10)	0.14404***	0.14151***	0.04734***	0.02517		
Mbulu (yellow '10)	0.24440***	0.28047***	0.24497***	0.28296***	0.27547***	

Significance levels: * <0.05; ** <0.01; *** <0.001

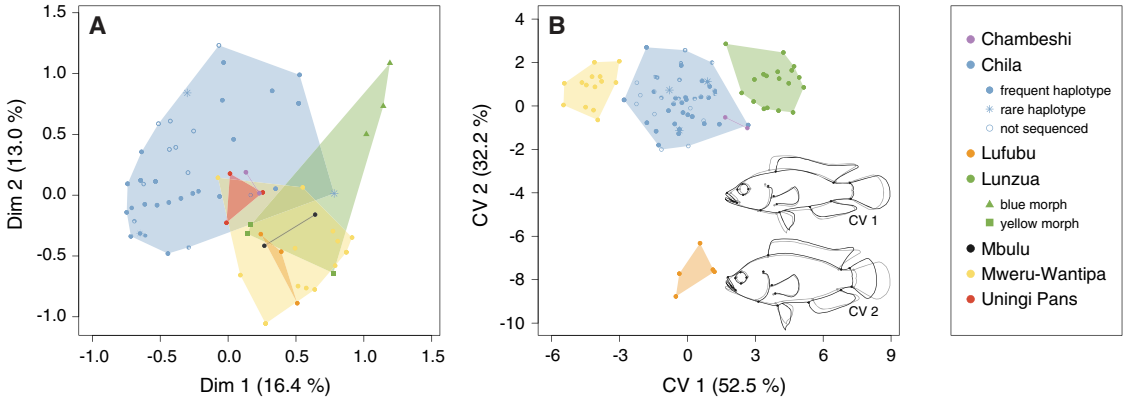

Fig. 2 **A** MCA based on nine male nuptial colouration traits. **B** CVA on male body shape based on 17 landmarks. *Green triangles* represent blue morphs and *green squares* yellow morphs from the Lunzua River (see Koblmüller et al. 2008b). *Filled blue circles* represent specimens assigned to the more frequent mitochondrial haplotype lineage; *blue stars* represent specimens assigned to the less frequent mtDNA lineage (*empty blue circles* represent individuals for which no mitochondrial sequence data was available)

explained 16 % and Dimension 2 explained 13 % of the variation. The traits explaining most of the variation were related to anal fin, pelvic fin and central body colouration (data not shown). The samples from Lake Chila show the widest distribution in trait-space; however, there were no distinct phenotypic clusters detectable within the population, e.g. with respect to mitochondrial lineage assignment (Fig. 2A). Specimens from Lake Mweru-Wantipa and the Lunzua River partly overlapped with Lake Chila phenotypes. Within the males from Lunzua River, blue and yellow morphs were separated along the axis of Dimension 2. Yellow morphs from Mbulu Creek clustered with yellow morphs from the Lunzua River. Specimens from the Lufubu, Chambeshi and Uningi Pans fell within the distribution range of samples from Lake Mweru-Wantipa and values did not overlap with the majority of the Lake Chila specimens (Fig. 2A). While the separation of colour morphs within the Lunzua River population is mainly due to blue and yellow central body colouration and the presence/absence of an anal fin blotch, phenotypic variation in the Lake Chila population is due to a more complex interplay of several traits (e.g. colour of anal fin blotch; colour of anal, dorsal, pelvic and caudal fin; ventral, dorsal and central body colouration). The MCA restricted to specimens from Lake Chila did not detect any clustering that would indicate the presence of distinct morphs (Fig. S1A).

Body shape

The CVA of the overall body shape of the sampled populations revealed a significant differentiation between all populations (Fig. 2B; all pairwise population comparisons $P < 0.05$). The main body shape changes are described by canonical variate 1 (CV1, accounting for 53 % of the variance), which shows mainly a prolongation of the head shape (with riverine Lunzua fish having longer heads and a more slender body shape), and CV2 (accounting for 32 % of the variance) describing additional changes in body shape and mouth position (with fish from the Lufubu River having longer caudal peduncles, more slender bodies and a more inferior position of the mouth). The PCA on body shape for the Lake Chila population only did not detect any clustering that would indicate the presence of distinct morphs (Fig. S1B).

Population structure

Bayesian clustering with Structure of the combined dataset (including population samples from the Lunzua River, Mbulu Creek and Lake Chila) based on five microsatellites revealed a clear geographic pattern. The most likely number of $K = 2$ separated one genotypic cluster comprising the two riverine populations from the cluster representing the Lake Chila stock (Fig. 3A). The separate Structure analysis for

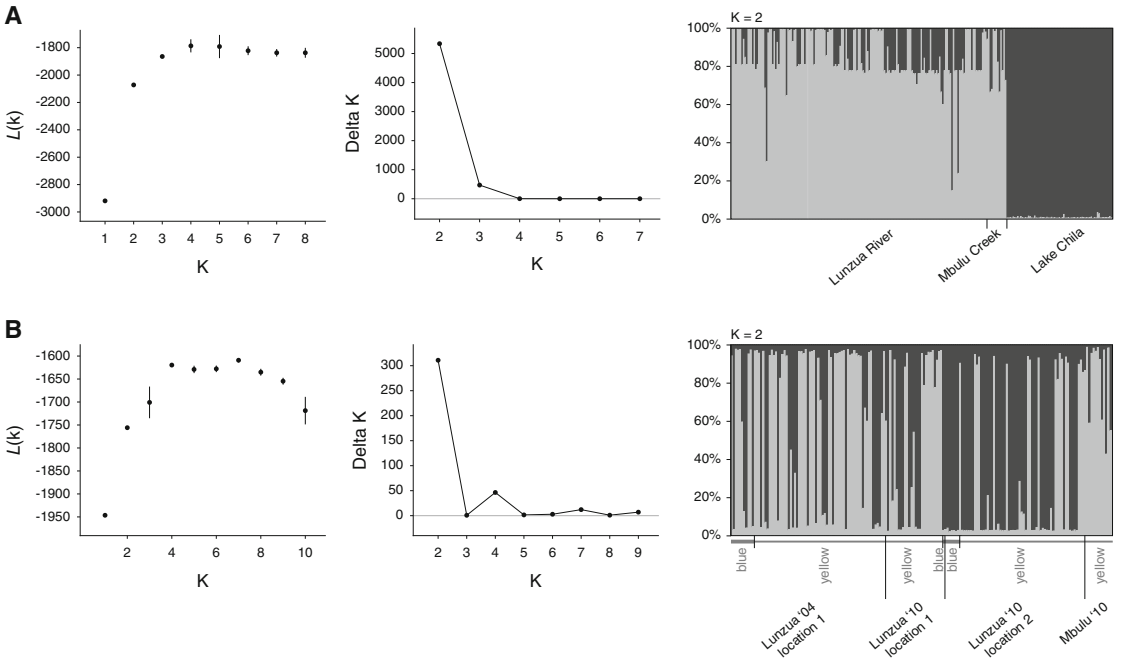

Fig. 3 Bayesian clustering analysis of *Pseudocrenilabrus* populations. **A** Dataset 1 (5 microsatellite loci) including samples from the Lunzua River, Mbulu Creek and Lake Chila. **B** Dataset 2 (6 microsatellite loci) including samples from the Lunzua River, Mbulu Creek and Lake Chila. *Left* mean likelihood ($L(K) \pm$ SD) over 10 runs assuming K clusters. *Middle* ΔK statistic (see Evanno et al. 2005). *Right* STRUCTURE plots for the most likely number of genetic clusters (K) as inferred from the ΔK statistic

the Lake Chila fish with seven microsatellites did not detect additional substructure within the population ($K = 1$, data not shown). The analysis of the dataset comprising only the Lunzua River and Mbulu Creek specimens based on six microsatellites, resulted in the most likely number of $K = 2$ (Fig. 3B). There was no clear genetic clustering detectable with regard to population or morph (Fig. 3B).

For the dataset including five microsatellite loci (Dataset 1, Table 1), pairwise comparisons revealed significant differentiation between morphs sampled in the years 2004 and 2010 (e.g. between yellow morphs from 2004 to 2010 from location 1) and between different sample locations (i.e. between Lunzua River locations 1 and 2; between Lake Chila and all other populations/morphs; between Mbulu Creek and all other populations/morphs), but not between blue and yellow morphs sampled within the same year.

Results from the Lunzua River/Mbulu Creek dataset (Dataset 2, Table 1) comprising 6 microsatellite loci (without the population from Lake Chila) are in line with those from the reduced dataset, with significant differentiation in all contrasts except between blue and yellow morphs sampled in the same year.

Phylogeography

Collapsing of sequences of the mitochondrial control region resulted in a total of 55 haplotypes (see Fig. 4; Table S1 for details). Our new BI phylogenetic reconstruction was largely in agreement with results from previous studies (Katongo et al., 2005; Koblmüller et al., 2012). The BI tree was rooted with *P.* sp. 'Lufubu A', which was identified as basal to all other *Pseudocrenilabrus* in previous phylogenetic studies (see Koblmüller et al., 2008a, 2012). Our new Lufubu River samples grouped with those downloaded from GenBank (Ht20 & Ht34; Fig. 4). The remaining haplotypes clustered into two major mitochondrial lineages: One comprised the Kafue–Zambezi clade and specimens from the Upper Luapula area: Lake

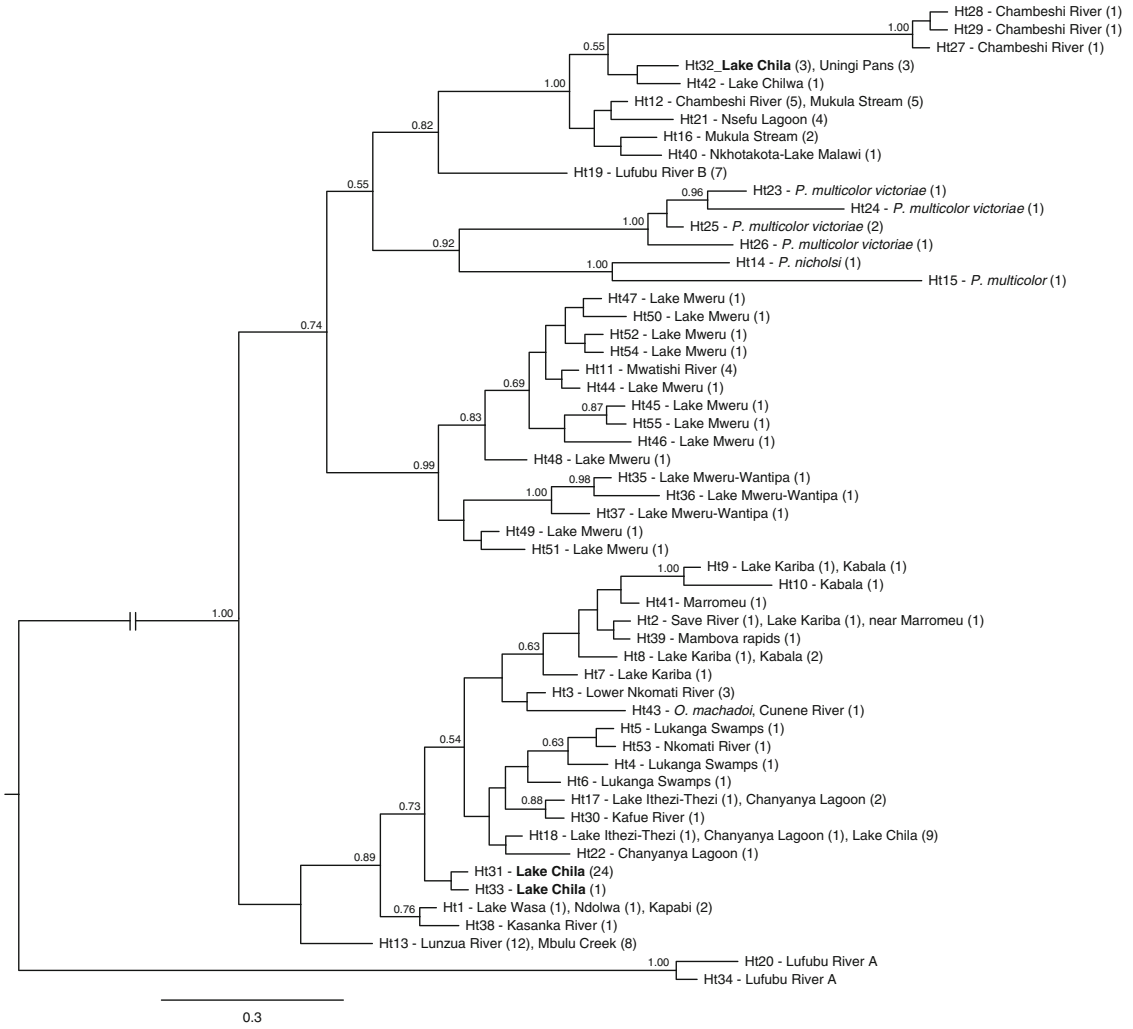

Fig. 4 Bayesian inference haplotype tree, rooted with *Pseudocrenilabrus* sp. 'Lufubu A'. Only posterior probabilities ≥0.50 are shown

Wasa, Kasanka River, Ndolwa and Kapabi in Kasanka NP (Ht1 & Ht38), as well as samples from further south (Cunene, Save and Nkomati basins). Our specimens from the Lunzua River and Mbulu Creek shared the haplotype with the previously published samples from the Lunzua River (Ht13) and formed the sister group to this lineage, although with very low posterior probabilities. '*Orthochromis' machadoi* and, interestingly, two *P.* cf. *philander* haplotypes from Lake Chila (Ht31 & Ht33) were resolved within the Kafue–Zambezi clade. In the other major mitochondrial lineage, *P.* sp. 'Lufubu B' (Ht19) was placed as sister group to the Chambeshi–Bangweulu clade. The newly sampled specimens from the Chambeshi River (Ht27, Ht28 & Ht29) grouped in this clade, as well as new individuals from Lake Chila and the Uningi Pans (Ht32) plus the two specimens from the geographically distant Malawi drainage and nearby basins (Lake Chilwa, Ht42 & Nkhotakota, Ht1). The samples from Lake Mweru-Wantipa (Ht35, Ht36 & Ht37) grouped within the Lake Mweru clade. Specimens from the Lake Victoria region, the remainder of the Congo drainage and the Nile, comprising the species *P. nicholsi* and *P. multicolour* (including the subspecies

P. m. victoriae), were placed as sister group to the Chambeshi–Bangweulu lineage, although with very low posterior probabilities.

Discussion

In this study, we reassessed the phylogeography of *Pseudocrenilabrus* in the watersheds of Zambia with a particular focus on a newly discovered lacustrine population of *Pseudocrenilabrus* cf. *philander* from Lake Chila, a small and shallow lake about 20 km south of LT. Males from this population displayed deeper bodies and more elaborate colour patterns compared to other known populations from the *P. philander* species complex. Interestingly, sequencing of the mitochondrial control region revealed the presence of two divergent mtDNA haplotype lineages in Lake Chila, with the more frequently sampled lineage (Ht31 & Ht33, ~90 % of Lake Chila mtDNA sequences) being associated with the Kafue–Zambezi clade, whereas the less frequent lineage (Ht32, ~10 % of Lake Chila mtDNA sequences) was placed within the Chambeshi clade (Fig. 4; Table S1). The exact origin of the two lineages remains unclear, and we cannot exclude the possibility that *Pseudocrenilabrus*, especially from the Zambezi–Kafue lineage, have been accidentally translocated in the course of a stocking event with *Oreochromis macrochir* (Thys van den Audenaerde, 1994; Lawrence Makasa, Fisheries Department Mpulungu, personal communication). However, this would not affect our conclusions about the maintenance of genetic and phenotypic diversity within Lake Chila.

We conducted a MCA based on nuptial colour traits of males to compare phenotypic diversity between different *Pseudocrenilabrus* populations. This analysis (and the MCA on the Lake Chila population only) did not result in the clustering of males with respect to mtDNA lineage assignment or any pattern that would indicate the presence of distinct morphs, but suggested a rather extensive colour pattern variation within the Lake Chila population, distinct from the other populations included in the analysis (Fig. 2A). Note, however, that males from Lake Chila that share the less frequent mtDNA haplotype with fish from the Uningi Pans (Ht32) showed a distinct phenotype (Fig. 2A), further rejecting an association between mtDNA lineage and nuptial colour pattern. The MCA separated blue and yellow morphs from the Lunzua River and revealed differences in nuptial colouration, although with overlapping distributions, among some of the included populations (e.g. Lake Mweru-Wantipa, Lake Chila and Lunzua River, see Fig. 2A).

The CVA on body shape detected significant population differentiation for all analysed populations, with the lacustrine populations from Lakes Mweru-Wantipa and Chila having shorter heads and deeper bodies compared to the riverine populations (Fig. 2B), indicating adaptation to different flow regimes in lake and riverine habitat (Webb, 1984). The PCA on the Lake Chila population did not reveal clustering of distinct phenotypes, rejecting the idea of eco-morphological divergence within the small lake. However, due to the bias in sample sizes of lake and stream populations, we cannot exclude that phenotypic variability of some of the included riverine populations may be underestimated.

In addition to a lack of discrete colour morphs within Lake Chila, the population assignment test with STRUCTURE (based on both datasets with 5 and 6 microsatellite loci) indicated no genetic substructure within the lake (Fig. 3), suggesting complete admixture between the two divergent mtDNA haplotype clades (the STRUCTURE analysis did infer distinct genetic clusters for Lake Chila and the populations from Lunzua River and Mbulu Creek; see Fig. 3).

Introgressive hybridisation between lineages has been proposed to facilitate the colonisation of new environments by increasing genetic variation and generating unique phenotypes via transgressive segregation (Kolbe et al., 2004; Seehausen, 2004). Such a genetically admixed 'hybrid swarm' often exceeds morphospace occupation when compared to parental populations (Lucek et al., 2010; Tobler & Carson, 2010). Thus, selection can act on a broadened working surface and new, adaptive trait combinations may enable the exploitation of previously not utilized niches (Seehausen, 2004).

Stelkens & Seehausen (2009) discovered two divergent mtDNA lineages in Lake Mweru, a rather large lake 150 km west of the southern end of LT (see Fig. 1). In Lake Mweru, one of the mitochondrial lineages was present in several distinct morphs (the study reports 'at least 13 distinct phenotypes'), whereas the other mtDNA lineage was represented by a single generalist phenotype only, and appeared to be generally very rare. The level of reproductive isolation between these morphs has been shown to

correlate positively with divergence in nuptial colour pattern and eco-morphological divergence, but not with genetic differentiation (Stelkens & Seehausen, 2009). Lake Mweru is much larger (131 km long and 56 km wide) and deeper (max. 27 m deep) than Lake Chila, and diversification in Lake Chila might be impeded due to the comparative long-term instability of the lake and the presumed recency (assuming that the Kafue–Zambezi haplotypes in Lake Chila result from unintentional stocking) of the admixture between the two distinct genetic lineages.

Given the small radiation in Lake Mweru, and the phenotypic and genetic variability in Lake Chila, it is puzzling why *Pseudocrenilabrus* did not diversify in any of the other lakes of the region despite its presence in most of the basins (Seehausen, 2006; Stelkens & Seehausen, 2009).

During several sampling trips to rivers draining into southern LT (Kalambo, Lunzua and Lufubu), we observed that *Pseudocrenilabrus* were present in the more upstream regions of these rivers, whereas the dominant cichlid species in the downstream areas was *Astatotilapia burtoni*. We never found the two species in sympatry in any of the rivers (see also Seegers, 1996; Theis et al., unpublished). In Lake Chila, however, the two species co-occur, although *Pseudocrenilabrus* are much more abundant and we only caught *A. burtoni* in very low numbers and in a restricted area. Further, *A. burtoni* were smaller in body size and less intensively coloured compared to populations from LT or inflowing rivers (Theis et al., unpublished). Lake Chila is located 1,600 m above sea level and *Pseudocrenilabrus* cf. *philander* is known to be tolerant to temperatures as low as 16 °C (Loiselle, 1982). It seems that under these conditions, *P.* cf. *philander* is able to compete against the apparently less temperature-tolerant *A. burtoni*. Competitive exclusion of the two generalist species in combination with differing temperature tolerance might also explain the mutually exclusive distribution ranges of *A. burtoni* and *P. philander* in Zambian rivers. Lake Mweru, to our knowledge, does not harbour any 'modern' haplochromine species, which could partly explain why *Pseudocrenilabrus* successfully utilized the provided ecological opportunities in this lake (Stelkens & Seehausen, 2009).

Our extended dataset on the *P. philander* species complex also provides new insights into the phylogeographic relationships of the genus. Overall, our mitochondrial phylogenetic reconstruction is largely in line with previous phylogenies from Katongo et al. (2005) and Koblmüller et al. (2012). However, an even more complex phylogeographic pattern emerges with the inclusion of additional samples. Our samples from the Lufubu River, which were assigned to *P.* sp. 'Lufubu A', grouped together with sequences from the most basal *Pseudocrenilabrus* lineage (Koblmüller et al., 2012; Fig. 4). The remaining taxa formed two major mitochondrial clades, one representing the Zambezi–Kafue drainage, and the other representing a lineage of mainly Congolese origin (see Figs. 1 and 4). The new samples from the Upper Luapula area (locations 19 and 20) were placed within the Zambezi–Kafue clade, indicating past connections of the Kafue/Zambezi and Chambeshi watersheds—in line with the presumed Zambezian influences of the ecoregion's ichthyofauna (Jackson, 1961, 1986; Balon, 1977; Scott, 2005). However, other specimens from locations 7, 10 and 11, which are part of the Chambeshi drainage, clustered with samples from Lake Mweru and Lake Mweru-Wantipa, which are part of the Congo drainage. The Bangweulu-Chambeshi subregion is known to harbour ichthyofaunal elements from both the Zambezi and Congo (Van Steenberge et al., 2014), and our phylogenetic inference demonstrates the occurrence of two mitochondrial lineages in the subregion, one belonging to the Zambezian and the other to the Congo drainage *Pseudocrenilabrus* clades. These phylogeographic patterns are in line with previous studies on other cichlid species (Joyce et al., 2005; Katongo et al., 2007) and African tigerfish (Goodier et al., 2011), all of which imply repeated and fairly recent faunal exchange between the Zambezi and Zambian Congo system by capture of entire river systems as well as small headwater creeks, despite the longstanding separation of the main courses (Stankiewizc & de Wit, 2006; Cotterill & de Wit, 2011).

The second *Pseudocrenilabrus* lineage found in the Lufubu, *P.* sp. 'Lufubu B', was placed as sister group to the Chambeshi clade, indicating a second wave of colonisation of the Lufubu river via the upper Congo system by a derived haplotype lineage (see also Koblmüller et al., 2012). Moreover, sequences from fish collected in Lake Chilwa and Nkhotakota/LM were resolved in this clade, which suggests a past connection between the upper Malawi and Chambeshi drainages, possibly via the Luangwa (note that

specimens from the Luangwa, Nsefu Lagoon also grouped in the same clade, Figs. 1, 4; see also Tweddle & Skelton, 2008).

Our specimens from the Lunzua River and Mbulu Creek all shared a single mitochondrial haplotype with previously published sequences (Koblmüller et al., 2008b, 2012) and were resolved, although weakly supported, as sister to the Zambezi clade. The dispersal route of this haplotype between the Lunzua River and Mbulu Creek is puzzling, given that the Uningi Pans, which contain a different haplotype, are located in between both river's headwaters (see Figs. 1, 4). The Lunzua and Mbulu, however, might have been connected downstream during a severe low surface level in LT (the two rivers enter LT in the Chituta Bay; McGlue et al., 2008)—or alternatively, gene flow between the two streams might have been enabled via past river capture of small headwaters. The two populations did show genetic differentiation at nuclear markers, as evidenced by significant pairwise θ_{ST} values (Table 1). We also detected genetic differentiation between the two sampling locations in Lunzua from 2010 and interestingly, also between specimens sampled from the same location in the years 2004 and 2010, corroborating the idea that genetic bottlenecks induced by strong seasonal variation of flood plains and small river confluences have a strong impact on the population dynamics of cichlid fish in general and on *Pseudocrenilabrus* in particular (Koblmüller et al., 2008b; Crispo & Chapman, 2010; Hermann et al., 2011). In contrast to Koblmüller et al. (2008b), blue and yellow morphs (both in 2004 and 2010) were not genetically differentiated (Table 1), which might be explained by the use of a different set of microsatellite markers.

Taken together, our study reveals a rather complex phylogeographic pattern and demonstrates introgression between distant mitochondrial lineages in a basal haplochromine cichlid, providing additional evidence for the role of hybridisation in the evolution of haplochromines (Joyce et al., 2011; Schwarzer et al., 2012). The occurrence of divergent mtDNA haplotypes and extensive morphological variation in Lake Chila, together with the small radiation in Lake Mweru, which contrast the low genetic and phenotypic diversity found in rivers, suggest that *Pseudocrenilabrus* are more prone to diversify in a lake habitat providing more ecological opportunity, especially when more derived 'modern' haplochromines are absent. That *Pseudocrenilabrus* did not (yet) diversify further in Lake Chila might be related to the small size and hence comparative long-term instability of Lake Chila and the presumed recency of the admixture between the two distinct genetic lineages.

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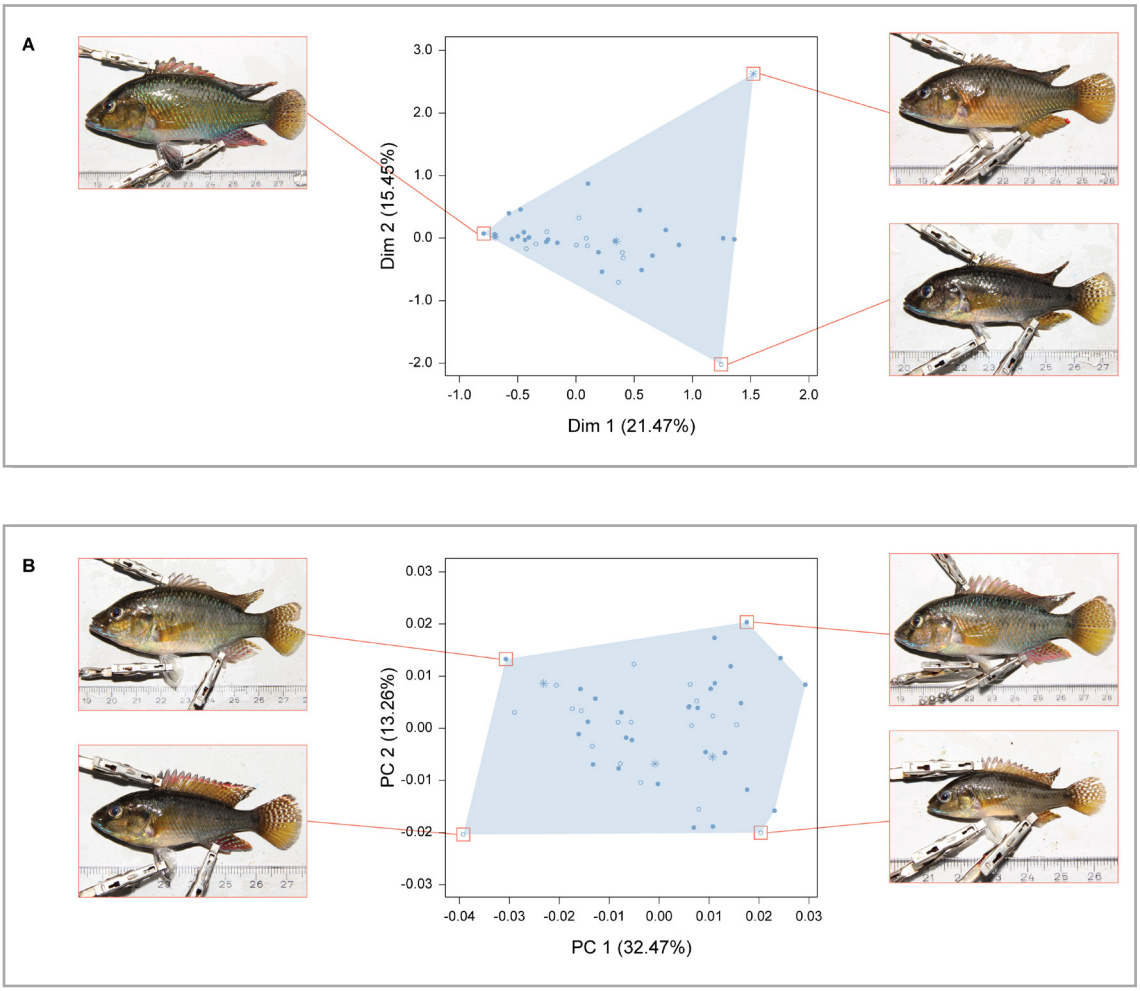

Figure S1 (A) MCA based on eight male nuptial colouration traits for the Lake Chila population. (B) PCA on male body shape based on 17 landmarks for the Lake Chila population. Photographs show the most extreme phenotypes in each analysis. Filled blue circles represent specimens assigned to the more frequent mitochondrial haplotype lineage; blue stars represent specimens assigned to the less frequent mtDNA lineage (empty blue circles represent individuals for which no mitochondrial sequence data was available).

Table S1 Specimens of Pseudocrenilabrus spp. And *'Orthochromis' machadoi*; included in the phylogenetic analysis of mitochondrial DNA sequences. Abbreviations for drainage systems are: LT, Lake Tanganyika drainage; CO, Congo Basin; MZR, middle Zambezi River; UZR, upper Zambezi River; LZR, lower Zambezi River; NI, Nile Basin; LV, Lake Victoria; CU, Cunene River; SA, Save River; NK, Nkomati River.

Sample ID	Nr.	Species	Locality	Location number (Fig. 1)	Drainage	Geographical coordinates	Reference (first pub.)	Collector	GeneBank accession number	Haplo-type number
Lake_Chila_DRA5	1	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872696	31
Lake_Chila_DRA6	2	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872697	31
Lake_Chila_DRA7	3	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872698	31
Lake_Chila_EFA1	4	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872699	32
Lake_Chila_EFA2	5	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872700	31
Lake_Chila_EFA3	6	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872701	18
Lake_Chila_EFA4	7	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872702	18
Lake_Chila_EFA5	8	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872703	31
Lake_Chila_EFA6	9	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872704	31
Lake_Chila_EFA7	10	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872705	18
Lake_Chila_EFA8	11	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872706	18
Lake_Chila_EFA9	12	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872707	31
Lake_Chila_EFB1	13	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872708	31
Lake_Chila_EFB2	14	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872709	18
Lake_Chila_EFB3	15	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872710	33
Lake_Chila_EFB4	16	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872711	31
Lake_Chila_EFB5	17	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872712	31
Lake_Chila_EFB7	18	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872713	32
Lake_Chila_EFB8	19	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872714	31
Lake_Chila_EFC1	20	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872715	18
Lake_Chila_EFC2	21	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872716	31
Lake_Chila_EFC3	22	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872717	31
Lake_Chila_EFC4	23	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872718	31
Lake_Chila_EFD2	24	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872719	31
Lake_Chila_EFD5	25	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872720	31
Lake_Chila_EFE2	26	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872721	31
Lake_Chila_EFE3	27	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872722	18
Lake_Chila_EFE4	28	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872723	31
Lake_Chila_EFE6	29	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872724	32
Lake_Chila_EFE7	30	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872725	31
Lake_Chila_EFF1	31	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872726	18
Lake_Chila_EFF2	32	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872727	18
Lake_Chila_EFI2	33	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872728	31
Lake_Chila_EFI3	34	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872729	31
Lake_Chila_EFI4	35	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872730	31
Lake_Chila_EFI7	36	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872731	31
Lake_Chila_EFI8	37	*Pseudocrenilabrus cf. philander*	Lake Chila	1	LT	8°50'5.02"S, 31°22'53.286"E	This study	Salzburger Lab	KJ872732	31
37E4_Mbulu	38	*Pseudocrenilabrus cf. philander*	Mbulu Creek	3	LT	8°51'27.83"S, 31°21'50.04"E	This study	Salzburger Lab	KJ872746	13
37E3_Mbulu	39	*Pseudocrenilabrus cf. philander*	Mbulu Creek	3	LT	8°51'27.83"S, 31°21'50.04"E	This study	Salzburger Lab	KJ872747	13
37D9_Mbulu	40	*Pseudocrenilabrus cf. philander*	Mbulu Creek	3	LT	8°51'27.83"S, 31°21'50.04"E	This study	Salzburger Lab	KJ872748	13
37D5_Mbulu	41	*Pseudocrenilabrus cf. philander*	Mbulu Creek	3	LT	8°51'27.83"S, 31°21'50.04"E	This study	Salzburger Lab	KJ872749	13
37D4_Mbulu	42	*Pseudocrenilabrus cf. philander*	Mbulu Creek	3	LT	8°51'27.83"S, 31°21'50.04"E	This study	Salzburger Lab	KJ872750	13
37D3_Mbulu	43	*Pseudocrenilabrus cf. philander*	Mbulu Creek	3	LT	8°51'27.83"S, 31°21'50.04"E	This study	Salzburger Lab	KJ872751	13
37D2_Mbulu	44	*Pseudocrenilabrus cf. philander*	Mbulu Creek	3	LT	8°51'27.83"S, 31°21'50.04"E	This study	Salzburger Lab	KJ872752	13
37D1_Mbulu	45	*Pseudocrenilabrus cf. philander*	Mbulu Creek	3	LT	8°51'27.83"S, 31°21'50.04"E	This study	Salzburger Lab	KJ872753	13
Uningi_pans_1	46	*Pseudocrenilabrus cf. philander*	Uningi Pans	2	LT	8°55'54.392"S, 31°21'45.984"E	This study	Salzburger Lab	KJ872739	32
Uningi_pans_2	47	*Pseudocrenilabrus cf. philander*	Uningi Pans	2	LT	8°55'54.392"S, 31°21'45.984"E	This study	Salzburger Lab	KJ872740	32
Uningi_pans_3	48	*Pseudocrenilabrus cf. philander*	Uningi Pans	2	LT	8°55'54.392"S, 31°21'45.984"E	This study	Salzburger Lab	KJ872741	32
Lunzua_54H3	49	*Pseudocrenilabrus cf. philander*	Lunzua River	6	LT	8°57'38"S, 31°10'38"E	This study	Salzburger Lab	KJ872734	13
Chambeshi_DRA1	50	*Pseudocrenilabrus cf. philander*	Upper Chambeshi River	7	CO	9°08'22.634"S, 31°22'31.097"E	This study	Salzburger Lab	KJ872692	27
Chambeshi_DRA2	51	*Pseudocrenilabrus cf. philander*	Upper Chambeshi River	7	CO	9°08'22.634"S, 31°22'31.097"E	This study	Salzburger Lab	KJ872693	28
Chambeshi_DRA3	52	*Pseudocrenilabrus cf. philander*	Upper Chambeshi River	7	CO	9°08'22.634"S, 31°22'31.097"E	This study	Salzburger Lab	KJ872694	29
Lufubu_DRD8	53	*Pseudocrenilabrus* sp. 'Lufubu A'	Lufubu River	4	LT	9°03'40.111"S, 30°55'35.694"E	This study	Salzburger Lab	KJ872733	34
Mweru-Wantipa_FBA1	54	*Pseudocrenilabrus cf. philander*	Lake Mweru-Wantipa	9	CO	8°31'30.708"S, 30°07'39.99"E	This study	Salzburger Lab	KJ872735	35
Mweru-Wantipa_FBA2	55	*Pseudocrenilabrus cf. philander*	Lake Mweru-Wantipa	9	CO	8°31'30.708"S, 30°07'39.99"E	This study	Salzburger Lab	KJ872736	36
Mweru-Wantipa_FBA3	56	*Pseudocrenilabrus cf. philander*	Lake Mweru-Wantipa	9	CO	8°31'30.708"S, 30°07'39.99"E	This study	Salzburger Lab	KJ872737	37
2355_Y_Lunzua	57	*Pseudocrenilabrus* sp. 'yellow'	Lunzua River	6	LT	8°57'21.383"S, 31°10'26.977"E	Katongo et al. 2005	Katongo et al.	AY612140	13
2800_Y_Lunzua	58	*Pseudocrenilabrus* sp. 'yellow'	Lunzua River	6	LT	8°57'21.383"S, 31°10'26.977"E	Katongo et al. 2005	Katongo et al.	AY612146	13
2802_Y_Lunzua	59	*Pseudocrenilabrus* sp. 'yellow'	Lunzua River	6	LT	8°57'21.383"S, 31°10'26.977"E	Katongo et al. 2005	Katongo et al.	AY612147	13
2819_Y_Lunzua	60	*Pseudocrenilabrus* sp. 'yellow'	Lunzua River	6	LT	8°57'21.383"S, 31°10'26.977"E	Katongo et al. 2005	Katongo et al.	AY612148	13
2830_Y_Lunzua	61	*Pseudocrenilabrus* sp. 'yellow'	Lunzua River	6	LT	8°57'21.383"S, 31°10'26.977"E	Katongo et al. 2005	Katongo et al.	AY612149	13
2831_B_Lunzua	62	*Pseudocrenilabrus* sp. 'blue'	Lunzua River	6	LT	8°57'21.383"S, 31°10'26.977"E	Katongo et al. 2005	Katongo et al.	AY612150	13
2832_B_Lunzua	63	*Pseudocrenilabrus* sp. 'blue'	Lunzua River	6	LT	8°57'21.383"S, 31°10'26.977"E	Katongo et al. 2005	Katongo et al.	AY612151	13
2834_B_Lunzua	64	*Pseudocrenilabrus* sp. 'blue'	Lunzua River	6	LT	8°57'21.383"S, 31°10'26.977"E	Katongo et al. 2005	Katongo et al.	AY612152	13
2835_B_Lunzua	65	*Pseudocrenilabrus* sp. 'blue'	Lunzua River	6	LT	8°57'21.383"S, 31°10'26.977"E	Katongo et al. 2005	Katongo et al.	AY612153	13
2837_B_Lunzua	66	*Pseudocrenilabrus* sp. 'blue'	Lunzua River	6	LT	8°57'21.383"S, 31°10'26.977"E	Katongo et al. 2005	Katongo et al.	AY612154	13
2838_B_Lunzua	67	*Pseudocrenilabrus* sp. 'blue'	Lunzua River	6	LT	8°57'21.383"S, 31°10'26.977"E	Katongo et al. 2005	Katongo et al.	AY615797	13
2344_Chambeshi_Bridge	68	*Pseudocrenilabrus philander*	Chambeshi-Chambeshi river	7	CO	10°55'32"S, 31°04'24"E	Katongo et al. 2005	Katongo et al.	AY612137	12
2345_Chambeshi_Bridge	69	*Pseudocrenilabrus philander*	Chambeshi-Chambeshi river	7	CO	10°55'32"S, 31°04'24"E	Katongo et al. 2005	Katongo et al.	AY612138	12
2346_Chambeshi_Bridge	70	*Pseudocrenilabrus philander*	Chambeshi-Chambeshi river	7	CO	10°55'32"S, 31°04'24"E	Katongo et al. 2005	Katongo et al.	AY612139	12
2840_Chambeshi_Bridge	71	*Pseudocrenilabrus philander*	Chambeshi-Chambeshi river	7	CO	10°55'32"S, 31°04'24"E	Katongo et al. 2005	Katongo et al.	AY612155	12
2841_Chambeshi_Bridge	72	*Pseudocrenilabrus philander*	Chambeshi-Chambeshi river	7	CO	10°55'32"S, 31°04'24"E	Katongo et al. 2005	Katongo et al.	AY612156	12
3637_Lufubu_A	73	*Pseudocrenilabrus* sp. 'Lufubu A'	Lufubu River	5	LT	9°15'36"S, 30°52'65"E	Koblmüller et al. 2012	Koblmüller et al.	JQ394794	20
3638_Lufubu_B	74	*Pseudocrenilabrus* sp. 'Lufubu B'	Lufubu River	5	LT	9°15'36"S, 30°52'65"E	Koblmüller et al. 2012	Koblmüller et al.	JQ394795	19
3639_Lufubu_B	75	*Pseudocrenilabrus* sp. 'Lufubu B'	Lufubu River	5	LT	9°15'36"S, 30°52'65"E	Koblmüller et al. 2012	Koblmüller et al.	JQ394795	19
3641_Lufubu_B	76	*Pseudocrenilabrus* sp. 'Lufubu B'	Lufubu River	5	LT	9°15'36"S, 30°52'65"E	Koblmüller et al. 2012	Koblmüller et al.	JQ394795	19
3642_Lufubu_A	77	*Pseudocrenilabrus* sp. 'Lufubu A'	Lufubu River	5	LT	9°15'36"S, 30°52'65"E	Koblmüller et al. 2012	Koblmüller et al.	JQ394794	20
6052_Lufubu_A	78	*Pseudocrenilabrus* sp. 'Lufubu A'	Lufubu River	6	LT	9°15'36"S, 30°52'65"E	Koblmüller et al. 2012	Koblmüller et al.	JQ394794	20
6053_Lufubu_A	79	*Pseudocrenilabrus* sp. 'Lufubu A'	Lufubu River	7	LT	9°15'36"S, 30°52'65"E	Koblmüller et al. 2012	Koblmüller et al.	JQ394794	20
6054_Lufubu_A	80	*Pseudocrenilabrus* sp. 'Lufubu A'	Lufubu River	8	LT	9°15'36"S, 30°52'65"E	Koblmüller et al. 2012	Koblmüller et al.	JQ394794	20
6055_Lufubu_A	81	*Pseudocrenilabrus* sp. 'Lufubu A'	Lufubu River	9	LT	9°15'36"S, 30°52'65"E	Koblmüller et al. 2012	Koblmüller et al.	JQ394794	20
3643_Lufubu_B	82	*Pseudocrenilabrus* sp. 'Lufubu B'	Lufubu River	5	LT	9°15'36"S, 30°52'65"E	Koblmüller et al. 2012	Koblmüller et al.	JQ394795	19
6051_Lufubu_B	83	*Pseudocrenilabrus* sp. 'Lufubu B'	Lufubu River	6	LT	9°15'36"S, 30°52'65"E	Koblmüller et al. 2012	Koblmüller et al.	JQ394795	19
6056_Lufubu_B	84	*Pseudocrenilabrus* sp. 'Lufubu B'	Lufubu River	7	LT	9°15'36"S, 30°52'65"E	Koblmüller et al. 2012	Koblmüller et al.	JQ394795	19
6057_Lufubu_B	85	*Pseudocrenilabrus* sp. 'Lufubu B'	Lufubu River	8	LT	9°15'36"S, 30°52'65"E	Koblmüller et al. 2012	Koblmüller et al.	JQ394795	19
2338_H_o_Mwatishi_River_Mweru_co	86	*Pseudocrenilabrus* sp. nov. 'orange'	Mwatishi River–Lake Mweru	8	CO	9°13'58"S, 28°48'02"E	Katongo et al. 2005	Katongo et al.	AY612133	11
2339_H_o_Mwatishi_River_Mweru_co	87	*Pseudocrenilabrus* sp. nov. 'orange'	Mwatishi River–Lake Mweru	8	CO	9°13'58"S, 28°48'02"E	Katongo et al. 2005	Katongo et al.	AY612134	11
2340_H_o_Mwatishi_River_Mweru_co	88	*Pseudocrenilabrus* sp. nov. 'orange'	Mwatishi River–Lake Mweru	8	CO	9°13'58"S, 28°48'02"E	Katongo et al. 2005	Katongo et al.	AY612135	11
2341_H_o_Mwatishi_River_Mweru_co	89	*Pseudocrenilabrus* sp. nov. 'orange'	Mwatishi River–Lake Mweru	8	CO	9°13'58"S, 28°48'02"E	Katongo et al. 2005	Katongo et al.	AY612136	11
Mweru_JQ950410	90	*Pseudocrenilabrus* sp.	Lake Mweru	8	CO	-	Wagner et al. 2012	Wagner et al.	JQ950410	44
Mweru_JQ950411	91	*Pseudocrenilabrus* sp.	Lake Mweru	8	CO	-	Wagner et al. 2012	Wagner et al.	JQ950411	45
Mweru_JQ950414	92	*Pseudocrenilabrus* sp.	Lake Mweru	8	CO	-	Wagner et al. 2012	Wagner et al.	JQ950414	46
Mweru_JQ950415	93	*Pseudocrenilabrus* sp.	Lake Mweru	8	CO	-	Wagner et al. 2012	Wagner et al.	JQ950415	47
Mweru_JQ950416	94	*Pseudocrenilabrus* sp.	Lake Mweru	8	CO	-	Wagner et al. 2012	Wagner et al.	JQ950416	48
Mweru_JQ950417	95	*Pseudocrenilabrus* sp.	Lake Mweru	8	CO	-	Wagner et al. 2012	Wagner et al.	JQ950417	49
Mweru_JQ950418	96	*Pseudocrenilabrus* sp.	Lake Mweru	8	CO	-	Wagner et al. 2012	Wagner et al.	JQ950418	50
Mweru_JQ950419	97	*Pseudocrenilabrus* sp.	Lake Mweru	8	CO	-	Wagner et al. 2012	Wagner et al.	JQ950419	51
Mweru_JQ950413	98	*Pseudocrenilabrus* sp.	Lake Mweru	8	CO	-	Wagner et al. 2012	Wagner et al.	JQ950413	52
Mweru_JQ950412	99	*Pseudocrenilabrus* sp.	Lake Mweru	8	CO	-	Wagner et al. 2012	Wagner et al.	JQ950412	54
Mweru_JQ950409	100	*Pseudocrenilabrus* sp.	Lake Mweru	8	CO	-	Wagner et al. 2012	Wagner et al.	JQ950409	55
2653_Mukula_Stream_Bangweulu	101	*Pseudocrenilabrus cf. philander*	Mukula Stream–Bangweulu	10	CO	11°26'03"S, 29°35'32"E	Katongo et al. 2005	Katongo et al.	AY612141	12
2654_Mukula_Stream_Bangweulu	102	*Pseudocrenilabrus cf. philander*	Mukula Stream–Bangweulu	10	CO	11°26'03"S, 29°35'32"E	Katongo et al. 2005	Katongo et al.	AY612143	16
2655_Mukula_Stream_Bangweulu	103	*Pseudocrenilabrus cf. philander*	Mukula Stream–Bangweulu	10	CO	11°26'03"S, 29°35'32"E	Katongo et al. 2005	Katongo et al.	AY612145	12
2656_Mukula_Stream_Bangweulu	104	*Pseudocrenilabrus cf. philander*	Mukula Stream–Bangweulu	10	CO	11°26'03"S, 29°35'32"E	Katongo et al. 2005	Katongo et al.	AY612160	16
2657_Mukula_Stream_Bangweulu	105	*Pseudocrenilabrus cf. philander*	Mukula Stream–Bangweulu	10	CO	11°26'03"S, 29°35'32"E	Katongo et al. 2005	Katongo et al.	AY612161	12
3067_Mukula_Stream_Bangweulu	106	*Pseudocrenilabrus cf. philander*	Mukula Stream–Bangweulu	10	CO	11°26'03"S, 29°35'32"E	Katongo et al. 2005	Katongo et al.	AY612142	12
3068_Mukula_Stream_Bangweulu	107	*Pseudocrenilabrus cf. philander*	Mukula Stream–Bangweulu	10	CO	11°26'03"S, 29°35'32"E	Katongo et al. 2005	Katongo et al.	AY612144	12
7_Pontoon_Kasanka	108	*Pseudocrenilabrus cf. philander*	Kasanka River	18	CO	12°34.347'S, 30°14.017'E	This study	Maarten Van Steenberge	KJ872738	38
2_Kapabi	109	*Pseudocrenilabrus cf. philander*	Kapabi-Kasanka River	18	CO	12°36.457'S, 30°12.836'E	This study	Maarten Van Steenberge	KJ872745	1
3_Kapabi	110	*Pseudocrenilabrus cf. philander*	Kapabi-Kasanka River	18	CO	12°36.457'S, 30°12.836'E	This study	Maarten Van Steenberge	KJ872744	1
4_Ndolwa	111	*Pseudocrenilabrus cf. philander*	Ndolwa-Kasanka River	18	CO	12°38.122'S, 30°14.528'E	This study	Maarten Van Steenberge	KJ872743	1
6_Lake_Wasa	112	*Pseudocrenilabrus cf. philander*	Lake Wasa	19	CO	12°33.297'S, 30°17.802'E	This study	Maarten Van Steenberge	KJ872687	1
3680_Nsefu_Lagoon_Luangwa	113	*Pseudocrenilabrus cf. philander*	Nsefu Lagoon-Luangwa River	12	MZR	12°56'11"S, 31°55'16"E	This study	Cyprian Katongo	KJ872754	21
3681_Nsefu_Lagoon_Luangwa	114	*Pseudocrenilabrus cf. philander*	Nsefu Lagoon-Luangwa River	12	MZR	12°56'11"S, 31°55'16"E	This study	Cyprian Katongo	KJ872755	21
3682_Nsefu_Lagoon_Luangwa	115	*Pseudocrenilabrus cf. philander*	Nsefu Lagoon-Luangwa River	12	MZR	12°56'11"S, 31°55'16"E	This study	Cyprian Katongo	KJ872756	21
3683_Nsefu_Lagoon_Luangwa	116	*Pseudocrenilabrus cf. philander*	Nsefu Lagoon-Luangwa River	12	MZR	12°56'11"S, 31°55'16"E	This study	Cyprian Katongo	KJ872757	21
2274_Lukanga_Swamps_KF	117	*Pseudocrenilabrus cf. philander*	Lukanga Swamps-Kafue River	16	MZR	14°27'13"S, 27°49'47"E	Katongo et al. 2005	Katongo et al.	AY612124	4
2275_Lukanga_Swamps_KF	118	*Pseudocrenilabrus cf. philander*	Lukanga Swamps-Kafue River	16	MZR	14°27'13"S, 27°49'47"E	Katongo et al. 2005	Katongo et al.	AY612125	5
2284_Lukanga_Swamps_KF	119	*Pseudocrenilabrus cf. philander*	Lukanga Swamps-Kafue River	16	MZR	14°27'13"S, 27°49'47"E	Katongo et al. 2005	Katongo et al.	AY612126	6

Sample ID	Nr.	Species	Locality	Location number (Fig. 1)	Drainage	Geographical coordinates	Reference (first pub.)	Collector	GeneBank accession number	Haplo-type number
3063_Ithezi_Kafue	120	*Pseudocrenilabrus cf. philander*	Lake Ithezi-Thezi-Kafue River	22	MZR	15°44′00″S, 26°00′54″E	Katongo et al. 2005	Katongo et al.	AY612158	17
3065_Ithezi_Kafue	121	*Pseudocrenilabrus cf. philander*	Lake Ithezi-Thezi-Kafue River	22	MZR	15°44′00″S, 26°00′54″E	Katongo et al. 2005	Katongo et al.	AY612159	18
3684_Chanyanya_Lagoon_Kafue	122	*Pseudocrenilabrus cf. philander*	Chanyanya Lagoon-Kafue River	24	MZR	15°40′43″S, 27°57′12″E	This study	Cyprian Katongo	KJ872758	17
3685_Chanyanya_Lagoon_Kafue	123	*Pseudocrenilabrus cf. philander*	Chanyanya Lagoon-Kafue River	24	MZR	15°40′43″S, 27°57′12″E	This study	Cyprian Katongo	KJ872759	23
3686_Chanyanya_Lagoon_Kafue	124	*Pseudocrenilabrus cf. philander*	Chanyanya Lagoon-Kafue River	24	MZR	15°40′43″S, 27°57′12″E	This study	Cyprian Katongo	KJ872760	18
3687_Chanyanya_Lagoon_Kafue	125	*Pseudocrenilabrus cf. philander*	Chanyanya Lagoon-Kafue River	24	MZR	15°40′43″S, 27°57′12″E	This study	Cyprian Katongo	KJ872761	17
Kafue_01D3	126	*Pseudocrenilabrus cf. philander*	Kafue - Kafue River	31	CO	15°47′12.499″S, 28°10′43.441″E	This study	Salzburger Lab	KJ872695	30
2330_Lake_Kariba_MZR	127	*Pseudocrenilabrus cf. philander*	Lake Kariba	14	MZR	16°32′07″S, 28°43′15″E	Katongo et al. 2005	Katongo et al.	AY612127	7
2331_Lake_Kariba_MZR	128	*Pseudocrenilabrus cf. philander*	Lake Kariba	14	MZR	16°32′07″S, 28°43′15″E	Katongo et al. 2005	Katongo et al.	AY612128	8
2332_Lake_Kariba_MZR	129	*Pseudocrenilabrus cf. philander*	Lake Kariba	14	MZR	16°32′07″S, 28°43′15″E	Katongo et al. 2005	Katongo et al.	AY612129	2
2333_Lake_Kariba_MZR	130	*Pseudocrenilabrus cf. philander*	Lake Kariba	14	MZR	16°32′07″S, 28°43′15″E	Katongo et al. 2005	Katongo et al.	AY612130	9
Zambezi_PSZ4 (Mambova rapids)	131	*Pseudocrenilabrus cf. philander*	Mambova rapids-Zambezi River	23	UZR	17°44′53.222″S, 25°11′15.587″E	This study	Salzburger Lab	KJ872742	39
2334_Kabala_UZR	132	*Pseudocrenilabrus cf. philander*	Kabala	15	UZR	17°33′09″S, 24°58′40″E	Katongo et al. 2005	Katongo et al.	AY612131	10
2335_Kabala_UZR	133	*Pseudocrenilabrus cf. philander*	Kabala	15	UZR	17°33′09″S, 24°58′40″E	Katongo et al. 2005	Katongo et al.	AY612132	9
3070_Kabala_UZR	134	*Pseudocrenilabrus cf. philander*	Kabala	15	UZR	17°33′09″S, 24°58′40″E	Katongo et al. 2005	Katongo et al.	AY612162	8
3071_Kabala_UZR	135	*Pseudocrenilabrus cf. philander*	Kabala	15	UZR	17°33′09″S, 24°58′40″E	Katongo et al. 2005	Katongo et al.	AY612163	8
JN119337_near_Nkhotakota_Malawi	136	*Pseudocrenilabrus cf. philander*	Nkhotakota-Lake Malawi	13	LZR	-	Genner et al. 2011	Genner et al.	JN119337	40
AY913860_Lake_Chilwa	137	*Pseudocrenilabrus cf. philander*	Lake Chilwa	27	LZR	-	Joyce et al. 2005	Joyce et al.	AY913860	42
3061_near_Marromeu_LZR	138	*Pseudocrenilabrus cf. philander*	near Marromeu-Zambezi River	26	LZR	18°16′50″S, 35°56′39″E	Katongo et al. 2005	Katongo et al.	AY612157	2
AY913859_Marromeu	139	*Pseudocrenilabrus cf. philander*	Marromeu-Zambezi River	26	LZR	-	Joyce et al. 2005	Joyce et al.	AY913859	41
2510_Pseu_mult (Nile) Ornamental	140	*Pseudocrenilabrus multicolor*	? (ornamental fish trade)	29	NI	-	Katongo et al. 2005	Katongo et al.	AY602995	15
AY929965_multiicolor_Kanyaboli	141	*Pseudocrenilabrus multicolor victoriae*	Lake Kanyaboli	28	LV	-	Salzburger et al. 2005	Salzburger et al.	AY929965	23
AY930026_multicolor_Kanyaboli	142	*Pseudocrenilabrus multicolor victoriae*	Lake Kanyaboli	28	LV	-	Salzburger et al. 2005	Salzburger et al.	AY930026	24
AY930027_multicolor_Kanyaboli	143	*Pseudocrenilabrus multicolor victoriae*	Lake Kanyaboli	28	LV	-	Salzburger et al. 2005	Salzburger et al.	AY930027	25
AY930028_multicolor_Kanyaboli	144	*Pseudocrenilabrus multicolor victoriae*	Lake Kanyaboli	28	LV	-	Salzburger et al. 2005	Salzburger et al.	AY930028	25
AY930029_multicolor_Kanyaboli	145	*Pseudocrenilabrus multicolor victoriae*	Lake Kanyaboli	28	LV	-	Salzburger et al. 2005	Salzburger et al.	AY930029	26
2451_Pseu_nich (Lualaba) Ornamental	146	*Pseudocrenilabrus nicholsi*	? (ornamental fish trade)	30	CO	-	Katongo et al. 2005	Katongo et al.	AY602996	14
AY913861_O_machadoi (Cunene river, CongoD)	147	*Orthochromis' machadoi*	Cunene River	25	CU	-	Joyce et al. 2005	Joyce et al.	AY913861	43
13425_Save_River	148	*Pseudocrenilabrus cf. philander*	Save River	21	SA	20°53.758′S, 34°38.643′E	This study	Radim Blazek & Martin Reichard	KJ872688	2
13426_Lower_Incomati_basin	149	*Pseudocrenilabrus cf. philander*	Lower Nkomati River	17	NK	25°03.728′S, 32°59.956′E	This study	Radim Blazek & Martin Reichard	KJ872689	3
13427_Lower_Incomati_basin	150	*Pseudocrenilabrus cf. philander*	Lower Nkomati River	17	NK	25°03.728′S, 32°59.956′E	This study	Radim Blazek & Martin Reichard	KJ872690	3
13428_Lower_Incomati_basin	151	*Pseudocrenilabrus cf. philander*	Lower Nkomati River	17	NK	25°03.728′S, 32°59.956′E	This study	Radim Blazek & Martin Reichard	KJ872691	3
AY913853_Nkomati	152	*Pseudocrenilabrus cf. philander*	Nkomati River	20	NK	-	Joyce et al. 2005	Joyce et al.	AY913853	53

Table S2 Specimens of Pseudocrenilabrus included in the population structure analysis with microsatellite markers. Abbreviations for drainage systems are: LT, Lake Tanganyika drainage; CO, Congo Basin; MZR, middle Zambezi River; UZR, upper Zambezi River; LZR, lower Zambezi River; NI, Nile Basin; LV, Lake Victoria; CU, Cunene River; SA, Save River; NK, Nkomati River.

Sample ID	Nr.	Species	Colour morph	Collection year	Locality	Location number (Fig.1)	Drainage	Geographical coordinates	Reference (first publ.)	Collector
Lunzua_T03-1-D20	1	*Pseudocrenilabrus cf. philander*	blue	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1D19	2	*Pseudocrenilabrus cf. philander*	blue	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1F20	3	*Pseudocrenilabrus cf. philander*	blue	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3H11	4	*Pseudocrenilabrus cf. philander*	blue	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T05-2A2	5	*Pseudocrenilabrus cf. philander*	blue	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T05-2A4	6	*Pseudocrenilabrus cf. philander*	blue	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T05-2A5	7	*Pseudocrenilabrus cf. philander*	blue	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3H12	8	*Pseudocrenilabrus cf. philander*	blue	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T05-2A8	9	*Pseudocrenilabrus cf. philander*	blue	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T05-2A9	10	*Pseudocrenilabrus cf. philander*	blue	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T05-2B10	11	*Pseudocrenilabrus cf. philander*	blue	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1-D17	12	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1-E1	13	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1-E4	14	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1-E5	15	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1-E7	16	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1-E8	17	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1-E9	18	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1-E10	19	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1-E11	20	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1-E12	21	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1-E13	22	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1-E14	23	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1-E15	24	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1-E16	25	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1E18	26	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1F5	27	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1G12	28	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1E19	29	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1F2	30	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1F3	31	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1F6	32	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1F12	33	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1F15	34	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1F17	35	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1G2	36	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1G3	37	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1G4	38	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1G5	39	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1G7	40	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1G13	41	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1G14	42	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3G20	43	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3H1	44	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3H2	45	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3H3	46	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3H4	47	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3H5	48	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3H7	49	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3H8	50	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3H9	51	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3H10	52	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3H14	53	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T04-6C19	54	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T05-2A10	55	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T05-2B3	56	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T05-2B4	57	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T05-2B5	58	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T05-2B6	59	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T05-2B7	60	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T05-2B9	61	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1F10	62	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1F7	63	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1F8	64	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1F11	65	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1F13	66	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1G1	67	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-1G8	68	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3G19	69	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3H6	70	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T03-3H13	71	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T04-6C20	72	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_T05-2A7	73	*Pseudocrenilabrus cf. philander*	yellow	2004	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	Koblmüller *et al.* 2008b	Koblmüller *et al.*
Lunzua_37B4	74	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_37B8	75	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_54C6	76	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_54C9	77	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_54D2	78	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_54D4	79	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_54D5	80	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_54D6	81	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_54D7	82	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_86A1	83	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_86A2	84	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_86A3	85	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_86A4	86	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_54D1	87	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_86A5	88	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_54E5	89	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_37A1	90	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_37A3	91	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_86A6	92	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_86A7	93	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_86A8	94	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_86B1	95	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_86B2	96	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_86B4	97	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab
Lunzua_86B5	98	*Pseudocrenilabrus cf. philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57'38''S, 31°10'38''E	This study	Salzburger Lab

Sample ID	Nr.	Species	Colour morph	Collection year	Locality	Location number (Fig.1)	Drainage	Geographical coordinates	Reference (first publ.)	Collector
Lunzua_54E4	99	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57′38″S, 31°10′38″E	This study	Salzburger Lab
Lunzua_54F3	100	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 1	6	LT	8°57′38″S, 31°10′38″E	This study	Salzburger Lab
Lunzua_54C7	101	*Pseudocrenilabrus* cf. *philander*	blue	2010	Lunzua River, location 1	6	LT	8°57′38″S, 31°10′38″E	This study	Salzburger Lab
Lunzua_54F5	102	*Pseudocrenilabrus* cf. *philander*	blue	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54H1A	103	*Pseudocrenilabrus* cf. *philander*	blue	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86F1	104	*Pseudocrenilabrus* cf. *philander*	blue	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86F2	105	*Pseudocrenilabrus* cf. *philander*	blue	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86F3	106	*Pseudocrenilabrus* cf. *philander*	blue	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54I2	107	*Pseudocrenilabrus* cf. *philander*	blue	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54F1	108	*Pseudocrenilabrus* cf. *philander*	blue	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54C8	109	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54D3	110	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54D8	111	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54F2	112	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54F4	113	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54F6	114	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54F7	115	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54F8	116	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54F9	117	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54G1	118	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86B6	119	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86B7	120	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86B8	121	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86C3	122	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86C4	123	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86C5	124	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86C6	125	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54G4	126	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86C7	127	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86C8	128	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86C9	129	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86D1	130	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86D2	131	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54G6	132	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86D3	133	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86D4	134	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86D5	135	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86D6	136	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86D7	137	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54G8A	138	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86D8	139	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86D9	140	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86E1	141	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86E2	142	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86E3	143	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54H2	144	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54H3	145	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86E4	146	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86E5	147	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86E6	148	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54H5	149	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86E7	150	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86E8	151	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86E9	152	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54H7A	153	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86F4	154	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86F5	155	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86F6	156	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86F7	157	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86F8	158	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86F9	159	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86G1	160	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86G2	161	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54I3	162	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54E6	163	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_54E7	164	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86B9	165	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86C1	166	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
Lunzua_86C2	167	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
37C7b_Mbulu	168	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Mbulu Creek	3	LT	8°51′27.83″S, 31°21′50.04″E	This study	Salzburger Lab
37C9_Mbulu	169	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Mbulu Creek	3	LT	8°51′27.83″S, 31°21′50.04″E	This study	Salzburger Lab
37D1_Mbulu	170	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Mbulu Creek	3	LT	8°51′27.83″S, 31°21′50.04″E	This study	Salzburger Lab
37D2_Mbulu	171	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Mbulu Creek	3	LT	8°51′27.83″S, 31°21′50.04″E	This study	Salzburger Lab
37D3_Mbulu	172	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Mbulu Creek	3	LT	8°51′27.83″S, 31°21′50.04″E	This study	Salzburger Lab
37D4_Mbulu	173	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Mbulu Creek	3	LT	8°51′27.83″S, 31°21′50.04″E	This study	Salzburger Lab
37D5_Mbulu	174	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Mbulu Creek	3	LT	8°51′27.83″S, 31°21′50.04″E	This study	Salzburger Lab
37D6_Mbulu	175	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Mbulu Creek	3	LT	8°51′27.83″S, 31°21′50.04″E	This study	Salzburger Lab
37D7_Mbulu	176	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Mbulu Creek	3	LT	8°51′27.83″S, 31°21′50.04″E	This study	Salzburger Lab
37D8_Mbulu	177	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Mbulu Creek	3	LT	8°51′27.83″S, 31°21′50.04″E	This study	Salzburger Lab
37D9_Mbulu	178	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Mbulu Creek	3	LT	8°51′27.83″S, 31°21′50.04″E	This study	Salzburger Lab
37E3_Mbulu	179	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Mbulu Creek	3	LT	8°51′27.83″S, 31°21′50.04″E	This study	Salzburger Lab
37E4_Mbulu	180	*Pseudocrenilabrus* cf. *philander*	yellow	2010	Mbulu Creek	3	LT	8°51′27.83″S, 31°21′50.04″E	This study	Salzburger Lab
Lake_Chila_DRA5	181	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_DRA6	182	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_DRA7	183	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_DRB1	184	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_DRB2	185	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_DRB3	186	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_DRB4	187	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_DRB5	188	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_DRB6	189	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_DRB7	190	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_DRB9	191	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_DRC2	192	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_DRC3	193	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_DRC5	194	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_DRC6	195	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFA1	196	*Pseudocrenilabrus* cf. *philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab

Sample ID	Nr.	Species	Colour morph	Collection year	Locality	Location number (Fig.1)	Drainage	Geographical coordinates	Reference (first publ.)	Collector
Lake_Chila_EFA2	197	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFA3	198	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFA4	199	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFA5	200	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFA6	201	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFA7	202	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFA8	203	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFA9	204	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFB1	205	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFB2	206	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFB3	207	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFB4	208	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFB5	209	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFB6	210	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFB7	211	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFB8	212	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFB9	213	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFC1	214	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFC2	215	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFC3	216	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFC4	217	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFC5	218	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFC6	219	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFC7	220	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFC8	221	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFC9	222	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFD1	223	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFD2	224	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFD3	225	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFD4	226	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFD5	227	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFD6	228	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFD7	229	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFD8	230	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFD9	231	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFE1	232	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFE2	233	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFE3	234	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFE4	235	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFE5	236	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFE6	237	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFE7	238	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFE8	239	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFE9	240	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFF1	241	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFF2	242	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFF9	243	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFI2	244	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFI3	245	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFI4	246	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFI5	247	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFI7	248	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
Lake_Chila_EFI8	249	*Pseudocrenilabrus cf. philander*	-	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab

Table S3 Specimens of Pseudocrenilabrus included in the colour matrix analysis (MCA) and/or in the morphometric analysis of body shape (CVA). Abbreviations for drainage systems are: LT, Lake Tanganyika drainage; CO, Congo basin.

Sample ID	Nr.	Colour matrix	Body shape	Species	Collection year	Locality	Location number	Drainage	Geographical coordinates	Reference (first pub.)	Collector
DRA5	1	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
DRA6	2	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
DRA7	3	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
DRB1	4	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
DRB2	5	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
DRB5	6	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
DRB6	7	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
DRB7	8	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
DRB9	9	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
DRC1	10		X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
DRC2	11	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
DRC4	12	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
DRC5	13	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFA1	14	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFA2	15	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFA3	16	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFA4	17	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFA5	18	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFA6	19	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFA7	20	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFA8	21	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFA9	22	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFB1	23	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFB2	24	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFB3	25	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFB4	26	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFB5	27	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFB6	28	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFB7	29	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFB8	30	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFB9	31	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFC1	32	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFC2	33	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFC3	34	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFC4	35	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFC5	36	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFD3	37	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFD5	38	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFD7	39	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFD9	40	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFE2	41	X		*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFE6	42	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFF9	43	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFI2	44	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFI3	45	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFI4	46	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFI7	47	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFI8	48	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFI9	49	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
EFE9	50	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Chila	1	LT	8°50′5.02″S, 31°22′53.286″E	This study	Salzburger Lab
DRA1	51	X	X	*Pseudocrenilabrus cf. philander*	2012	Upper Chambeshi River	7	CO	9°08′22.634″S, 31°22′31.097″E	This study	Salzburger Lab
DRA2	52	X	X	*Pseudocrenilabrus cf. philander*	2012	Upper Chambeshi River	7	CO	9°08′22.634″S, 31°22′31.097″E	This study	Salzburger Lab
DRD6	53	X	X	*Pseudocrenilabrus* sp.'Lufubu A'	2012	Lufubu River	4	LT	9°03′40.111″S, 30°55′35.694″E	This study	Salzburger Lab
DRD7	54	X	X	*Pseudocrenilabrus* sp.'Lufubu A'	2012	Lufubu River	4	LT	9°03′40.111″S, 30°55′35.694″E	This study	Salzburger Lab
DRD8	55	X	X	*Pseudocrenilabrus* sp.'Lufubu A'	2012	Lufubu River	4	LT	9°03′40.111″S, 30°55′35.694″E	This study	Salzburger Lab
DRD9	56		X	*Pseudocrenilabrus* sp.'Lufubu A'	2012	Lufubu River	4	LT	9°03′40.111″S, 30°55′35.694″E	This study	Salzburger Lab
DRE1	57		X	*Pseudocrenilabrus* sp.'Lufubu A'	2012	Lufubu River	4	LT	9°03′40.111″S, 30°55′35.694″E	This study	Salzburger Lab
FJA8	58		X	*Pseudocrenilabrus cf. philander* 'yellow'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJA9	59		X	*Pseudocrenilabrus cf. philander* 'yellow'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJB1	60		X	*Pseudocrenilabrus cf. philander* 'yellow'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJB2	61		X	*Pseudocrenilabrus cf. philander* 'blue'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJB3	62		X	*Pseudocrenilabrus cf. philander* 'yellow'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJB4	63		X	*Pseudocrenilabrus cf. philander* 'yellow'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJB5	64		X	*Pseudocrenilabrus cf. philander* 'yellow'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJB6	65		X	*Pseudocrenilabrus cf. philander* 'yellow'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJB7	66		X	*Pseudocrenilabrus cf. philander* 'yellow'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJB8	67		X	*Pseudocrenilabrus cf. philander* 'yellow'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJC1	68		X	*Pseudocrenilabrus cf. philander* 'yellow'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJC2	69		X	*Pseudocrenilabrus cf. philander* 'yellow'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJC3	70		X	*Pseudocrenilabrus cf. philander* 'yellow'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJC4	71		X	*Pseudocrenilabrus cf. philander* 'blue'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJC5	72		X	*Pseudocrenilabrus cf. philander* 'yellow'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJC6	73		X	*Pseudocrenilabrus cf. philander* 'yellow'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJC8	74		X	*Pseudocrenilabrus cf. philander* 'blue'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
FJC9	75		X	*Pseudocrenilabrus cf. philander* 'yellow'	2013	Lunzua River	6	LT	8°57′32.598″S, 31°10′15.608″E	This study	Salzburger Lab
54C7	76	X		*Pseudocrenilabrus cf. philander* 'blue'	2010	Lunzua River, location 1	6	LT	8°57′38″S, 31°10′38″E	This study	Salzburger Lab
54F1	77	X		*Pseudocrenilabrus cf. philander* 'blue'	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
54F4	78	X		*Pseudocrenilabrus cf. philander* 'yellow'	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
54F5	79	X		*Pseudocrenilabrus cf. philander* 'blue'	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
54F7	80	X		*Pseudocrenilabrus cf. philander* 'yellow'	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
54I2	81	X		*Pseudocrenilabrus cf. philander* 'blue'	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
54I3	82	X		*Pseudocrenilabrus cf. philander* 'yellow'	2010	Lunzua River, location 2	6	LT	8°57′17.57″S, 31°9′42.70″E	This study	Salzburger Lab
37D2	83	X		*Pseudocrenilabrus cf. philander*	2010	Mbulu Creek	3	LT	8°51′27.83″S, 31°21′50.04″E	This study	Salzburger Lab
37D9	84	X		*Pseudocrenilabrus cf. philander*	2010	Mbulu Creek	3	LT	8°51′27.83″S, 31°21′50.04″E	This study	Salzburger Lab
FBA1	85	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Mweru Wantipa	9	CO	8°31′30.708″S, 30°07′39.99″E	This study	Salzburger Lab
FBA2	86	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Mweru Wantipa	9	CO	8°31′30.708″S, 30°07′39.99″E	This study	Salzburger Lab
FBA3	87	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Mweru Wantipa	9	CO	8°31′30.708″S, 30°07′39.99″E	This study	Salzburger Lab
FBA4	88	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Mweru Wantipa	9	CO	8°31′30.708″S, 30°07′39.99″E	This study	Salzburger Lab
FBA5	89	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Mweru Wantipa	9	CO	8°31′30.708″S, 30°07′39.99″E	This study	Salzburger Lab
FBA7	90	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Mweru Wantipa	9	CO	8°31′30.708″S, 30°07′39.99″E	This study	Salzburger Lab
FBA8	91	X		*Pseudocrenilabrus cf. philander*	2012	Lake Mweru Wantipa	9	CO	8°31′30.708″S, 30°07′39.99″E	This study	Salzburger Lab
FBA9	92	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Mweru Wantipa	9	CO	8°31′30.708″S, 30°07′39.99″E	This study	Salzburger Lab
FBB7	93	X	X	*Pseudocrenilabrus cf. philander*	2012	Lake Mweru Wantipa	9	CO	8°31′30.708″S, 30°07′39.99″E	This study	Salzburger Lab

Sample ID	Nr.	Colour matrix	Body shape	Species	Collection year	Locality	Location number	Drainage	Geographical coordinates	Reference (first pub.)	Collector
FBB8	94	X	X	*Pseudocrenilabrus* cf. *philander*	2012	Lake Mweru Wantipa	9	CO	8°31'30.708"S, 30°07'39.99"E	This study	Salzburger Lab
FBB9	95	X	X	*Pseudocrenilabrus* cf. *philander*	2012	Lake Mweru Wantipa	9	CO	8°31'30.708"S, 30°07'39.99"E	This study	Salzburger Lab
FBC1	96	X	X	*Pseudocrenilabrus* cf. *philander*	2012	Lake Mweru Wantipa	9	CO	8°31'30.708"S, 30°07'39.99"E	This study	Salzburger Lab
FBC3	97	X	X	*Pseudocrenilabrus* cf. *philander*	2012	Lake Mweru Wantipa	9	CO	8°31'30.708"S, 30°07'39.99"E	This study	Salzburger Lab
FBC4	98	X	X	*Pseudocrenilabrus* cf. *philander*	2012	Lake Mweru Wantipa	9	CO	8°31'30.708"S, 30°07'39.99"E	This study	Salzburger Lab
FBC5	99	X	X	*Pseudocrenilabrus* cf. *philander*	2012	Lake Mweru Wantipa	9	CO	8°31'30.708"S, 30°07'39.99"E	This study	Salzburger Lab
IMG_6984	100	X		*Pseudocrenilabrus* cf. *philander*	2012	Uningi Pans	2	LT	8°55'54.392"S, 31°21'45.984"E	This study	Salzburger Lab
IMG_7006	101	X		*Pseudocrenilabrus* cf. *philander*	2012	Uningi Pans	2	LT	8°55'54.392"S, 31°21'45.984"E	This study	Salzburger Lab
IMG_7009	102	X		*Pseudocrenilabrus* cf. *philander*	2012	Uningi Pans	2	LT	8°55'54.392"S, 31°21'45.984"E	This study	Salzburger Lab

3.2 A fitness benefit for mouth dimorphism in a scale-eating cichlid fish

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3.2.1 Manuscript: p. 167 - 175
3.2.2 Figures & Tables: p. 176 - 177
3.2.3 Supporting information: p. 178 - 179

I helped during fieldwork with building the cages, stocking the cages with fishes and with data acquisition in the lab. I analysed the data and designed the figures and tables. Together with AI, I wrote the first draft of the manuscript, which was refined together with WS and BE.

A fitness benefit for mouth dimorphism in a scale-eating cichlid fish

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Abstract

Random asymmetry, the co-existence of left- and right-sided (or -handed) individuals within a population, is a particular case of natural variation; what triggers and maintains such dimorphisms remains unknown in most cases. Here, we report a field-based cage experiment in the scale-eating Tanganyikan cichlid *Perissodus microlepis*, which occurs in two morphs in quasi-equal frequencies in nature, left-skewed and right-skewed individuals with respect to mouth orientation. We first confirm that, under semi-natural conditions, left-skewed scale-eaters preferentially attack the right flank of their prey, whereas right-skewed individuals feed predominantly from the left side. Importantly, we demonstrate that scale-eaters have a higher probability for successful attacks when kept in dimorphic experimental populations (left- AND right-skewed morphs together) as compared to monomorphic populations (left- OR right-skewed morphs), most likely because prey fishes fail to accustom to strikes from both sides. The significantly increased probability for attacks appears to be the selective agent responsible for trait divergence in *P. microlepis*.

Introduction

Variation in morphology between individuals plays a crucial role in the adaptive evolution of natural populations (e.g. Darwin 1859; Nosil 2012). This morphological variation is most often manifested in a symmetrical and continuous trait variance among individuals within populations, but there are also cases where the natural symmetry is broken and morphological asymmetries exist (Palmer 1994, 2010). In many cases, these are random asymmetries, meaning that both right- and left-sided (or: right- and left-'handed') individuals occur within a population at certain frequencies; as opposed to dextral and sinistral asymmetries, where only right- respectively left-sided individuals are present (Van Valen 1962; Palmer 2009, 2010). Examples for random morphological asymmetries are, among many others, the claws of American lobsters (about half of the individuals have the larger crusher claw on the right side and the other half on the left) (Govind 1989; Palmer 2005), the eyes of some flatfish (either the right or the left eye migrates, during ontogeny, to the other, then upside, sphere of the face) (Friedman 2008; Schreiber 2006), or the mouths of several fish species (either opening to the right or to the left side) (Hori 1993). In most of these cases, the selective regimes maintaining the random asymmetry in natural populations are unknown (Palmer 2009, 2010).

One of the most fascinating examples of random mouth asymmetry in fish is found in several species of scale-eating cichlids endemic to East African Lake Tanganyika, which show an extensive left/right mouth dimorphism and have become a textbook example for behavioural and morphological laterality (Fryer & Iles 1972; Futuyama 2009) as well as for frequency dependent selection (Hori 1993; Takeuchi et al. 2012). These scale-eaters belong to the Perissodini, a relatively species poor cichlid lineage counting nine described species (Liem & Stewart 1976; Koblmüller et al. 2007) but exhibiting a particularly specialized feeding mode in that they live, to various degrees, on scales and epidermis of other fishes (Marlier & Leleup 1954; Takahashi et al. 2007a, 2007b). To this end, scale-eaters have evolved remarkable adaptations such as asymmetry of mouth opening (Fig. 1A), hook-like teeth, as well as sophisticated attack strategies including aggressive mimicry (Hori & Watanabe 2000; Boileau et al. 2015).

Perissodus microlepis is the most common and perhaps the most specialized lepidophagous cichlid in Lake Tanganyika, and feeds almost exclusively upon scales of other fishes (Takahashi et al. 2007a, 2007b; Muschick et al. 2012). It hunts and feeds by ambushing its prey fish from the rear, instantly attacks the flanks of its victim, and bites out a single or a bunch of scales together with epidermis. For a long time, it has been noted that *P. microlepis* come in two versions with respect to mouth morphology (Fig. 1A), ones with a mouth opening to the left side ('left-skewed'; the right upper jaw bow is elongated) and ones with a mouth opening to the right side ('right-skewed'; the left upper jaw bow is elongated) (Fryer & Iles 1972). (Note, however, that a more recent study based on external examinations suggested a more continuous distribution of this trait (Kusche et al. 2012).)

The mouth dimorphism in *P. microlepis* has long been implicated with a lateralized feeding behaviour. Hori (1993) observed that individuals with a left-skewed mouth preferentially attack the right flank of prey fish, while individuals with a right-skewed mouth mainly feed from the prey's left flank. Hori (1993) further showed that natural populations of *P. microlepis* fluctuate around a 50:50 left-to-right-skewed-ratio (with an amplitude of 0.15 and a wavelength of about five years) and postulated negative frequency dependent selection as responsible mechanisms maintaining this polymorphism: the rare morph would persistently have a selective advantage over the common one, as prey fish would accustom to being attacked more often from one side and would become more alert on that side, creating a relatively higher feeding success for the rare attacker (Hori 1993). That left-skewed and right-skewed individuals indeed feed predominantly from the right and left flanks of a prey fish, respectively, has been confirmed in experiments following one-predator:one-prey settings (Lee et al. 2012; Takeuchi et al. 2012); the latter authors further suggested that an asymmetric mouth enables individual scale-eaters to attack from steeper rear angles thereby increasing overall feeding success as prey species have a lower probability of perceiving and avoiding the attacker (Takeuchi et al. 2012). Using simulated trophic level food webs Nakajima et al. (2004) had already shown an evolutionary advantage for dimorphic populations of scale-eaters, causing the persistence and fluctuation of this dimorphism. However, no empirical study exists to date which attributes a greater foraging success to individual scale-eaters living in dimorphic populations with respect to mouth morphology (i.e. left- and right-skewed fish together) as compared to scale-eaters in monomorphic populations (i.e. either left-skewed or right-skewed) – a main prediction if maintaining the dimorphism in natural populations is adaptive.

In this study we report a field-based enclosure experiment with the scale-eating cichlid fish *P. microlepis* under semi-natural conditions and with interacting communities in Lake Tanganyika. We used underwater cages stocked with *P. microlepis* of differential mouth orientation as well as natural prey fish in order to (*i*) confirm the asymmetrical attack strategies of left-skewed and right-skewed scale-eaters under semi-natural conditions; and (*ii*) test the hypothesis that dimorphic scale-eater populations would have an overall higher feeding success and, hence, a selective advantage over monomorphic ones. In addition, we assessed the potential influence of habitat structures (rocky *versus* sandy) on the attack strategies as well as on the overall feeding success. All together we aimed to disentangle causalities in the evolution of this system, and to demonstrate the selective advantage of dimorphic mouth opening and attack strategy in scale-eaters, which is necessary to explain how such asymmetries have evolved and can be maintained in natural populations.

Materials and Methods

Experimental Setup

All experiments were carried out at Rift Valley Tropicals (S 8° 37' 25.99"; E 31° 12' 2.86") on the southern shore of Lake Tanganyika in northern Zambia during two field seasons in September 2012 and 2013. We used the scale-eating cichlid fish *Perissodus microlepis* as predator and the common algae grazing cichlids *Interochromis loocki* and *Tropheus moorii* as prey (Figure 1B). Experimental fishes were caught

by the authors and by local fishermen using mono-filamentous gillnets with a mesh size of 6 mm; fishes were carefully chased into the nets on snorkelling or on SCUBA and immediately removed from the nets to reduce the risk of damage to the scale cover. Prior to the experiments, fishes were kept species-wise in concrete ponds (1 x 1 x 1 m) for several days to allow them to settle and to ensure that the scale-eaters had emptied their intestines before being utilized in the experiments. *Perissodus microlepis* individuals were scored by eye and separated according to mouth orientation into two discrete groups, those with a mouth opening to the left (left-skewed) and those with a mouth opening to the right (right-skewed). Scoring was carried out independently by three examiners (AI, AT and WS), and fish were only used for the experiment if laterality was clearly visible and all three examiners agreed upon mouth orientation.

The experimental setup (Fig. 1C, D) consisted of 6 equally sized underwater cages (2 x 2 x 2 m) made of a hollow steel frame covered by a sturdy net with 6 mm mesh size. The cages were open to the bottom to allow for the interaction of the experimental fishes with the natural substrate. The cages were installed around 30 m off shore in a water depth of 6 to 9 m. Three cages were placed on a homogeneous sandy ground, while the other three were equipped with natural rocks providing potential hiding places for prey and predator fish.

In an initial round of experiments, we carried out two trial runs to get familiar with the experimental procedure. During these trials, the condition of the experimental fishes was inspected regularly to assess attack rates of the scale-eaters. From this data, we defined the most suitable density of predator and prey fishes as well as the optimal duration of the experiment to avoid an effect of oversaturation.

For the actual experiment, consisting of three consecutive rounds in which all six cages were used, we stocked each cage with 20 prey specimens (10 *I. loocki* and 10 *T. moorii*) and 14 predators (*P. microlepis*). Within each habitat type (rocky *versus* sandy bottom), one cage was stocked with exclusively left-skewed *P. microlepis* (L), one with solely right-skewed individuals (R), and one with a dimorphic population (7 left-skewed and 7 right-skewed individuals; M) (Fig. 1C). In doing so, we created two types of experimental population setups with respect to mouth morphology of the scale-eaters: monomorphic experimental populations (L and R) and dimorphic experimental populations (M). The assignment of these populations to individual cages was altered in rotation to avoid cage position effects. Within each experimental round, prey fish and predators were distributed according to body size among the six cages to secure a homogenous size distribution. Cages were immediately sealed upon stocking with predator and prey fish. Each experimental round lasted for three days, after which all fishes were re-caught using SCUBA and 6 mm mesh sized gillnets. Fishes were immediately euthanized with an overdose of clove-oil, and permanently stored in 96% Ethanol for transportation to and long-term storage at the Zoological Institute, University of Basel.

Data assessment

In a first step, we examined whether or not the attack strategy of the scale-eaters correlates with mouth asymmetry (that is, we tested whether left-skewed fish feed more from the right body side of prey fish and right-skewed fish feed more from the left side). To this end, we inspected all prey fish for missing scales on each body side in the laboratory using Leica S6E binoculars with LeicaL2 light sources. The number of missing scales was determined by two examiners (AI and AT), and the average of the two counts was taken for further transformation in order to minimize count errors. On a few prey fish, larger parts of the scale cover were missing. In these cases, we excluded the data of the respective area on both sides of the prey fish to avoid introducing a possible bias possibly caused from handling the fish after the experiment.

In a second step, we quantified the feeding success of *P. microlepis* in relation to the different experimental conditions (that is, we compared feeding success of the scale-eaters between the mono- and dimorphic experimental populations). To this end, we dissected the ethanol-preserved scale-eaters and inspected their intestinal tracts. We first determined whether a predator fish was able to feed at all ('feeding event'; scales present in the stomach or gut), and, in cases where predators had eaten, counted the amount of scales in the intestinal tract ('scale count'). Since very little is known about the mode as

well as the rate of digestion of scales in *P. microlepis*, and since digested scales form a homogenous mass more downstream in the gut, we only counted intact or slightly digested scales from recent feeding events, which were still recognizable as discrete entities. Scale-counts could only be performed once and by one examiner (AI), since the specimens and their intestines were damaged during dissection.

Statistical analyses

All statistical analyses were done using the statistical software R (v.3.0.2; R Core Team 2013). To test for a putative correlation between attack strategy and mouth morphology (left-skewed *versus* right-skewed), we categorized – in all monomorphic experimental populations – the absolute number of missing scales into the attack strategies 0 and 1 as follows: If more scales were missing on the left flank of the prey fish than on its right side, we coded the attack strategy as 1 (that is, the predators' strategy is to preferentially attack the left flank of the prey), whereas if fewer scales were missing on the left than on the right flank, we coded it as 0 (that is, the predators' strategy is to preferentially attack the right flank of the prey). Data had to be categorized in this way since the distribution of counts turned out to be random so that it was not possible to transform the data. The attack strategy categories were used as response variable, together with the fixed effects mouth morph (left-skewed *versus* right-skewed) and habitat (rocky *versus* sandy), in a generalized linear mixed model (GLMM) with a logistic link function in the R package LME4 (Bates et al. 2014) (see Supplementary Table 1A). The factor 'cage' was included as a random effect to account for within cage dependence of the data. We then calculated the modelled proportion of prey with more scales missing on the left side of the body when kept in the cages with either only left-skewed or only right-skewed individuals, using the probability-logit-inverse function PLOGIS.

To analyse the feeding success of *P. microlepis* with respect to the composition of the experimental population (mono- *versus* dimorphic), we applied a hurdle model with the package GLMMADMB (Fournier et al. 2012; Skaug et al. 2013) (see Supplementary Table 1B, C, D). This model separates the data into two sets to disentangle (*i*) if the experimental populations showed, in general, different proportions of feeding events, and (*ii*) if the number of scales in the intestinal tract ('scale count') differed among the ones with scales present in their stomach. For the first part of the hurdle procedure describing the probability for feeding events, we fitted a model to the binary part of the data, which means that all zeroes (no scales in stomach) were coded as 0 and all non-zeroes (one or more scales in stomach) were coded as 1. In a GLMM with logistic link function we then tested if feeding events correlate with the experimental populations setup (mono- *versus* dimorphic) as a fixed effect and the factor 'cage' as a random effect (see Supplementary Table 1B). Due to the fact that neither standard length (SL) nor habitat (rocky/sandy) improved the model significantly (ANOVA model comparison; $\chi^2_{\text{with SL}} = 0.174$, $p_{\text{with SL}} = 0.6766$; $\chi^2_{\text{with habitat}} = 0.012$, $p_{\text{with habitat}} = 0.9128$), these parameters were not included as additional fixed effects.

In the second part of the hurdle procedure, to compare the intestinal scale count of *P. microlepis* among the experimental populations setups (mono-/dimorphic), a truncated negative binomial distribution (NB1) was fitted to the non-zero outcomes of the counted intestinal scales. Additionally to the experimental populations, SL and habitat were included as fixed effects. The factor 'cage' was again included as a random effect (see Supplementary Table 1C). The model was also repeated with the logarithmic prey:predator ratio as an offset (see Supplementary Table 1D) after checking for a correlation between prey:predator ratio and experimental population setups (mono- *versus* dimorphic). This correlation was performed with a GLMM with a logistic link function in LME4 (Bates et al. 2014), using the additional fixed effect 'habitat' and the random effect 'cage' (see Supplementary Table 1E).

Results

Overall, more than two thirds of the experimental fish were recovered at the end of the 3-day trials. Of the initially stocked 252 specimens of *P. microlepis*, 162 were recaptured at the end of the trials; of the

360 stocked prey individuals, 260 were recaptured (*T. moorii*: 118 of 180; *I. loocki*: 142 of 180; for cage-specific sample sizes see Supplementary Table 2). In addition, six non-stocked individuals were found, which were also included in further analysis since they served as prey as well. Despite the reduction in sample size, the size distribution was stable throughout the cages (mean SL ± sd; *P. microlepis* = 78.9 ± 9.0; prey = 74.9 ± 12.2; for cage-specific SL distribution see Supplementary Table 2).

All 207 recaptured prey individuals from L and R experimental populations featured missing scales. The number of missing scales was highly variable between prey specimens, ranging from 1 to 109 per specimen (mean number of missing scales ± sd = 16.589 ± 15.094; for cage-and experimental population-specific information see Supplementary Table 2). In most cases, missing scales were detected on both sides of the preys' body; only 8 individuals showed missing scales exclusively on one body side. The proportion of prey with more scales missing on the left than on the right body side and *vice versa* were significantly influenced by mouth orientation of the predator (GLMM; n = 207, z = 6.309, $p < 0.0001$; Fig. 2A) and therefore seem to correlate with the attack strategies of *P. microlepis* - with left-skewed fish attacking from the right side whereas right-skewed ones attacked from the left side in the majority of cases. Contrarily to mouth morph, no effect of habitat on attack strategy was found (GLMM; n = 207, z = 1.513, p = 0.13).

In the second part we tested whether *P. microlepis* of dimorphic experimental populations were more successful than monomorphic ones with regard to feeding events and the number of ingested scales. The dissection of the 162 *P. microlepis* intestines revealed that 106 individuals were able to succeed at a recent feeding event and therefore contained intestinal scales (monomorphic experimental populations: 66 of 111 individuals; dimorphic experimental populations: 40 of 51 individuals). *Perissodus microlepis* therefore had a higher probability for feeding events if they were kept in cages with dimorphic experimental populations than the ones in the cages with only monomorphic experimental populations (GLMM; n = 162, z = -2.32, p = 0.0204; Fig. 2B).

Between 1 and 44 scales per intestinal tract were recovered in the 106 successfully feeding *P. microlepis* (mean intestinal scales ± sd, range; monomorphic experimental populations = 7.5 ± 8.1, 1-44; dimorphic experimental populations = 7.0 ± 6.8, 1-31; for details on intestinal scale count information per cage see Supplementary Table 2). The intestinal scale count was only significantly influenced by SL, but not by experimental population setup nor by habitat (GLMM; n = 106, $z_{\text{experimental population setup}} = 0.32$, $p_{\text{experimental population setup}} = 0.7470$; $z_{SL} = -2.13$, $p_{SL} = 0.033$; $z_{habitat} = -1.36$, $p_{habitat} = 0.1730$). These results must be taken with caution, though, as 'scale count' could be influenced by the variable prey:predator ratio observed between the cages (Supplementary Table 2). These differences in the ratio between prey and predator fishes arose through varying sample sizes per cage due to unequal loss of experimental individuals, which is difficult to avoid in a semi-natural setting such as ours. Main reasons for losses in our experiment might be problems with recompression (note that fishes had to be brought to a depth of 6 to 9 m) and territorial fights within cages. When correcting for variable prey:predator ratios, we found that scale-eaters in the dimorphic experimental populations do have a higher feeding rate compared to the ones in monomorphic experimental populations (GLMM; n = 106, z = -3.17, p = 0.0015). Again, feeding rate was significantly influenced by SL here, but not by habitat (GLMM; n = 106; $z_{SL} = -2.81$, $p_{SL} = 0.0049$; $z_{habitat} = -1.75$, $p_{habitat} = 0.0801$). We note, however, that correcting for prey:predator ratio might itself introduce a bias by acting as a confounding factor. The average prey:predator ratio was – probably coincidentally – lower in dimorphic experimental populations than in monomorphic ones, which was not explainable by habitat (GLMM; n= 106, $z_{\text{experimental population setup}} = 3.131$, $p_{\text{experimental population setup}} = 0.0017$; $z_{habitat} = 0.059$, $p_{habitat} = 0.9530$). Therefore, the correlations of prey:predator ratio with the response variables intestinal scale count and the fixed effect experimental population setup cannot be disentangled. We would also like to note that the models with and without offset (i.e. correction for prey:predator ratio) resulted in nearly identical AIC values and should thus both be taken into account.

Disscusion

In this study we report a field based enclosure experiment in a semi-natural environment to assess attack strategies and feeding success of the scale-eating cichlid *Perissodus microlepis* in Lake Tanganyika in East Africa. In a first step, by examining the missing scales on prey fishes exposed to scale-eaters in underwater cages, we confirm previous findings on the attack strategy of *P. microlepis* (Takeuchi et al. 2012; Lee et al. 2012) and show that also under semi-natural circumstances and with community interactions, the two mouth morphs show a feeding preference on the respectively most suitable flank of the prey (that is, left-skewed fish feed preferably from the right flank of prey fishes, while right-skewed individuals attack more often the left side) (Fig. 2A). However, in contrast to previous work reporting relatively few (ca. 20% in Takeuchi et al. 2012) or no (in Lee et al. 2012) attacks to the 'wrong' flank of the prey, our field- and community-based experiments with monomorphic populations revealed that scale-eaters regularly feed from the 'wrong' side of the prey as well; notably, only eight out of 207 prey fish in the monomorphic populations had been attacked at only one side. The difference between previous studies and our present work is most likely explained by the different experimental settings: while Takeuchi et al. (2012) and Lee et al. (2012) used one-predator:one-prey setups, we opted for a community setting with several predator and prey fishes in semi-natural conditions using underwater cages in the natural habitat of scale-eaters. It thus seems likely that scale-eaters depart from their optimal hunting strategy (the one uncovered in one-predator:one-prey experiments) under semi-natural or natural conditions, where fishes encounter each other in differing orientations and on multiple occasions. Another explanation might be that the continuous rather than discreet nature of the trait might weaken the behavioural effect seen in natural populations. This could be the case even though we only chose unambiguous individuals for the experiment, since this does not completely eradicate trait variation (Kusche et al. 2012). Alternatively, the relatively high rate of attacks to the 'wrong' flank might be an indication that our setup provided the scale-eaters with more opportunities for strikes, e.g. due to the slightly elevated prey density compared to natural communities and the lack of dilution by other species (Sturmbauer et al. 2008).

In a second step, by counting the scales from the intestinal tracts of *P. microlepis*, we determined the feeding success of scale-eaters in mono- and dimorphic populations, whereby feeding success is composed of two factors that were analysed separately here: (*i*) the opportunity to feed as defined by whether or not an actual feeding event has taken place, and (*ii*) the number of ingested scales in the intestinal tract of a scale-eater. Importantly, the probability to feed was greater in scale-eaters living in a dimorphic experimental population than in individuals in monomorphic populations (Fig. 2B). This seems to be attributable to the fact that – in a dimorphic population – prey specimens have a lowered chance to adapt to the attack strategies of the scale-eater (as strikes occur towards both flanks) as compared to a monomorphic population (where strikes occur towards to one flank with higher frequency). Another possible explanation is that dimorphic scale-eater populations have access to a larger area of prey surface when deploying their optimal hunting strategy, as opposed to a purely monomorphic population. The intestinal scale counts provided a less clear picture as to whether dimorphic scale-eater populations have a selective advantage over monomorphic ones. Only when correcting for differing prey:predator ratios in the different cages did we find that scale-eaters had a significantly higher feeding rate in the dimorphic experimental populations. Interestingly, the habitat structure (sandy *versus* rocky) did not have any effect on neither the attack strategy nor on feeding success. This could be an effect of the limited sample size, nevertheless it is somewhat surprising, given that the rocky habitat provides ample opportunity for prey fishes to hide from predators as well as for predators to ambush their prey. Furthermore the evidence that smaller sized scale-eaters feature a higher feeding rate than larger one's, might be due to the lower detectability or the diminished intimidation effect of smaller predators giving them higher feeding event probability.

Taken together, our study is the first experimental demonstration that scale-eaters have a significantly increased chance of striking an attack when living in dimorphic compared to monomorphic populations,

suggesting that the higher probability for feeding – possibly resulting in a higher feeding rate – is the selective agent responsible for the initial phase of trait divergence in *P. microlepis*. Negative frequency dependent selection, as postulated by Hori (1993), would then be the stabilizing force responsible for maintaining the mouth dimorphism at a quasi-equal ratio in natural populations. The big unknown in this system is whether or not mouth 'handedness' is a heritable trait, and if so, how it is inherited (see e.g. Hori et al. 2007; Takahashi & Hori 2008; Lee et al. 2010, 2012; Van Dooren et al. 2010; Kusche et al. 2012). In any case, the two mouth morphs of *P. microlepis* can be viewed as two divergent natural groups with respect to attack strategy that, based on our results, persist within a single interbreeding species, for the reason that the selective advantage of the trait in question arises primarily through its intrinsic bimodality.

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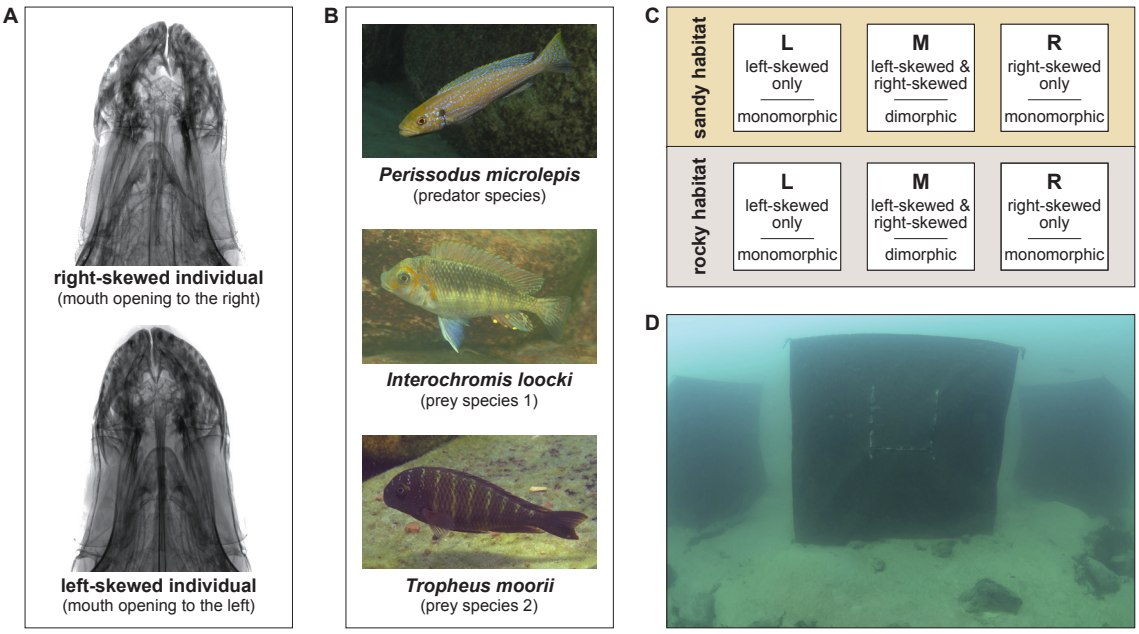

Figure 1 Experimental setup. (A) X-ray images showing the head region of *P. microlepis* with different mouth morphs in dorsal view. Above: 'right-skewed' (where the left upper jaw bow is elongated); below: left-skewed (where the right upper jaw bow is elongated) (pictures: Heinz Büscher). (B) Underwater photographs of the predator and prey species in their natural habitat (pictures: Adrian Indermaur). (C) Scheme of the experimental setup showing the cages (squares) with the distribution of the different mouth morphs (left-skewed/right-skewed), experimental populations (L/M/R), experimental population setups (mono-/dimorphic) and habitats (rocky/sandy bottom). (Note that the experimental arrangement was randomly rotated within habitat in every trial). (D) Underwater photograph of the experimental cages (picture: Angel M. Fitor).

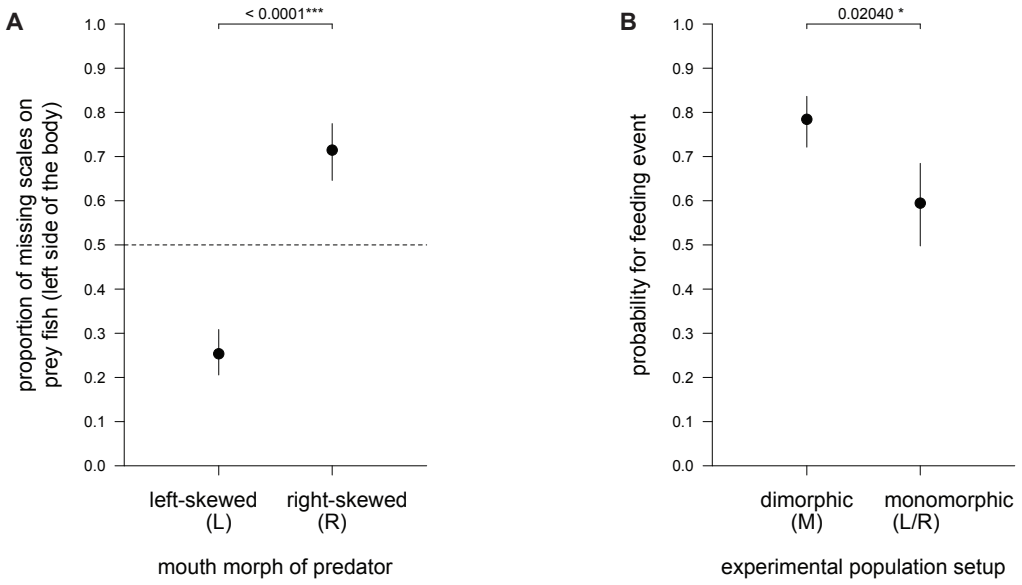

Figure 2 Attack strategies and feeding events in experimental scale-eater populations. (A) Attack strategy as the proportions of missing scales on the prey species' left body side for the separate mouth morphs. (B) Probability for a feeding event in dimorphic or monomorphic experimental population setups.

Table S1 Models used to test for a correlation of attack strategy and mouth morph (L *versus* R) (A), probability for a feeding event and experimental population setup (mono- *versus* dimorphic) (B), scale count and experimental population setup (mono- *versus* dimorphic) without a correction for prey:predator ratio (C) and with a correction for prey:predator ratio (D) as well as for a correlation of prey:predator ratio and experimental population setup (mono- *versus* dimorphic) (E).

A	glmer(attack_strategy ~ experimental_population + habitat + (1|cage), data = missing_scales_binomial, family = "binomial")
B	glmmadmb(feeding_event ~ experimental_population_setup + (1|cage), data = intestinal_scales_binomial, family = "binomial")
C	glmmadmb(intestinal_scale_count ~ experimental_population_setup + SL + (1|cage), data = subset(intestinal_scale_count_numeric > 0), family = "truncnbinom1")
D	glmmadmb(intestinal_scale_count ~ experimental_population_setup + SL + offset(log(prey_predator_ratio)) + (1|cage), data = subset(intestinal_scale_count_numeric > 0), family = "truncnbinom1")
E	glmer(cbind(prey_number, predator_number) ~ experimental_population_setup + habitat + (1|cage), data = subset(intestinal_scale_count_numeric > 0), family="binomial")

Table S2 Cage- and experimental population-specific information on sample sizes for predator and prey specimen, prey:predator ratios, left-to-right-skewed-ratios, standard lengths and scale information (missing scales on the prey's body side as well as intestinal scale counts for *P. microlepis*).

cage information				sample size *P. microlepis*			sample size prey				prey:predator ratio	left-to-right-skewed-ratio		standard length (mm) average (mean ±sd) range (min - max)		scale informations average (mean ±sd) range (min - max)					
number	experimental population	experimental population setup	habitat	survivals (successful feeders) / original stock			stocked prey survivals / original stock		non-stocked prey	total						missing scales on the prey's body side				intestinal scale counts of *P. microlepis*	
				left-skewed	right-skewed	total	*I. loocki*	*T. moori*				wanted	achieved	*P. microlepis*	prey	left	right	difference (left - right)	total (left + right)	all individuals	successful feeders only
1	L	monomorphic	rocky	8 (5) / 14	-	8 (5) / 14	10 / 10	4 / 10	2	16	2.0000	100 : 0	100 : 0	78.875 ± 9.672 67.0 - 92.0	79.250 ± 10.415 61.5 - 98.5	3.719 ± 2.966 0.0 - 11.0	6.219 ± 4.091 0.0 - 14.0	-2.500 ± 4.037 -11.0 - 5.0	9.938 ± 5.896 2.5 - 25.0	3.625 ± 4.207 0.0 - 12.0	5.800 ± 3.899 2.0 - 12.0
2	L	monomorphic	rocky	11 (8) / 14	-	11 (8) / 14	9 / 10	9 / 10	0	18	1.6364	100 : 0	100 : 0	73.909 ± 10.358 61.0 - 97.0	76.722 ± 11.030 60.0 - 98.0	11.972 ± 16.607 2.5 - 70.5	16.111 ± 15.590 0.0 - 64.0	-4.139 ± 12.678 -31.5 - 32.0	28.083 ± 29.613 3.5 - 109.0	8.000 ± 9.154 0.0 - 25.0	11.000 ± 9.055 2.0 - 25.0
3	L	monomorphic	rocky	11 (6) / 14	-	11 (6) / 14	9 / 10	10 / 10	1	20	1.8182	100 : 0	100 : 0	79.818 ± 11.898 63.0 - 103.0	71.650 ± 11.930 50.5 - 96.0	4.925 ± 3.711 0.5 - 14.0	9.325 ± 7.836 0.5 - 29.0	-4.400 ± 6.504 -22.5 - 4.5	14.250 ± 10.394 3.5 - 42.0	7.091 ± 13.308 0.0 - 44.0	13.000 ± 16.186 1.0 - 44.0
4	L	monomorphic	sandy	11 (7) / 14	-	11 (7) / 14	9 / 10	8 / 10	0	17	1.5455	100 : 0	100 : 0	76.273 ± 6.166 64.0 - 85.0	77.382 ± 11.608 62.0 - 102.5	4.118 ± 2.281 1.0 - 8.5	5.500 ± 4.287 1.5 - 19.5	-1.382 ± 3.706 -11.0 - 3.5	9.618 ± 5.781 3.5 - 28.0	4.455 ± 7.062 0.0 - 22.0	7.000 ± 7.895 1.0 - 22.0
5	L	monomorphic	sandy	12 (9) / 14	-	12 (9) / 14	7 / 10	5 / 10	0	12	1.0000	100 : 0	100 : 0	80.667 ± 11.171 64.0 - 98.0	79.417 ± 10.460 64.0 - 98.0	11.125 ± 9.684 1.0 - 34.0	12.708 ± 8.484 1.0 - 25.0	-1.583 ± 5.571 -11.5 - 9.0	23.833 ± 17.334 3.0 - 59.0	2.833 ± 2.855 0.0 - 8.0	3.778 ± 2.682 1.0 - 8.0
6	L	monomorphic	sandy	11 (3) / 14	-	11 (3) / 14	8 / 10	9 / 10	0	17	1.5455	100 : 0	100 : 0	78.727 ± 8.844 63.0 - 92.0	70.559 ± 12.240 50.0 - 90.5	10.735 ± 9.141 1.0 - 30.0	14.588 ± 12.412 1.0 - 42.5	-3.853 ± 7.295 -20.5 - 9.0	25.324 ± 20.544 2.0 - 65.0	1.818 ± 5.076 0.0 - 17.0	6.667 ± 8.963 1.0 - 17.0
	total L			**64 (38) / 84**	**-**	**64 (38) / 84**	**52 / 60**	**45 / 60**	**3**	**100**	**1.5625**	**100 : 0**	**100 : 0**	**78.047 ± 9.782 61.0 - 103.0**	**75.500 ± 11.618 50.0 - 102.5**	**7.595 ± 9.420 0.0 - 70.5**	**10.700 ± 10.421 0.0 - 64.0**	**-3.105 ± 7.344 -31.5 - 32.0**	**18.295 ± 18.459 2.0 - 109.0**	**4.656 ± 7.872 0.0 - 44.0**	**7.842 ± 8.937 1.0 - 44.0**
7	R	monomorphic	rocky	-	8 (6) / 14	8 (6) / 14	10 / 10	7 / 10	2	19	2.3750	0 : 100	0 : 100	82.500 ± 9.636 72.0 - 98.0	76.500 ± 11.897 61.5 - 96.0	9.553 ± 7.774 1.0 - 28.5	7.342 ± 12.147 0.5 - 55.5	2.211 ± 9.631 -27.0 - 17.0	16.895 ± 17.979 2.5 - 84.0	7.000 ± 10.650 0.0 - 32.0	9.333 ± 11.518 2.0 - 32.0
8	R	monomorphic	rocky	-	9 (6) / 14	9 (6) / 14	10 / 10	7 / 10	0	17	1.8889	0 : 100	0 : 100	82.444 ± 8.218 72.0 - 101.0	75.912 ± 10.263 60.5 - 95.0	9.029 ± 3.233 5.0 - 16.0	4.765 ± 2.768 0.0 - 10.0	4.265 ± 3.981 -3.0 - 10.5	13.794 ± 4.514 7.0 - 24.5	5.556 ± 7.970 0.0 - 24.0	8.333 ± 8.595 2.0 - 24.0
9	R	monomorphic	rocky	-	9 (5) / 14	9 (5) / 14	10 / 10	9 / 10	0	19	2.1111	0 : 100	0 : 100	83.444 ± 7.502 74.0 - 95.0	71.632 ± 12.533 51.5 - 93.5	6.368 ± 3.872 1.0 - 16.0	6.868 ± 9.853 0.0 - 41.5	-0.500 ± 8.288 -27.5 - 12.5	13.237 ± 12.468 3.5 - 57.5	1.556 ± 1.944 0.0 - 5.0	2.800 ± 1.789 1.0 - 5.0
10	R	monomorphic	sandy	-	7 (4) / 14	7 (4) / 14	10 / 10	10 / 10	1	21	3.0000	0 : 100	0 : 100	81.286 ± 8.995 69.0 - 93.0	75.429 ± 12.225 57.0 - 104.0	9.238 ± 3.750 3.0 - 19.5	4.310 ± 3.520 0.0 - 17.0	4.929 ± 4.597 -3.5 - 17.0	13.548 ± 5.637 5.0 - 30.5	4.714 ± 5.090 0.0 - 11.0	8.250 ± 3.594 3.0 - 11.0
11	R	monomorphic	sandy	-	6 (3) / 14	6 (3) / 14	8 / 10	5 / 10	0	13	2.1667	0 : 100	0 : 100	77.167 ± 3.251 74.0 - 82.0	71.716 ± 9.671 60.5 - 90.0	11.231 ± 7.568 4.5 - 30.5	6.077 ± 3.605 2.0 - 12.0	5.154 ± 7.072 -5.0 - 18.5	17.308 ± 9.514 7.5 - 42.5	4.667 ± 5.645 0.0 - 12.0	9.333 ± 3.786 5.0 - 12.0
12	R	monomorphic	sandy	-	8 (4) / 14	8 (4) / 14	8 / 10	10 / 10	0	18	2.2500	0 : 100	0 : 100	79.625 ± 6.567 68.0 - 86.0	66.417 ± 9.345 50.5 - 83.5	8.750 ± 5.735 1.0 - 24.5	7.250 ± 5.048 0.0 - 19.0	1.500 ± 4.159 -6.5 - 8.5	16.000 ± 9.972 1.0 - 43.5	2.000 ± 2.828 0.0 - 8.0	4.000 ± 2.828 2.0 - 8.0
	tota R			**-**	**47 (28) / 84**	**47 (28) / 84**	**56 / 60**	**48 / 60**	**3**	**107**	**2.2766**	**0 : 100**	**0 : 100**	**81.319 ± 7.619 68.0 - 101.0**	**73.054 ± 11.490 50.5 - 104.0**	**8.911 ± 5.543 1.0 - 30.5**	**6.084 ± 7.219 0.0 - 55.5**	**2.827 ± 6.816 -27.5 - 18.5**	**14.995 ± 10.919 1.0 - 84.0**	**4.191 ± 6.405 0.0 - 32.0**	**7.036 ± 7.010 1.0 - 32.0**
13	M	dimorphic	rocky	4 (3) / 7	3 (1) / 7	7 (4) / 14	7 / 10	3 / 10	0	10	1.4286	50 : 50	57 : 42	73.857 ± 6.012 70.0 - 84.0	78.400 ± 14.734 53.5 - 99.5	6.400 ± 3.414 3.0 - 15.0	6.650 ± 4.750 1.5 - 17.5	-0.250 ± 6.763 -12.0 - 13.5	13.050 ± 4.764 6.5 - 23.0	6.571 ± 7.458 0.0 - 20.0	11.500 ± 5.972 6.0 - 20.0
14	M	dimorphic	rocky	4 (3) / 7	5 (4) / 7	9 (7) / 14	4 / 10	1 / 10	0	5	0.5556	50 : 50	44 : 55	74.000 ± 4.717 67.0 - 80.0	76.400 ± 15.690 63.0 - 95.5	27.200 ± 10.474 12.5 - 37.5	21.600 ± 17.387 7.5 - 48.5	5.600 ± 11.518 -11.0 - 21.5	48.800 ± 26.293 20.0 - 86.0	3.444 ± 3.644 0.0 - 11.0	4.429 ± 3.552 1.0 - 11.0
15	M	dimorphic	rocky	4 (1) / 7	6 (6) / 7	10 (7) / 14	3 / 10	8 / 10	0	11	1.1000	50 : 50	42 : 58	80.400 ± 13.218 62.0 - 102.0	71.409 ± 11.256 59.0 - 90.5	17.455 ± 10.734 3.0 - 37.5	11.818 ± 11.660 3.5 - 42.5	5.636 ± 9.521 -13.0 - 26.0	29.273 ± 20.291 7.5 - 72.0	4.800 ± 7.084 0.0 - 23.0	6.857 ± 7.669 1.0 - 23.0
16	M	dimorphic	sandy	5 (4) / 7	4 (3) / 7	9 (7) / 14	10 / 10	8 / 10	0	18	2.0000	50 : 50	55 : 44	79.889 ± 5.011 71.0 - 89.0	78.556 ± 13.885 57.0 - 107.0	7.972 ± 7.480 0.5 - 25.5	8.417 ± 6.274 0.0 - 23.5	-0.444 ± 5.418 -14.5 - 7.0	16.389 ± 12.700 0.5 - 49.0	8.444 ± 9.838 0.0 - 31.0	10.857 ± 9.924 2.0 - 31.0
17	M	dimorphic	sandy	4 (4) / 7	6 (6) / 7	10 (10) / 14	5 / 10	2 / 10	0	7	0.7000	50 : 50	40 : 60	76.000 ± 10.985 59.0 - 95.0	82.143 ± 16.525 62.5 - 101.0	19.714 ± 7.588 8.5 - 32.5	9.929 ± 3.359 4.5 - 15.0	9.786 ± 9.827 -6.5 - 23.0	29.643 ± 6.414 23.5 - 42.0	6.600 ± 6.022 1.0 - 15.0	6.600 ± 6.022 1.0 - 15.0
18	M	dimorphic	sandy	3 (3) / 7	3 (2) / 7	6 (5) / 14	5 / 10	3 / 10	0	8	1.3333	50 : 50	50 : 50	82.500 ± 5.612 78.0 - 93.0	76.429 ± 15.034 59.5 - 103.0	9.250 ± 6.840 1.0 - 20.0	4.625 ± 2.888 1.0 - 9.0	4.625 ± 9.330 -8.0 - 19.0	13.875 ± 4.816 8.5 - 21.0	2.167 ± 1.722 0.0 - 5.0	2.600 ± 1.517 1.0 - 5.0
	total M			**24 (18) / 42**	**27 (22) / 42**	**51 (40) / 84**	**34 / 60**	**25 / 60**	**0**	**59**	**1.1569**	**50 : 50**	**48 : 52**	**77.667 ± 8.856 59.0 - 102.0**	**77.164 ± 13.937 53.5 - 107.0**	**12.669 ± 10.051 0.5 - 37.5**	**9.534 ± 8.956 0.0 - 48.5**	**3.136 ± 8.619 -14.5 - 26.0**	**22.203 ± 16.975 0.5 - 86.0**	**5.490 ± 6.655 0.0 - 31.0**	**7.000 ± 6.779 1.0 - 31.0**
	TOTAL L + R (monomorphic only)			**64 (38) / 84**	**47 (28) / 84**	**111 (66) / 168**	**108 / 120**	**93 / 120**	**6**	**207**	**1.864865**	**-**	**-**	**79.432 ± 9.030 61.0 - 103.0**	**74.236 ± 11.589 50.0 - 104.0**	**8.275 ± 7.674 0.0 - 70.5**	**8.314 ± 9.185 0.0 - 64.0**	**-0.039 ± 7.659 -31.5 - 32.0**	**16.589 ± 15.094 1.0 - 109.0**	**4.459 ± 7.260 0.0 - 44.0**	**7.500 ± 8.126 1.0 - 44.0**
	TOTAL L + R + M (mono- & dimorphic)			**88 (56) / 126**	**74 (50) / 126**	**162 (106) / 252**	**142 / 180**	**118 / 180**	**6**	**266**	**1.641975**	**-**	**-**	**78.877 ± 8.993 59.0 - 103.0**	**74.877 ± 12.174 50.0 - 107.0**	**9.250 ± 8.440 0.0 - 70.5**	**8.585 ± 9.132 0.0 - 64.0**	**0.665 ± 7.975 -31.5 - 32.0**	**17.835 ± 15.677 0.5 - 109.0**	**4.784 ± 7.071 0.0 - 44.0**	**7.311 ± 7.616 1.0 - 44.0**

Discussion

The work presented in my doctoral thesis focuses on phenotypic and functional diversity of naturally and sexually selected traits in haplochromine cichlid fishes, thus contributing to elucidate the mechanisms responsible for their astonishing radiations. The first part of my thesis investigated the evolutionary origin **(1.1)** and the function **(1.2 & 1.3)** of egg-spots, a putative key innovation of haplochromine cichlids. The second part focused on the natural diversity of egg-spots **(2.1)** as well as body shape and other ecologically relevant traits in a newly established lake-stream system **(2.2)**. Further, phylogeographic relationships and genetic as well as morphometric diversity were assessed in a broader set of *Astatotilapia burtoni* populations from the southern part of Lake Tanganyika **(2.2)** and in *Pseudocrenilabrus cf. philander* populations from Lake Chila and surrounding rivers **(3.1)**. The last project examined the possible selective advantage of mouth asymmetry in a scale-eating cichlid fish **(3.2)**.

In the first manuscript entitled **a sensory bias has triggered the evolution of egg-spots in cichlid fishes (1.1)** we could show that *Pseudocrenilabrus multicolor* females prefer computer-animated photographs of males with an artificially added egg-spot over images showing unaltered males (with no true egg-spots). The experiments on colour preference uncovered a bias for egg-spot-like colours (yellow, orange or red) in the cichlid species examined, suggesting that this bias has evolved before the emergence of the first haplochromines. Taken together, these results indicate that sensory exploitation of a pre-existing bias was most probably involved in the evolution of anal fin egg-spots in haplochromine cichlids. Since the preference for egg-spot-like dots is prevalent in male and female cichlids, and, also, in substrate spawners basal to the haplochromines, the bias is most probably directed towards high-quality (carotenoid-enriched) food instead of the previously proposed affinity to detect own eggs as such (Wickler 1962; Tobler 2006).

The studies testing for a function of egg-spots in *A. burtoni* **(1.2 The function of anal fin egg-spots in the cichlid fish *Astatotilapia burtoni*)** and *Astatotilapia calliptera* **(1.3 Egg-spot pattern and body size asymmetries influence male aggression in haplochromine cichlid fishes)** found no evidence for an influence of egg-spots on female preference or fertilization rate. In both species, egg-spots are rather used in male-male interactions to assess the strength of an opponent. This is in line with the general observation that throughout the animal kingdom reddish signalling traits are used to signal strength in combats (e.g. Bakker & Sevenster 1983; Evans & Norris 1996; Pryke et al. 2002). However, the two tested species elicited an opposite attack strategy based on this assessment: Territorial males of *A. burtoni* seemed to be intimidated by stimulus males bearing egg-spots and directed more attacks against the presumably weaker intruder (males with artificially removed egg-spots). Males of *A. calliptera* on the other hand, adopted a high-risk attack strategy, launching more attacks against seemingly stronger intruders (represented by higher egg-spot numbers and larger body sizes). This discrepancy in attack strategy between the two species could be due to differences in the intimidation effect of the egg-spot itself, an interplay of egg-spots with other colour patterns, or differences in resource value.

To the best of my knowledge, these are the first studies demonstrating a direct function of egg-spots in male aggression behaviour. Further, these findings in two generalist haplochromine species support the hypothesis that the process of intrasexual selection on male colouration may have played an important role in the astonishing radiations of haplochromine cichlids (reviewed in Dijkstra & Groothuis 2011), in addition to the often mentioned intersexual selection (e.g. Seehausen et al. 1997; Kocher 2004; Maan et al. 2004; Genner & Turner 2005). In contrast to the results presented here, in some species females base their mating preference on high egg-spot number (Hert 1989, 1991) or enlarged egg-spot size with constraint number (Couldridge 2002). Even though the latter support Wickler's egg-mimicry hypothesis (1962) with regard to courtship behaviour, none of the above mentioned studies on the evolution and function of egg-spots implied that egg-spots necessarily mimic true eggs of the corresponding species, and none of these studies found evidence that egg-spots increased the fertilization rate. In summary, haplochromine egg-spots supposedly evolved via a female sensory bias, which suggests an ancestral function in female choice, with a subsequent evolution to multiple functions, e.g. in male interactions and/or species recognition.

The multiple functions of egg-spots illustrate that we should exercise caution generalizing the function of visual signals and interpreting their function based on their appearance to the human eye and sense. A traditional division of secondary sexual traits into 'weapons' acting in male interactions and 'ornaments' having an effect in female preference (Darwin 1871) seems thus out-dated. It is well known that an ornament's function can alter from female choice to male-male competition and vice versa or can have a dual function (reviewed in Berglund et al. 1996).

Future experiments could be conducted to test more haplochromine species on both, intra- and intersexual selection, to gain more insights how the function of this trait diverged and if it reveals associations between their function, pattern, colouration, the species' mating behaviour and environment. Further, as mentioned above, up to now, no influence of egg-spots on fertilization rate was found. Future experiments should thus test if egg-spots could have an influence on fertilization rate in habitats with increased water turbidity and/or water current. Additionally, the trials with altered environmental conditions should also test for a functional change of egg-spots, e.g. if egg-spots in turbid water function as mate- or species-recognition signals. A change of egg-spot function with environmental changes would be especially interesting with respect to the observed differences in egg-spot phenotype in different locations and environmental conditions in the field.

Along these lines, the study on the **variation of anal fin egg-spots along an environmental gradient in a haplochromine cichlid fish (2.1)** revealed sex- and habitat-specific differences in egg-spot characteristics among lake and stream populations in *A. burtoni*. The egg-spot phenotype differed substantially between sexes, with males possessing larger and more conspicuous egg-spots than females, which is likely explained by their function in sexual selection. In addition to the more general differences between sexes, habitat-specific differences in egg-spot phenotype suggest adaptations to the respective environments. Even though the four lake-stream systems did not consistently show the same differences

Figure 2 Male-male interaction in *Astatotilapia burtoni*.

in egg-spot characteristics between lake and stream populations, there is a general trend of increasing conspicuousness of egg-spots from lake towards riverine populations, with the latter generally showing fewer, but larger egg-spots with a more intense colouration and a higher egg-spot to fin contrast. Testing for an association between egg-spot phenotypes and environmental as well as physiological parameters revealed that underwater light environment seems to influence egg-spot colouration, whereas egg-spot number and relative average egg-spot area correlate with immune activity. The fact that different egg-spot characteristics may be influenced by variable environmental factors illustrates that several replicates need to be examined to elucidate the causes for variation in such a complex trait. Nevertheless, it can be stated that haplochromine egg-spots are a sexually selected visual signal that features the potential to adapt to the respective underwater light environment, and is traded-off with the investment into the immune system. The great phenotypic and functional diversity of this trait provides further support for the assumption that egg-spot patterns played an important role in the divergence of the exceptionally colourful and most species-rich cichlid tribe, the haplochromines.

The question if ecological factors could also be responsible for the diversity in egg-spots among species is part of an ongoing study, in which we examine the natural variation of egg-spots in the East African Great Lakes.

To establish the *A. burtoni* setting as the first replicate lake-stream divergence system in cichlids, the next chapter focuses on the **adaptive divergence between lake and stream populations of an East African cichlid fish (2.2)**. This study revealed the presence of several divergent lake and stream populations positioned at different stages of the speciation continuum, which follow the same morphological and ecological trajectories along this environmental gradient. Lake fish show deeper bodies, a more superior mouth position, longer gill rakers and more slender pharyngeal jaws, and feed on a plant/algae and zooplankton-biased diet, whereas stream fish feed more on benthic food such as snails, insects and plant seeds. Phenotypic divergence in body shape is most likely associated with different flow regimes in lake and stream habitats, whereas shifts in trophic traits are linked to differential resource use. Reproductive isolation experiments between closely related lake and stream populations did not detect population-assortative mating. Analyses of body shape and gill raker length of F1 offspring reared under common garden conditions indicate, however, that the morphological differences observed in natural populations do not constitute pure plastic responses to different environmental conditions, but also have a genetic basis. The high morphological diversity in body shape was also detected in a broader sample of over twenty populations from southern Lake Tanganyika. Phylogeography and population genetics of these populations revealed extensive population structure and an unexpectedly high degree of genetic diversity in *A. burtoni*, which is similar to or even exceeds the diversity across the entire haplochromine cichlid assemblage of Lake Victoria (Verheyen et al. 2003). This diversity in *A. burtoni*, even across small geographic scales, thus suggests a long coalescence time and, consequently, the presence of this species in the study area over long time periods.

This characterization of replicate lake-stream population pairs in *A. burtoni* at different stages of the 'speciation continuum' enables subsequent projects to examine the early phases of adaptive divergence in this system. In a follow-up project, we used RAD sequencing (Egger et al. in preparation); and a more in-depth analysis based on whole genomes, geometric morphometric analyses, ecological, physiological and immunological assessment as well as mate choice experiments are ongoing. Here, the main questions are how many loci are involved in the adaptation along the lake-stream environmental gradient and how these are distributed across the genome. Even though *A. burtoni* represents one of the five cichlid species whose genome was recently sequenced (Brawand et al. 2014) and the reference genome is available on GenBank, the density of SNP markers as revealed by RADseq is by far not sufficient to link the outlier genomic regions to certain genes or their regulatory regions. This is why it is planned to move towards sequencing entire genomes. Currently, we perform further mate choice experiments to test for assortative mating between lake and stream *A. burtoni* populations, which are genetically more distinct from each other than the previously tested ones.

The **phylogeographic and phenotypic assessment of a basal haplochromine cichlid fish from Lake Chila, Zambia (3.1)** revealed a complex phylogeographic pattern and demonstrated admixture of two distant mitochondrial lineages, producing a 'hybrid swarm' with substantial phenotypic variability. Males from this population from Lake Chila occupy a much larger portion of the morphospace with respect to body size and colouration. Lake Chila fish displayed deeper bodies and more elaborate colour patterns compared to riverine populations from the *P. cf. philander* species complex. This extensive genetic and morphological variation in Lake Chila is in contrast to the rather low diversity found in rivers and suggests that *Pseudocrenilabrus spp*. are more prone to diversify in a lake habitat providing more ecological opportunity (especially when more derived 'modern' haplochromines are absent). The setting seems to be a nice example of introgressive hybridisation between lineages facilitating the colonisation of new environments by increasing genetic variation and generating unique phenotypes (Kolbe et al. 2004; Seehausen 2004).

In the last chapter, we investigated a special case of mouth asymmetry by conducting a field based assessment of attack strategies and feeding success in the scale-eating cichlid fish *Perissodus microlepis* **(3.2 A fitness benefit for mouth dimorphism in a scale-eating cichlid fish)**. We first confirm previous findings on the attack strategy of *P. microlepis* (Lee et al. 2012; Takeuchi et al. 2012) and show that also under semi-natural circumstances and with community interactions, the two mouth morphs show a feeding preference on the respectively most suitable flank of the prey: left-skewed scale-eaters preferentially attack the right flank of their prey, whereas right-skewed individuals feed predominantly from the left side. Additionally, we were able to empirically demonstrate that the probability to feed was greater in scale-eaters living in a dimorphic experimental population than in individuals in monomorphic populations. This resulting ecological advantage might be the selective agent responsible for the initial phase of trait divergence in *P. microlepis* and other asymmetrical scale-eaters from Lake Tanganyika.

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Divergence between lake and stream populations in an East African cichlid fish

2013 Poster presentation at the meeting of the European Society for Evolutionary Biology (ESEB), Lisbon, Portugal.
Variation of anal fin egg-spots along an environmental gradient in a haplochromine cichlid fish

Talk at the Swiss Biological Science meeting (Biology13), Basel, Switzerland.
Lake-stream population pairs in cichlids

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Lake-stream population pairs in cichlids

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Talk at the cichlid meeting (CichlidScience 2010), Basel, Switzerland.
The function of the anal fin egg-spots in the haplochromine cichlid fish *Astatotilapia burtoni*

FIELDWORK EXPERIENCE

2014 Lake Tanganyika, Zambia (two weeks)
Cichlids, reproductive isolation experiment

2013 Lake Tanganyika, Zambia (four weeks)
Cichlids, sampling lake-stream systems

2012 Lake Tanganyika, Zambia (three weeks)
Cichlids, sampling lake-stream systems

2011 Lake Tanganyika, Zambia (nine weeks)
Cichlids, sampling lake-stream systems

2010 Lake Tanganyika, Zambia (four weeks)
Cichlids, sampling lake-stream systems

2009 Lake Xiloa, Nicaragua (two weeks)
Cichlids, project on breeding behaviour of convict cichlids

2008 Tvärminne, Finland (two weeks)
Independent project development in the field

Tarifa, Spain (ten days)
Marine biology, project on overfishing and aquacultures

St. Bauzille de Putois, France (one week)
Botany, project in dendrology

2007 Erquy, France (one week)
Marine biology, project on water chemistry fluctuation in rock pools

2006 Banyuls, France (two weeks)
Marine biology, project on echinoderms

2005 Bimini, Bahamas (three months)
Marine biology, voluntary work on the migratory behaviour of lemon sharks

SUPERVISION OF STUDENTS

2012 – 2015 Co-supervision of four Master theses, Zoological Institute, University of Basel, Switzerland

2015 Co-supervision of two student projects in the Zoology block course for students in the third year of the B.Sc., University of Basel, Switzerland

2013 Co-supervision of three student projects in the Zoology block course for students in the third year of the B.Sc., University of Basel, Switzerland

2011 Co-supervision of one student project in the Zoology block course for students in the third year of the B.Sc, University of Basel, Switzerland

REVIEW ACTIVITIES

PLoS ONE

SCIENTIFIC MEMBERSHIPS

2013 FAG (Freiwillige Akademische Gesellschaft)

ESEB (European Society for Evolutionary Biology)

SZG (Swiss Zoological Society)

COURSES

2012 Statistics course and workshop *Data analyses in life sciences using linear models with R*, Vogelwarte, Sempach, Switzerland

OUTREACH ACTIVITIES

2009 - present Assistant at the Café Scientifique, KidsLab, University of Basel, Switzerland

ADDITIONAL SKILLS

Advanced IT-knowledge	R, JMP, MorphoJ
Presentation softwares	Photoshop, Illustrator, InDesign, Keynote
Languages	German (mothertongue), English (fluent in spoken and written)

RESEARCH AND TRAVEL GRANTS

2014 Travel grant, field trip to Lake Tanganyika, Zambia
Basler Stiftung für experimentelle Zoologie - 1'300 CHF

2013 Research grant, extension of my PhD
FAG (Freiwillige Akademische Gesellschaft) - 14'000 CHF

Travel grant, meeting of the European Society for Evolutionary Biology (ESEB)
University of Basel - 668 CHF

Travel grant, field trip to Lake Tanganyika, Zambia
SZG (Swiss Zoological Society) -1'500 CHF

2012 Travel grant, International Behavioral Ecology Congress (ISBE)
University of Basel - 1'256 CHF

Travel grant, statistics course *Data analyses in life sciences using linear models with R*
University of Basel - 722 CHF

2011 Travel grant, meeting of the European Society for Evolutionary Biology (ESEB)
University of Basel - 326 CHF

2010 Travel grant, field trip to Lake Tanganyika, Zambia
SCNAT (Swiss Academy of Science) – 1'700 CHF

PUBLICATIONS (in peer-reviewed Journals)

published

(1) Egger B*, Klaefiger Y*, Theis A and Salzburger W (2011) A sensory bias has triggered the evolution of egg-spots in cichlid fishes. *PLoS ONE* 6(10): e25601.

(2) Theis A, Salzburger W and Egger B (2012) The function of anal fin egg-spots in the cichlid fish *Astatotilapia burtoni*. *PLoS ONE* 7(1): e29878.

(3) Egger B, Kläfiger Y, Indermaur A, Koblmüller S, Theis A, Egger S, Näf T, Van Steenberge M, Sturmbauer C, Katongo C and Salzburger W (2014) Phylogeographic and phenotypic assessment of a basal haplochromine cichlid fish from Lake Chila, Zambia. *Hydrobiologia* 748(1): 171-184.

(4) Theis A*, Ronco F*, Indermaur A, Salzburger W and Egger B (2014) Adaptive divergence between lake and stream populations of an East African cichlid fish. *Molecular Ecology* 23: 5304-5322.

(5) Theis A, Bosia T, Roth T, Salzburger W and Egger B (2015) Egg-spot pattern and body size asymmetries influence male aggression in haplochromine cichlid fishes. *Behavioral Ecology* 26(6): 1512-1519.

(6) Boileau N, Cortesi F, Egger B, Muschick M, Indermaur A, Theis A, Büscher HH and Salzburger W (2015) A complex mode of aggressive mimicry in a scale-eating cichlid fish. *Biology Letters* 11: 20150521.

(7) Göppert C, Harris RM, Theis A, Boila A, Hohl S, Rüegg A, Hofmann HA, Salzburger W and Böhne A (2016) Inhibition of aromatase induces partial sex change in a cichlid fish: distinct functions for sex steroids in brains and gonads. *Sexual Development* 10(2): 97-110.

accepted

(8) Theis A, Roth O, Cortesi F, Ronco F, Salzburger W and Egger B. Egg-spot pattern and body size asymmetries influence male aggression in haplochromine cichlid fishes. *accepted in Evolution*

submitted

(9) Indermaur A, Theis A, Egger B and Salzburger W. A fitness benefit for mouth dimorphism in a scale-eating cichlid fish. *submitted to Proceedings of the Royal Society B*