



Signal and preference divergence among populations of the non-endemic basal Lake Malawi cichlid fish *Astatotilapia calliptera* (Perciformes: Cichlidae)

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In Lake Malawi and Lake Victoria, cichlid fishes have diversified into hundreds of species, many reproductively isolated by mate choice. Territorial males tend to be more aggressive to similar-coloured males, facilitating coexistence of divergent colour morphs or species. Behavioural mate choice and aggression biases of species and allopatric populations of specialized rocky shore cichlids are influenced by divergent signals such as male colour. Believed to be basal to the Lake Malawi haplochromine radiation, and inhabiting shallow weedy areas of the lake and neighbouring water bodies, *Astatotilapia calliptera* also shows allopatric variation in colour. Here, it is demonstrated that such signal divergence is associated with tendencies of females to mate with males of their own population and also for males to preferentially attack males of their own population, indicating that preference divergence related to signal divergence in allopatry may have operated throughout the adaptive radiation of the Malawian cichlids. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, **110**, 180–188.

ADDITIONAL KEYWORDS: aggression – allopatric divergence – Malawi – mate preference.

INTRODUCTION

Haplochromine cichlid fish from the East African Great Lakes have been studied extensively because of their exceptional morphological and ecological diversification and their species richness. Although natural selection and ecological diversification are thought to play a major role in driving diversification after the invasion of a new habitat (Coyne & Orr, 2004; Baker, 2005; Turner, 2007), sexual selection acting on signal traits may also influence speciation and coexistence of species in sympatry (Seehausen *et al.*, 1998; Seehausen & Schluter, 2004; Kaneshiro, 2006). Among the many sympatric species of cichlid fish found on the rocky shores of these lakes, some share many aspects of their basic ecology, such as grazing on and sheltering and breeding among the algae-covered rocks (e.g. Lake Malawi mbuna, Genner & Turner, 2005). Closely related, ecologically similar sympatric species have been shown to be capable of

producing viable hybrid offspring in laboratory no-choice conditions, but are naturally isolated by female preference for mating with conspecific males (Seehausen, 1997; Knight *et al.*, 1998; van Oppen *et al.*, 1998; Seehausen *et al.*, 1998). This is often associated with distinctive male courtship signals, such as colour (Seehausen & van Alphen, 1998; Couldridge & Alexander, 2002; Jordan *et al.*, 2003; Jordan, 2008). Many cichlid populations differ from allopatric populations in traits, such as male colour, reminiscent of the differences among sympatric species (see Konings, 2007). There is no clear-cut rule for assigning species status to allopatric populations. African lake cichlids are no exception: whereas some genera contain many allopatric ‘species’ largely delimited by colour, others contain fewer species, each comprising numerous, often distinctive, geographical variants or races (Turner *et al.*, 2001; Genner *et al.*, 2004, sometimes referred to as ‘morphs’, e.g. Salzburger *et al.*, 2006). Laboratory experiments have shown that some of these allopatric populations have divergent mate preferences leading to partial reproductive isolation (Seehausen, 1997; Knight &

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Turner, 2004; Salzburger *et al.*, 2006; Egger *et al.*, 2008; Egger, Mattersdorfer & Sefc, 2010; Pauers, Ehlinger & McKinnon, 2010).

Female mate preference is not the only means whereby sexual selection may favour divergence in male colour; differences between closely related species may also reduce aggression between heterospecifics in intrasexual encounters (Andersson, 1994). This may benefit individuals not only through reducing their frequency of being attacked, but also through a reduction in the energy they expended attacking individuals that are not in direct competition for mates. Many cichlid species are highly aggressive and males defend territories which may often be prerequisite for breeding. Aggression biases towards males of the same species or colour morphs were shown for Lake Victoria cichlid fishes (Dijkstra, Seehausen & Groothuis, 2005) and it was suggested that this may facilitate species coexistence or even sympatric speciation (Mikami, Kohda & Kawata, 2004; van Doorn, Dieckmann & Weissing, 2004). With Lake Malawi cichlids, there have been demonstrations that aggressive competition is greatest between conspecifics (Genner, Turner & Hawkins, 1999; Pauers *et al.*, 2008), that similar-looking species interact more aggressively than visually different species (e.g. Pauers *et al.*, 2008; Young, Whitman & Turner, 2009), and that coevolved species compete less than those brought into secondary contact (Young *et al.*, 2009).

In summary, it seems that there will often be a preference by females to mate with males of their own population or species. Males will tend to attack similar-looking males, which will generally be those of their own species or population. Thus, male signal trait differentiation may facilitate coexistence of closely related or incipient species through assortative mating and reduced aggressive competition (Seehausen & Schluter, 2004).

To date, studies of population divergence of mate preferences and aggression biases have been carried out on members of clades of specialized rocky-shore species that are believed to have arisen relatively recently within the lake radiations (Moran, Kornfield & Reinthal, 1994; Day, Cotton & Barraclough, 2008). This leaves open the possibility that divergence of mating signals and preferences may play a significant role in speciation only in such specialized forms. Indeed, it has been proposed that African cichlid radiations follow a three-phase model, initially diversifying on the basis of gross habitat preferences, then on fine-scale trophic adaptation and only later through divergence in sexual signals and preferences (Danley & Kocher, 2001). Here, we carry out similar behavioural preference experiments with populations of the widely distributed *Astatotilapia calliptera*

(Günther 1894). This species is believed to be the sister group of the Lake Malawi haplochromine radiation as a whole, although another interpretation is that it may have invaded the lake on a number of occasions, giving rise to different radiating subclades, which in turn may have later undergone partial or complete introgression within the lake (Joyce *et al.*, 2011).

METHODS

STUDY SPECIES

Astatotilapia calliptera is one of the few species found in Lake Malawi that is also found in the surrounding water bodies (Konings, 2007) – streams, swamps, ponds, and along the margins of rivers and smaller lakes. Related or conspecific populations are also found in the catchments of rivers flowing into the Indian Ocean, including the Zambezi and Rovuma (Joyce *et al.*, 2011). In this study, three allopatric populations were used for the investigation of female mate preference and male aggression biases. A population from the vicinity of Salima was used as a representative of the common colour variant from the main lake, in which territorial and courting males are bright yellow. A second population with similar colour was collected from Lake Chilingali – a satellite lake 11.5 km from the nearest shoreline of Lake Malawi. Males of most known populations of this species share this yellow breeding colour, but those of the third study population are blue-grey: these came from Chizumulu Island, an offshore island in the middle of Lake Malawi. All Lake Chilingali fish were wild caught, but individuals from Salima and Chizumulu were a mixture of wild caught and laboratory bred fish.

GENERAL MATE CHOICE EXPERIMENTAL METHODS

Within 2-m-long aquaria connected to a recirculating system maintained at 25 °C, male territories of 300 × 300 mm were bounded by plastic mesh grids or 'partial partitions' (Turner *et al.*, 2001), preventing the larger males from fighting and interfering with each other, while allowing the smaller females to enter and leave freely, and allowing full sensory contact during spawning with chosen males. These fish are maternal mouthbrooders, females pick up eggs immediately after spawning and brood the offspring in their mouths for around 3 weeks. In our experiment, the floor of each male compartment was made of a mesh grid (10 × 10 mm), which meant that before females could pick them up, most eggs fell through the floor into an inaccessible underfloor chamber. The front wall of this chamber was made of transparent plastic, allowing us to determine when

eggs had been laid, remove and count them, thereby assigning female preference among the candidate males. Experimental tanks contained plastic plants to provide some cover for the females and from external disturbances. Fish were fed twice a day on a varied diet of flake, frozen daphnia, and pea and prawn mix. When not being used in the experiment males were kept in individual compartments, with visual contact with other males from the same population; females were returned to mixed-sex, single-population stock tanks.

THREE-POPULATION THREE-WAY MATE CHOICE

Females of one population at a time were given the choice of spawning with one male from each of the three populations. Eight males of each population were used, making eight unique sets. Multiple females (of one population) were left in the tanks, until a spawning occurred, whereupon preference was scored and all of the males were changed. This was repeated until one female from each population had spawned with each set of males. A total of 24 spawnings were scored (from eight females of each population) over a 7-month period. Females were not removed after they had spawned because we could not always tell which ones had spawned and experience suggested that if female numbers were reduced, female–female aggression might result in excessive harassment of the weaker fish. At the beginning of the experiment all males were weighed and measured, ranging from 79 to 105 mm SL. We attempted to size match them within sets, generally keeping differences to within 5 mm and 5 g, but with a maximum difference of 26 mm and 13.48 g between males. Each time the males were changed each population occupied a different compartment.

TWO-POPULATION SIX-WAY MATE CHOICE

Multiple paternity proved to be fairly common in the first experiment: half of the spawning events involved more than one male. Perhaps females have a requirement for polyandrous mating that masks any tendency to prefer males of their own population, when there is only a single male of each population available? Then, if females were given a chance to mate polyandrously with males of their own population, the level of population-specific mating may be higher. Thus, females were given the choice of six males at a time, three from each of the two populations. Multiple females of a single population at a time were left in the tanks, and again, once a spawning occurred the males were changed with populations assigned to a new chamber. This was repeated until one female from each population had spawned with each set of

males. Males were weighed and measured before each use. Due to the large number of males needed for each trial, size matching all the males of each set was not possible. As a result of the first set of experimental results, in this experiment, only two out of the three possible population pair were tested. In all cases one of the males was of the widespread yellow phenotype, from Salima on the shore of the main Lake Malawi. The other male type was either blue from Chizumulu Island or yellow from Lake Chilingali. In each case, the females used were either from Salima or the other population used in the trial. The expectation from this second experiment was therefore an increase in preference for own-population males, when compared with the first experiment. For the choice between different-coloured males, 17 Salima and 20 Chizumulu males were used. Six sets of males were used for six spawnings by Salima females (individual males re-used 0–3 times) and 12 sets of males were used for 13 spawnings by Chizumulu females (individual males re-used 0–5 times). For the choice between same-coloured males, 16 Salima and 17 Chilingali males were used. Eight sets of males were used for eight spawnings by Salima females (individual males re-used 0–3 times) and seven sets of males were used for eight spawnings by Chilingali females (individual males re-used 0–3 times).

MALE AGGRESSION EXPERIMENT

Pairwise simulated intruder choice tests were carried out in two replicate sets of aquaria, each consisting of a central 30-litre tank (490 × 430 × 140 mm) with a refuge (plastic pipe) for the focal fish, with adjacent tanks (200 × 430 × 140 mm) for the stimulus fish. All tanks were part of a large re-circulating system, but each with its own inflow and outflow, preventing direct water exchange between the focal and stimulus. Focal and stimulus fish were separated by a 5-mm-thick non-perforated glass partition glued in place with silicone sealant, eliminating olfactory communication and probably attenuating auditory signals, but allowing transmission of visual cues (i.e. colour and shape and behaviour). Each focal male was tested against two sets of stimulus fish from the same pair of populations; intruder populations were presented on alternate sides to eliminate any possible side bias. Aggressive or display behaviours (lunge/butt, bite, frontal display, lateral display, quiver) directed towards each stimulus fish were recorded during a 5-min observation period. Recording began after the focal male had emerged from the central territory and reacted to both of the stimulus fish. To avoid pseudoreplication, scores from the two trials were averaged before analysis, giving one result for each focal male. Preliminary analyses indicated that

frequencies of individual behaviour types showed no particular pattern and subsequent analyses were carried out on the sum of all aggressive and display behaviours per trial.

We had already analysed the mate choice experiment and shown that females of the Salima/Chilingali populations, with similar-looking yellow males, showed no significant preferences among these populations (see Results). Thus, the experiment was carried out with males of the Salima (yellow) and Chizumulu (blue) populations only. Thirteen stimulus pairs, comprising one male of each population, were used to test aggression biases of 12 focal males of each population. Each pair was used as a stimulus twice, apart from two pairs that were used once each.

DATA ANALYSIS

Analyses were carried out on the number of eggs laid with different males during the mate choice experiments and the total number of aggressive behaviours performed towards the stimulus males in the aggression experiments. All data were tested for deviations from homoscedasticity and normality before parametric tests were carried out. If the data violated these assumptions, transformations were attempted; if these failed to rectify the problem, non-parametric equivalents were used. For the three-way female mate choice trials, preference by females from each population was tested using a three-way Friedman test. Where a significant difference in the number of eggs laid with males from the different populations was observed, post-hoc Wilcoxon signed-rank tests were carried out to identify between which populations the preference was significant. Paired *t*-tests were used for the two-population female mate choice trials. Fisher's combined probabilities test was used to test for overall significance across the two experiments. For the male aggression experiment, paired *t*-tests were used to compare the number of aggressive behaviours displayed with the two stimulus types by males of each focal population.

RESULTS

THREE-POPULATION MATE CHOICE

When given the choice of one male from each of the three populations, only Chizumulu females showed a significant preference (Friedman test $N = 8$, $\chi^2 = 7.200$, d.f. = 2, $P = 0.027$), preferring males of their own population over Chilingali (Wilcoxon signed-rank tests $Z = -2.100$, $P = 0.036$) and Salima males ($Z = -2.176$, $P = 0.030$), but not differentiating among the two populations with yellow males ($Z = -0.105$, $P = 0.917$). Females from Salima (Friedman test: $N = 8$, $\chi^2 = 3.769$, d.f. = 2, $P = 0.152$) and

Chilingali ($N = 8$, $\chi^2 = 1.500$, d.f. = 2, $P = 0.472$) did not show a significant preference, although they laid more eggs with males from their own populations (Fig. 1A).

TWO-POPULATION MATE CHOICE

Although there was a slight increase in the mean proportion of eggs laid with own-population males when given the choice between three Salima and three Chilingali males there was still no significant preference shown by Chilingali females (paired *t*-test: $t = -0.359$, d.f. = 7, $P = 0.730$) or Salima females ($t = -1.148$, d.f. = 7, $P = 0.289$; Fig. 1B).

Salima females still showed no significant preference when given the choice between three Chizumulu and three own-population males ($t = 0.208$, d.f. = 5, $P = 0.843$). Although they did lay many more eggs with own-population males, the preference by Chizumulu females was not significant ($t = -1.673$, d.f. = 12, $P = 0.120$; Fig. 1B). However, by combining the probabilities from the two experiments, a significant overall preference for own-population males was found (Fisher's combined probabilities test: $-2\sum \ln P = 11.464$, d.f. = 4, $P < 0.025$).

MALE AGGRESSION EXPERIMENT

Following square-root transformation to eliminate significant heteroscedasticity, territorial males from both Salima (paired *t*-test: $t = 3.622$, d.f. = 11, $P = 0.004$) and Chizumulu ($t = -2.453$, d.f. = 11, $P = 0.032$) showed a significant tendency to bias aggressive behaviour towards males from their own population in dyadic simulated intruder choice tests (Fig. 1B).

DISCUSSION

Allopatric populations of *Astatotilapia calliptera* sometimes differ in male courtship colour. We have shown that this can be correlated with divergent mate preferences by females and aggression biases by males. Thus, it seems that the potential for divergence in signals and behavioural responses is present in the generalized riverine fishes that are believed to represent the sister group of the specialized Lake Malawi endemic haplochromines, and so this process may have facilitated the divergence of cichlid species throughout this and other adaptive radiations.

Based on morphology, biogeography and mitochondrial DNA sequences, *A. calliptera* has long been proposed as a candidate sister species for some or all of the Lake Malawi haplochromine radiation (Meyer, 1993; Moran *et al.*, 1994). The situation has been complicated by later molecular studies (Shaw *et al.*,

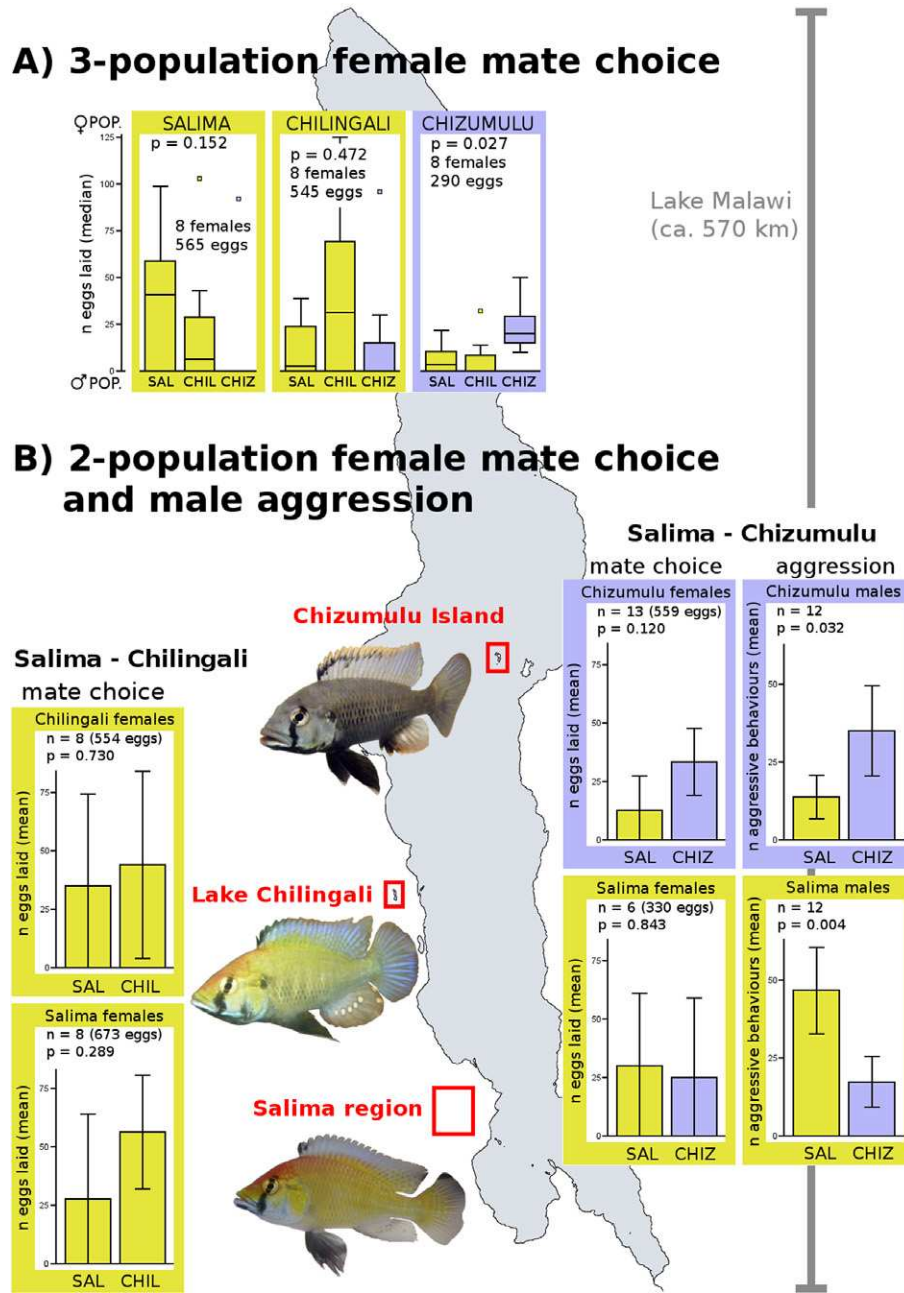


Figure 1. Map of Lake Malawi with locations of *Astatotilapia calliptera* study populations and results from female mate choice and male aggression experiments. The y-axes show either number of eggs laid with males in the female mate choice experiments or number of aggressive behaviours towards stimulus males in the males aggression experiments. A, when given the choice between males from three different populations, Chizumulu females showed a significant (Friedman test and post-hoc Wilcoxon signed-rank tests) preference for males from their own population. Salima and Chilingali females showed non-significant (Friedman tests) trends towards preference for males from their own populations. Charts show median number of eggs laid and error bars show inter-quartile range. B, when given the choice between males from two populations, with multiple males from each population present, there was still no significant preference between Salima and Chilingali (paired *t*-tests). Despite the observation that, on average, Chizumulu females still laid many more eggs with own-population males compared with other-population males, there was also no significant preference between the Salima and Chizumulu populations (paired *t*-tests). However, Fisher’s combined probabilities test shows an overall preference, by Chizumulu females, when the two experiments are pooled. Males from the Salima and Chizumulu populations both showed a significant tendency to preferentially attack males from their own population (*t*-tests). Charts show mean number of eggs laid or number of aggressive behaviours per 5-min trial and error bars show 95% confidence intervals.

2000; Seehausen *et al.*, 2003; Joyce *et al.*, 2011; Genner & Turner, 2012). The most comprehensive recent study suggests that allopatric populations of this species harbour diverse mitochondrial lineages, often apparently fixed and indicating recent gene exchange with sections of the endemic haplochromine radiation. Largely nuclear amplified fragment length polymorphism (AFLP) markers cluster all populations together and place them basal to the Lake Malawi endemic haplochromine radiation (Joyce *et al.*, 2011). However, a single specimen incorporated into another recent phylogenetic study showed the reverse, being placed within the endemic radiation by AFLP but basal by mitochondrial sequence analysis (Genner & Turner, 2012). What is clear is that *A. calliptera* is genetically close to the Malawi endemic haplochromines and that it is found not only in shallow weedy areas along the lake shore but also in ponds, small lakes, and river margins in the Lake Malawi catchment and also much more widely in south-east Africa. These observations are consistent with the proposal that *A. calliptera* can be regarded as the best available model for the ancestor of the Lake Malawi radiation.

Our study considered yellow and blue male colour forms. These may be analogous to the thoroughly studied blue and red sympatric endemic haplochromines (*Pundamilia*) from Lake Victoria (Seehausen, 1997; Seehausen & van Alphen, 1998; Seehausen *et al.*, 1998; Seehausen & Schluter, 2004) and to similar forms among the *Pseudotropheus zebra* complex from Lake Malawi (Knight *et al.*, 1998; van Oppen *et al.*, 1998; Knight & Turner, 2004). Complete or partial assortative mating has also been demonstrated among geographically isolated populations of specialized rocky shore endemics in Lake Tanganyika (Egger *et al.*, 2008; 2010). Studies on such endemic rocky shore specialists have led to proposals that sexual selection acting on male signal traits, most obviously courtship colour, has probably played a major role in the adaptive radiation of cichlid fishes (Seehausen, 1997; Knight *et al.*, 1998). Blue and yellow sympatric colour morphs are also known in *Astatotilapia burtoni* from the Lake Tanganyika catchment, where they seem to represent morphs within a single gene pool (Fernald & Hirata, 1977). Similar forms are reported to be distinct species of the *Astatotilapia pseudopaludinosus* complex in Lake Rukwa (Seegers, 1996), although molecular and behavioural studies are lacking.

Thus, our study represents the first published demonstration of behavioural correlates of population differentiation in colour among 'riverine' haplochromine cichlids, such as *Astatotilapia*. While we cannot claim that these populations represent the unchanged ancestors that originally colonized Lake Malawi, they

are probably the nearest proxy available. These findings could at least suggest a greater scepticism may be warranted about the widely accepted three-phase model of cichlid radiations (Danley & Kocher, 2001). That model is based on a molecular phylogeny indicating that the deepest splits within the Lake Malawi radiation are between groups where most taxa live on different habitats (e.g. rock-dwellers vs. sand-dwellers), that the next level of splits are generally among genera of differing feeding habits and associated trophic morphology, and that the most recent splits are among ecomorphologically similar congeners that differ in signal traits such as male courtship colour. From this, it is deduced that three separate speciation processes must have dominated the radiation in the same sequence: habitat differentiation, trophic differentiation, and signal differentiation. However, our results suggest that perhaps riverine haplochromines sometimes possess the potential to undergo speciation by divergence in signal traits associated with sexual selection. This implies an alternative explanation of the phylogenetic evidence: that haplochromine speciation has always been largely associated with signal divergence. It is possible that either divergence in ecomorphological traits and habitat preferences are slower processes gradually accumulating in specialized lineages long after speciation, or perhaps more plausibly, that each took place in conjunction with signal divergence, as suggested by the sensory drive speciation model of Seehausen and colleagues (Seehausen *et al.*, 2008).

Observations from a diverse range of taxa, including invertebrates (Hawaiian *Drosophila*, Kaneshiro, 2006) fish (sticklebacks *Gasterosteus*, Rafferty & Boughman, 2006; swordtails *Xiphophorus*, Ryan & Wagner, 1987), and mammals (pocket gophers *Geomys*, Bradley, Davies & Baker, 1991), suggest that signal divergence in allopatry may not always occur along with corresponding divergence in behavioural preference. Often, females of ancestral species/populations discriminate against males of more derived species/populations, while females from derived species/populations mate readily with males from ancestral species/population. Our results indicate the opposite.

The blue colour of the territorial male appears to be unique to the narrowly endemic Chizumulu population, and so is likely to be a derived trait. Thus, it seems that in *A. calliptera*, females of the derived phenotype have population-specific preferences, while females of the more ancestral form mate randomly with regard to male colour. Asymmetries in mate preferences among species of Hawaiian *Drosophila* tend to stem from differences in courtship behaviour. It has been suggested that the acceptance of a simplified display and relaxation of sexual selection may

accompany founder events because successful establishment of the new population is likely to be enhanced when the behavioural pattern is simplified (Kaneshiro, 2006). In the case of haplochromine cichlids male behavioural courtship signals remain conserved throughout even the most distantly related taxa (McElroy & Kornfield, 1990). These differences between previously observed patterns of asymmetries and the patterns found in this study could be down to the reasons for the asymmetries. Rather than being based on divergence of behavioural courtship displays and the acceptance of simplified displays in derived populations they could also be due to divergence of a single (colour) rather than multiple (e.g. colour and behavioural display) signals. It is often differences in courtship displays that maintain reproductive isolation between species that would show asymmetric isolation if choice was based solely on the presence or absence of single ancestral traits (e.g. Ryan & Wagner, 1987; Rafferty & Boughman, 2006).

The reason for allopatric colour and preference divergence remains unclear: there are no reported field studies of these populations. Candidate explanations could include differences in abiotic environmental factors such as the visual environment, differences in fish community compositions, or different histories of sexual selection perhaps triggered by mutation or founder events in the geographically isolated Chizumu population.

Females from the population with males of the probably derived colour prefer males of their own population, indicating a possible early stage in speciation. However, this colour difference appears to make no difference to the attractiveness of these males to females of the ancestral and most widespread form. Due to the lack of complete reproductive isolation between these allopatric populations, the result of secondary contact would probably be introgression. Speciation of the study populations would only be possible if further divergence were to take place: for example, if blue males became sufficiently different in colour or some other trait that the females of the populations with yellow males no longer accept them.

The ancestor of the Lake Malawi haplochromine radiation may have passed on the propensity for divergence of colour and corresponding preferences to the species-rich lacustrine taxa. The haplochromine cichlids are thought to have originated in Lake Tanganyika and spread through river systems to the younger lakes, as they formed, where they then radiated within the newly found lacustrine environments (Salzburger *et al.*, 2005). Haplochromine flocks make up the majority of species in Lakes Malawi and Victoria (Meyer, 1993; Moran *et al.*, 1994; Turner, 2007). Similar divergences in traits and preferences may not

be limited to the ancestor of the Lake Malawi radiation and the species flock that has arisen from it, but rather be part of the 'key innovations' (traits present in invading ancestral lineages) that have played a role throughout the diversification of lacustrine cichlids and allowed lacustrine radiations to become so speciose. Sexual dimorphism and maternal mouthbrooding allow great potential for speciation by sexual selection and colour divergence when specialization to patchy lacustrine habitats prevents gene flow between populations distributed around a lake. However, generalist species may not realize this potential as easily as their specialist endemic descendants which more readily become isolated due to specialization to patchily distributed habitats.

CONCLUSIONS

Patterns of evolution involving allopatric colour divergence, partial reproductive isolation, and a tendency for males to preferentially attack other males of the same colour are found in endemic specialist cichlid fishes in different lakes. We have demonstrated similar patterns among populations of the non-endemic Lake Malawi lineage *Astatotilapia calliptera*, suggesting that similar processes may have acted throughout the history of the lacustrine radiations, rather than being confined to a 'third phase' after initial divergence due to habitat and dietary specialization, as proposed in an influential model.

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