

Peripheral Isolate Speciation of a Lake Malawi Cichlid Fish from Shallow Muddy Habitats

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Abstract Much of the exceptional diversity of cichlid fishes in the African Great Lakes can be explained by geographic variation among isolated populations of species specialised to live on patchily distributed rocky habitat. However, there are also many endemic species that are not specialised for rocky shores. These appear to experience weaker geographic isolation. Major decreases in lake volume may have segregated such populations in isolated refugia in the distant past, but subsequent range changes have likely eliminated most of the phylogeographic signal of these events. Divergence in currently isolated peripheral water bodies may be more amenable to the study of recent processes of allopatric divergence. We investigate a haplochromine cichlid fish, here referred to as *Lethrinops* sp. ‘chilingali’, isolated in a small satellite lake near Lake Malawi, and the candidate sister taxon, *Lethrinops lethrinus*, which inhabits shallow muddy habitats in the main lake and associated water bodies. The satellite lake form from Lake Chilingali showed significant morphological differentiation, with a less ventrally-placed mouth and shorter snout, associated with a shift in diet from a diverse

range of benthic invertebrates towards specialisation on mid-water chaoborid larvae and pupae. The Lake Chilingali population showed substantially reduced mitochondrial DNA diversity and no haplotype sharing was observed with populations from the main lake system. In laboratory experiments, putative species showed a high degree of assortative mating and territorial males were significantly more aggressive towards intruders of their own population. This study adds to the evidence that rapid evolution of novel phenotypes in peripheral habitats can add to the diversity of lacustrine cichlids through the evolution of at least partial reproductive isolation in allopatry.

Keywords Aggression · Allopatric · Cichlid · Lake Malawi · Mate choice · Peripheral isolate · Speciation

Introduction

Geographical separation of populations is important for divergence and speciation (Mayr 1963; Rice and Hostert 1993; Barraclough and Vogler 2000; Coyne and Orr 2004; Dawson and Hamner 2008); reproductive isolation may develop as a by-product of the genetic differences that accrue during divergent adaptation to local environments and genetic drift that occurs after the division of a population by geological barriers that prevent gene flow (Mayr 1963; Rice and Hostert 1993; Coyne and Orr 2004). In Lakes Malawi, Tanganyika and Victoria, cichlid fish have diversified into over 1,000 endemic species (Genner et al. 2004). Most research has focussed on species specialised to live on shallow rocky habitats, which are patchily distributed within each lake. These rock specialist exhibit high habitat fidelity, lack a dispersal phase, and show strong

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population genetic differentiation. On the basis of phenotypic divergence, particularly in male colour, numerous local endemic forms have been identified and variously classed as species or geographic colour variants (Ribbink et al. 1983; Genner and Turner 2005). The presence of so many geographic variants indicates a potential for intralacustrine allopatric speciation, which is sometimes regarded as the likely dominant mode of speciation in endemic cichlid species in the larger lakes (but see, for example, Seehausen and van Alphen 1999).

A review of the taxonomic status of all reported cichlids from these three lakes suggests that the number of geographically variable rock-restricted taxa is collectively of the order of 185–445, leaving some 870 species which are either habitat generalists or adapted to non-rocky habitats (Konings 2007). Within Lake Malawi, non-rock restricted species show little indication of geographic phenotypic variation and little or no population structuring over large geographic distances (Genner et al. 2007; Pereyra et al. 2004; Shaw et al. 2000; Taylor and Verheyen 2001; but see Anseeuw et al. 2008). Therefore, for the majority of species that are not tightly associated with rocky shores, alternative speciation models must be considered. One such possibility is peripatric or peripheral isolate speciation, which may occur when a small population becomes isolated in a habitat island at the margin of the main range of the species (Barraclough and Vogler 2000; Stuart et al. 2012). It has been proposed that peripheral isolation may increase the probability of speciation via several mechanisms. There may be a greater reduction in gene flow due to impassable, rather than inhospitable, barriers that prevent dispersal. There may also be greater opportunity for ecological specialization resulting from a lack of competition. Additionally, a greater degree of habitat differentiation could lead to a faster rate of neutral genetic change in smaller populations, particularly in the early stages of establishment via founder effects. For aquatic organisms, isolated water bodies have been proposed to act in a similar manner to islands for terrestrial organisms (Dawson and Hamner 2008), promoting molecular and morphological divergence among taxa that normally have high dispersal capacity and weak population structuring, such as pelagic Lake Malawi cichlid fish in a satellite lake (Genner et al. 2007) or jellyfish confined to marine lakes on the island of Palau (Dawson and Hamner 2005).

Lake Nabugabo, a small satellite lake near Lake Victoria, has long been known to contain endemic haplochromines closely related to those of the main lake (Trewavas 1933) and it has been proposed that allopatric speciation in this and similar lakes, such as Lake Kanyaboli, may have played a major role in the radiation of Lake Victoria cichlid fishes (Brooks 1950; Greenwood 1965; Stager et al. 2005; Odhiambo et al. 2011, 2012). We

are unaware of any test of reproductive isolation between the Nabugabo/Kanyaboli and Victoria species; however, reproductive isolation by assortative mating among size-matched individuals was shown in laboratory trials of the pelagic Lake Malawi haplochromine *Rhamphochromis longiceps* and a morphologically similar form apparently endemic to the small satellite Lake Chilingali (Genner et al. 2007). In contrast, *Astatotilapia calliptera* from Lake Chilingali showed no significant assortative mating when tested against fish from the main lake (Tyers and Turner 2013). This species is very closely related to the Malawian endemic haplochromines, but is also found in lakes and river systems not currently connected to Lake Malawi (Joyce et al. 2011).

Here we aim to investigate morphological, ecological, genetic and behavioural divergence between a benthic haplochromine cichlid *Lethrinops lethrinus* and a morphologically similar taxon from Lake Chilingali. Along with the *Rhamphochromis*, this *Lethrinops* appears to be the only other representative of a genus endemic to Lake Malawi that has been recorded from this satellite lake. If main lake fish and their satellite lake counterparts from both of these genera were to show significant genetic and morphological differentiation as well as a high level of assortative mating, this would add to the evidence that ecologically driven allopatric divergence of lacustrine endemics in peripheral water bodies has played a role in the evolution of biological diversity and speciation of cichlids.

Methods

Study Species and Populations

The genus *Lethrinops* is endemic to the catchment of Lake Malawi: the last taxonomic review listed 24 species (Eccles and Trewavas 1989), but at least 66 are informally recognised (Konings 2007). Mitochondrial DNA studies do not support monophyly (Turner et al. 2004). One group of species, mainly inhabiting shallow water, appears to belong to the large clade of benthic species which are mostly habitat generalists or adapted to sandy or muddy habitats, variously known as ‘non-mbuna’ (Shaw et al. 2000), ‘utaka’ (Sturmbauer et al. 2001) or ‘benthic’ (Genner and Turner 2012). Other species, mainly from deeper water, are placed in a different clade, dominated by the rocky-shore mbuna species, but also including deep-water or crepuscular species of the genera *Alticorpus* and *Aulonocara* and some of the non-monophyletic genus *Placidochromis*. These groups are however generally morphologically similar and are closely related based on nuclear markers (Loh et al. 2008; Genner and Turner 2012). The mito-nuclear discordance may therefore indicate hybridisation

between two groups of habitat specialists (mbuna and sand-dwellers) during the emergence of the dark-adapted bentic group, rather than being an indication of phylogenetic relationships (Genner and Turner 2012). *Lethrinops lethrinus* is distinguished from other species of the genus by the distinctive melanin pattern seen in females and immature or non-territorial males: a horizontal stripe along the mid-line of the flank with a row of blotches, sometimes partially fused into a stripe, roughly half way between the horizontal stripe and the upper surface of the body (Fig. 3a). Species of the genus *Protomelas* have similar markings, but have a different arrangement of teeth in the lower jaws (Eccles and Trewavas 1989). Thus, unusually for a Lake Malawi haplochromine cichlid fish, *L. lethrinus* is a relatively distinctive species. It has been reported as frequently occurring in shallow vegetated areas and the mouths of inflowing rivers, but may also be found to depths of 34 m in some areas (Eccles and Trewavas 1989). *Lethrinops lethrinus* is also known from Lake Malombe and the Upper and Middle Shire River (Turner 1996). Konings (2007) states that the species has a lake-wide distribution and there are reports of collections from as far north as Nkhata Bay (Ngatunga 2000). Like other haplochromine cichlid fishes, *Lethrinops lethrinus* is a maternal mouthbrooder and shows strong sexual dimorphism in size, colour and behaviour. Males attain lengths of 160 mm SL, are more brightly coloured (Fig. 3a) and are territorial during the breeding season, building large, rather complex, bowers out of soft muddy sand (Konings 2007). Otherwise, the fishes are drab (Fig. 3a) and live in loose shoals foraging in soft, muddy sediments. Limited investigations of stomach contents suggest a diet of chironomid larvae and copepods (Turner 1996; Ngatunga 2000), at least sometimes mixed with detritus and sand (Turner 1996), which is consistent with benthic foraging. No geographic variation in morphology or colour has been noted; statements to this effect (e.g. Eccles and Lewis 1978) appear to have been due to confusion of specimens with those of *Lethrinops leptodon* (Ngatunga 2000). For the present study, specimens of *L. lethrinus* were obtained from the south eastern arm of Lake Malawi and the nearby Lake Malombe, which is connected to the south of the main lake by a short stretch of deep, slow-flowing river known as the Upper Shire.

During surveys in 2004, specimens of a fish very similar to *L. lethrinus* were obtained from Lake Chilingali (12°57'46"S, 34°12'49"E), a small (ca. 5 × 1 km with a maximum depth of 5.1 m) satellite lake located 11.5 km from the western shore of, and approximately 30 m above the current level of, Lake Malawi. The water levels in the Great Lakes have fluctuated over time, and there is evidence to show that they have been both lower (Sturmbauer et al. 2001) and higher (Dixey 1927; van Bocxlaer et al. 2012) than their present day levels. Given the proximity of

Lake Chilingali to Lake Malawi, it is therefore considered to be not unlikely that at one time, during a high water period, Lake Chilingali existed as a lagoon similar to those found along the lake shore today (e.g. Chia to the south and Unaka to the north). Currently, there is a seasonal outflow which drains into Lake Malawi through the surrounding rivers, but no substantial inflow (Genner et al. 2007). Females and immature or non-territorial males of *Lethrinops* sp. 'chilingali' have a similar melanin pattern to *L. lethrinus*, but the mid-line stripe is generally discontinuous (Fig. 3a). The stripe pattern is not visible in territorial or courting males of either form.

Specimens of *Lethrinops* sp. 'chilingali' were collected from local fishers in 2010; live fish were transported to the holding facility of an aquarium fish exporter and supplemented by later visits to Lake Chilingali by their catching team. Other fish were euthanised by MS222 anaesthetic overdose and fixed in formalin for later morphological analysis after washing and transfer to 70 % ethanol or dissected for analysis of stomach contents (stored in ethanol). Morphological samples of *L. lethrinus* for quantitative study for this project were obtained from local fishers in Lake Malombe (14°37'36"S, 35°15'6"E). Comparisons with *L. lethrinus* material from other locations, from sampling expeditions for previous projects and from existing museum collections, indicated that Lake Malombe *L. lethrinus* were representative of the species throughout its range. Tissue samples for molecular analysis were obtained by clipping the fins of euthanised fish and storing these in ethanol. For aquarium experiments, live individuals of *L. lethrinus* were obtained from an aquarium fish exporter, who gave the collecting location as Mazinzi Reef in the south eastern arm of Lake Malawi.

Morphological Analysis

Thirty alcohol-preserved individuals from each population (Lake Chilingali and Lake Malombe) were photographed against a standard background with a scale for calibration. Images were converted to the appropriate data format (tps files from jpeg files) using Tpsutil (Rohlf 2004). TpsDig (v.1.37, Rohlf 2001) was used to map landmarks, 21 for each individual (Fig. 1), and digitised using the generalised least squares method (Rohlf and Slice 1990). Thin plate spline interpolation was used to visualise shape differences on deformation grids using TpsRelw (v.1.31, Rohlf 2003). Relative warp scores generated on TpsRelw were converted using MODICOS software (Carvajal-Rodríguez and Rodríguez 2005) into a format that could be used in Microsoft Excel and SPSS. Levene's test did not indicate a significant deviation from homoscedasticity and statistical comparisons of populations on relative warp scores were carried out in SPSS using one-way analysis of variance.

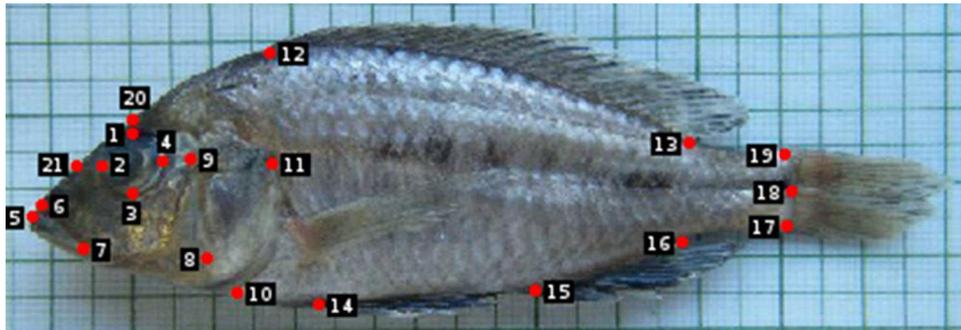


Fig. 1 Landmarks used for geometric morphometric analysis: 1–4 upper, anterior, lower and posterior point of eye; 5 tip of rostrum; 6 tip of upper lip; 7 posterior tip of upper jaw; 8 most posterior and ventral extent of gill cover; 9 origin of gill cover; 10 base of isthmus; 11 most posterior part of operculum; 12, 13 beginning and end of

dorsal fin; 14 anterior origin of pelvic fin; 15, 16 beginning and end of anal fin; 17–19 lower, mid and upper point of caudal peduncle; 20 point directly vertical from point one at limit of head; 21 point directly parallel to point 2 on the front of the snout (20 and 21 show the slope of the head)

Dietary Analysis

Stomach content analysis was carried out on 51 *L. sp.* ‘chilingali’ individuals, some of which were the same individuals used for the morphological analysis, and the rest were caught during the same sampling trip. These specimens ranged from 18 to 110 mm SL and were divided into three size classes of 17 individuals each: small (18–35 mm SL), medium (40–55 mm SL) and large (56–110 mm SL). Volumes of items were estimated for a subsample of at least 10 individual items and used to extrapolate estimated percentage volumes for each individual. Lake Malawi *L. lethrinus* dietary data were obtained from an unpublished PhD thesis (Darwall 2003) based on a large survey of hundreds of species, which was carried out over 2–3 years. It is likely, therefore, that this set of samples contained individuals from several Lake Malawi populations and although detailed information regarding the timings of sampling were not recorded for each species, statistical analysis showed that there was only significant seasonal variation in the diets of species from the zooplanktivorous guild, which does not include *L. lethrinus*.

Molecular Analysis

In total, 61 *Lethrinops* mtDNA sequences (GenBank) from three populations were used for population genetic analysis: two from within the Lake Malawi system (south eastern arm of Lake Malawi and Lake Malombe to the south of Lake Malawi) as well as the isolated Lake Chilingali (Fig. 4a). Tissue samples for DNA extraction for sequencing came either from fish caught for use in other parts of this study, or from fin samples preserved from trawl catches in the South Eastern Arm of Lake Malawi.

DNA Extraction and Sequencing

DNA was isolated from ethanol preserved fin tissue by salt extraction (Aljanabi and Martinez 1997). An approximately 1,000 bp section of the mtDNA control region was amplified using the primers HapThr–2+4 and Fish12S (Joyce et al. 2005). PCR was performed in 25 µl reactions containing 1 µl DNA, 2.5 µl 5× PCR buffer, 2.5 µl dNTPs (1 mM), 1 µl each primer (10 µM), 1.6 µl MgCl (25 mM), 1 unit Taq, 15.2 µl SDW H₂O. PCR conditions were: 1 min at 95 °C, followed by 34 cycles of 30 s at 95 °C, 30 s at 50 °C, 60 s at 72 °C and finally 5 min at 72 °C. Cleaned PCR products were sequenced by MacroGen using the forward primer HapThr–2+4.

Genetic Diversity, Molecular Differentiation and Phylogenetic Analysis

Sequences were checked by eye and edited using Chromas Lite v.2.1.1 (Technelysium). A final alignment of 550 base pairs (including gaps) was prepared using ClustalW in DAMBE v.5.3.15 (Xia and Xie 2001). DnaSP v.5.10.01 (Librado and Rozas 2009) was used to make inferences of haplotype diversity and sharing. Two separate analyses were carried out in Arlequin v.3.5.1.2 (Excoffier et al. 2005): firstly a pairwise comparison to test for significant genetic differentiation between *L. sp.* ‘chilingali’ and *L. lethrinus* from the Lake Malawi system; secondly, an AMOVA, with post-hoc pairwise permutation tests, was carried out to test for significant genetic structure between the three lakes. Invariable sites were removed and sites with gaps not considered. A minimum spanning network (MSP) was created in PopArt v.1 (<http://popart.otago.ac.nz/index.shtml>).

Mate Choice Experiment

Dichotomous female mate choice experiments were carried out in two replicate 2 m long tanks (one for females of each population) to test for female recognition of and preference for mating with males from their own population. Experimental tanks were filtered externally on a recirculating flow-through system maintained at ca. 25 °C. In nature, breeding in *L. lethrinus* is seasonal and takes place on a lek, where males attempt to attract passing females to spawn on their bowers (Konings 2007). Females choose a mate from among the many males available. After spawning, as with many haplochromine cichlids, females brood young in their buccal cavity for approximately 3 weeks. Therefore, in experiments where full contact and spawning was allowed, maternity is always known and paternity may be determined by microsatellite DNA allele sizing of potential fathers and offspring. Pilot studies indicated that, in common with other fishes found in open habitats, such as *Rhamphochromis* (Genner et al. 2007), *Lethrinops* females would not readily pass through mesh barriers to mate with preferred males. Therefore, a partial partition experimental design (Turner et al. 2001) was not possible. However, given large enough tanks, aggression between males was found to be much less of an issue than it would be for mbuna species or *Astatotilapia*. Although there were no physical barriers and full contact was possible between all males and females within the experiment, males were encouraged to occupy one end of the tank each by the use of three offset baffles which divided the tank, preventing visual contact and reducing interference once each male had settled into a territory (Fig. 2). As preliminary observations found that these fish prefer to spawn on raised surfaces, platforms were provided at each end of the tank to act as a focal point for territoriality; these were made from tiles resting on upturned terracotta plant pots. ‘Dither’ fish (*Rhamphochromis* sp.) were also present throughout the experiment, as this was found to help the *Lethrinops* to settle down (i.e. show independent movement and

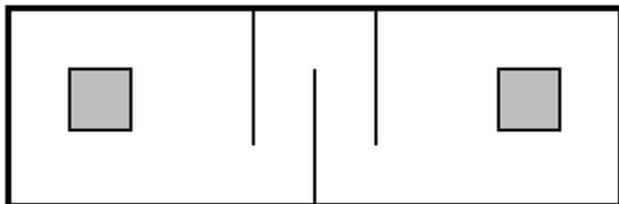


Fig. 2 Plan view of mate choice experimental tank set up. Three offset baffles in the middle of the 2 m long tank separated the tank into two distinct areas to encourage co-existence of two males by reducing visual contact once territoriality had been established. Platforms at each end provided a focal point for territorial behaviour and a raised surface on which to spawn

territorial behaviour, rather than shoaling as a group). Due to the limited availability of wild caught fish, this experiment was carried out with both wild-caught fish ($n = 29$) and 1st generation laboratory-bred fish ($n = 28$), which came from 8 broods (2 Malawi and 6 Chilingali). Wild caught adult male pairs were size matched to within 9 mm/10.5 g (7.5/24.9 % of mean size), while the smaller lab-bred fish were matched to within 4 mm/1.2 g (5.8/13.7 % of mean size). One wild-caught Chilingali male was re-used, paired with a different Malawi male, but otherwise each male was only used once. On two occasions females lost their eggs (probably through ingestion) a day or two after spawning. This is not uncommon in cichlids, even those kept in single species/population stock tanks (pers. obs.). Brood loss was deemed unlikely to be the result of inviable hybrid offspring: in the one case of heterospawning that occurred, offspring were viable. Additionally, viable hybrids are produced by crosses of even distantly related cichlids and have been produced by crossing *A. calliptera* and *L. sp.* ‘chilingali’ in a no-choice situation in mixed species stock tanks (pers. obs.). Females that experienced brood loss were therefore left in the experiment and likely spawned in a later trial with a different pair of males present.

DNA Extraction and Genotyping

Males and females were swabbed for DNA samples. Broods were taken from the mouths of the females approximately 10 days after spawning and euthanised, after which they were preserved in ethanol and tissue samples taken for DNA extraction. Salt extraction was used for all DNA samples. Females were left in isolation after brood removal and swabbed a few days later then returned to mixed-sex stock tanks. Where DNA samples were not available for females, due to some initial difficulties with extraction from swabs, it was still possible to assign paternity to offspring based on male samples alone. DNA samples from fry, potential fathers (and the mother in some cases) were used for assigning paternity to offspring by allele sizing of three microsatellite markers (Ppun5, Ppun7, Ppun21: Taylor et al. 2002), used in a PCR multiplex approach (Qiagen multiplex kit). Genotyping of the amplified samples was carried out on an Applied Biosystems (ABI) 3130xl genetic analyser using LIZ 500(–250) (ABI) size standard. Genotypes were determined manually using the Genemapper software v.4.0.

Data Analysis

Clutch size ranged from 9 to 111 (24–111 for the larger wild caught females and 9–33 for the smaller lab-bred females) and 10 offspring from each brood were genotyped

(apart from one brood which only contained 9 offspring). A total of 219 offspring from 22 (15 Chilingali and 7 Malawi) broods were genotyped. No cases of multiple paternity were detected and it was possible to assign paternity unambiguously in all cases based on at least one of the loci. Whole broods were therefore assigned to a father and a binomial test was used.

Male Aggression Experiment

To test for aggression biases among males of the Lake Malawi and Lake Chilingali populations, pairwise simulated intruder choice test were carried out. Two replicate sets of aquaria were used for this experiment and the males were from the same 1st generation lab-bred stocks that were used in the mate choice experiment. These mate choice and aggression experiments were run concurrently and the timing of usage of males in each was therefore haphazard. In total, the aggressive behaviours of eleven focal/territorial males of each population were recorded using eleven stimulus pairs, comprised of one male of each population. Males were tested one at a time. The ‘resident’ or focal male inhabited a 0.9 m long free-standing tank with an internal heater, air-driven box filter, a central plant pot to act as a refuge and gravel as substrate. Within this tank, smaller glass tanks (measuring 0.3 m × 0.2 m) were placed, one at each end, to hold the ‘intruder’ or stimulus fish. These internal glass tanks had their own filtration and water was not allowed to mix between these chambers and the main tank, eliminating olfactory communication and probably attenuating auditory signals, but allowing transmission of visual cues (i.e. colour and shape and behaviour). Focal fish were allowed 48 h to settle before the introduction of the stimulus fish. Throughout this experiment all stimulus fish, although settled and interacting with the focal fish, displayed the horizontal black stripe characteristic of non-territorial individuals (Fig. 3a) meaning some visual differences in patterning were apparent even to the human observer. Each focal male was tested against two pairs of stimulus fish; intruder populations were presented on alternate sides to eliminate any possible side bias. Trials were recorded using a video camera and behaviour scored from the videos. Aggressive attack (lunge/butt and bite) and display (frontal display, lateral display and quiver) behaviours directed towards each stimulus fish were recorded during a 10 min observation period. Recording began after the focal male had emerged from the central refuge 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 were carried out to compare overall aggressiveness of the two populations and to investigate the possibility of differential use of display

and attack behaviour before pooling all aggressive behaviours to test for aggression biases.

Data Analysis

All data were tested for deviations from homoscedasticity and normality before parametric tests were carried out. One-way ANOVAs were used to test for differences in overall aggressiveness and differences in the use of display and attack behaviours (display:attack ratio) between the two populations. Two-tailed paired *t* tests were carried out to compare the number of aggressive behaviours (total, displays and attacks) displayed to the two stimulus types by males of each focal population.

Results

Morphological Analysis

Lethrinops lethrinus and *L. sp.* ‘chilingali’ differed in body shapes, as indicated by the significant difference in the mean values of relative warp 1 (ANOVA, $F_{1,58} = 28.75$; $p < 0.001$). Sample variances in relative warp 2 were significantly different (Levene’s test, $F_{1,58} = 7.00$; $p = 0.01$), with *L. lethrinus* clearly showing much greater variation in body shape (Fig. 3c). Deformation grids show that the main difference among the populations lies in the head shape and in particular in the position of the mouth, which is relatively lower on the body in *L. lethrinus* compared to *L. sp.* ‘chilingali’ (Fig. 3b).

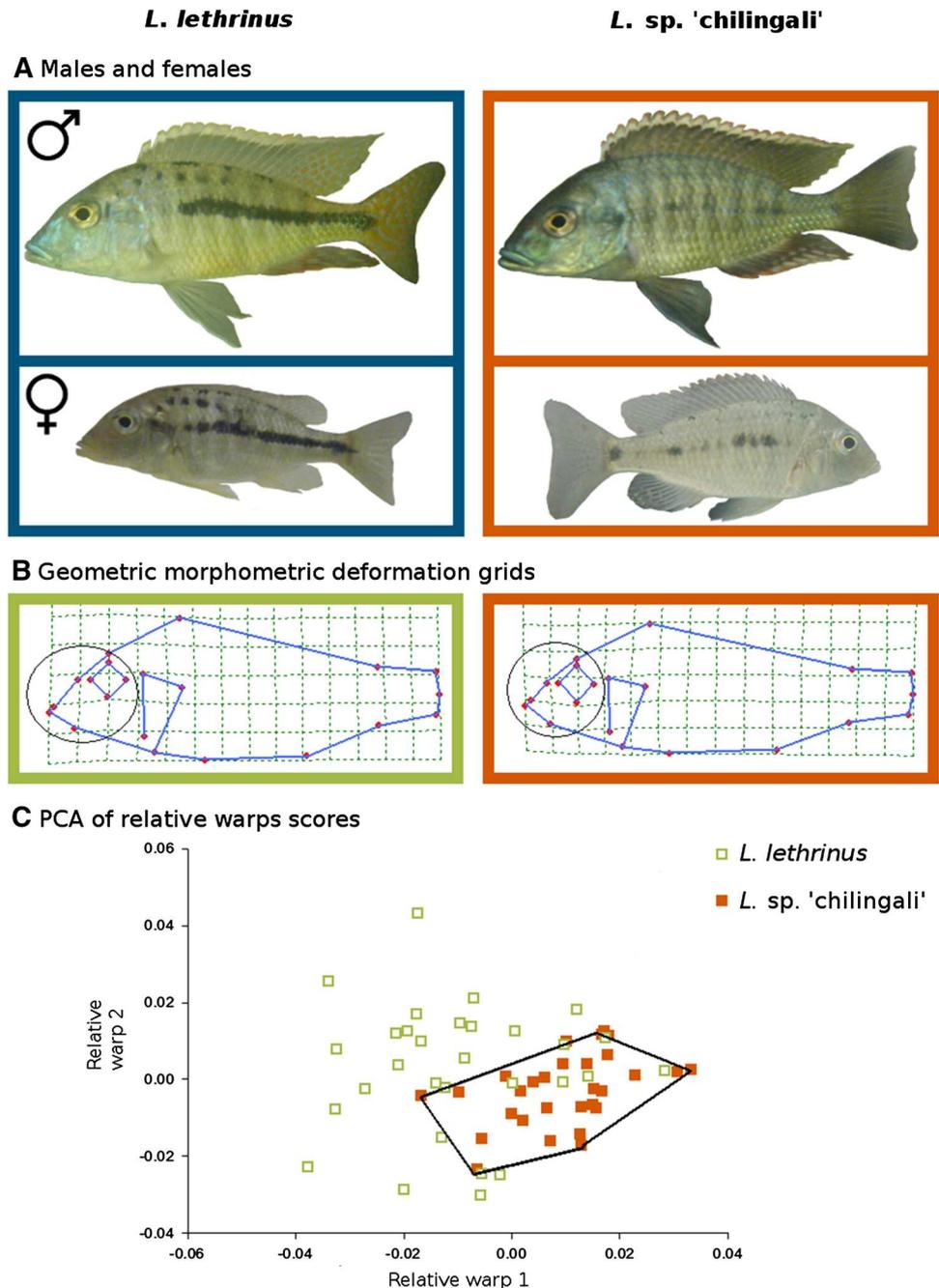
Dietary Analysis

For all sizes of *L. sp.* ‘chilingali’ examined, dipteran larvae/pupae represented more than 80 % of the stomach contents by volume (Table 1). In all cases where identification was possible, these were found to be immature stages of *Chaoborus* spp. Cladocera (crustacea) comprised almost 10 % of the stomach content volume of the smallest size class of fish, but were much less significant in larger size classes. Among the larger fishes, odonata (dragonfly nymphs) were the only other major component by volume, comprising less than 10 %. Stomach contents contained little or no detritus. By contrast, diets of *L. lethrinus* were much more diverse (Table 1) and comprised a wide range of benthic organisms and a reasonable amount of detritus.

Molecular Analysis

In the 61 *Lethrinops* sequenced there were 14 haplotypes. All six haplotypes found within Lake Chilingali were unique to that population. Initial pairwise analysis found a

Fig. 3 Phenotypic differences between *L. lethrinus* and *L. sp. 'chilingali'*. **a** Males and females. **b** Deformation grids from geometric morphometric analysis (for landmarks see Fig. 1) show that the main difference among the populations from the main Lake Malawi system and Lake Chilingali lies in the head shape and in particular in the position of the mouth, which is relatively lower on the body in *L. lethrinus* compared to *L. sp. 'chilingali'*. **c** Geometric morphometric PCA plot: *L. lethrinus* from Lake Malombe and *L. sp. 'chilingali'* were significantly different in body shapes (ANOVA relative warp 1). Sample variances in relative warp 2 were significantly different (Levene's test), with *L. lethrinus* showing much greater variation in body shape



significant difference between *L. sp. 'chilingali'* and *L. lethrinus* (pairwise $F_{ST} = 0.779$, $p < 0.001$). Of the remaining eight haplotypes, one was shared by 30 of the 39 specimens from Lakes Malawi (18 out of 24 individuals) and Malombe (12 out of 15 individuals). A highly significant component of variation among sequences was explained by differences among the three lakes (AMOVA: $F_{ST} = 0.727$, $p < 0.001$). In pairwise comparisons, a significant difference was found between all three populations, although the difference and significance was greater between Lake Chilingali and the two Lake Malawi system

populations (Table 2). All but one of the 22 *L. sp. 'chilingali'* specimens were resolved as falling into a single clade in the minimum spanning network (Fig. 4b).

Mate Choice Experiment

Twenty-two experimental broods were produced, each from a different female. A total of 219 offspring were genotyped for parentage analysis: 10 from each brood, with the exception of one brood which only contained 9 offspring. No cases of multiple paternity of broods were

Table 1 Comparison of percentage volumes of stomach contents of three size classes of *Lethrinops* sp. ‘chilingali’ with data for *Lethrinops lethrinus* from Darwall (2000), sampled from Lake Malawi

Size and <i>n</i> Standard Length (mm)	<i>L. sp</i> ‘chilingali’			<i>L. lethrinus</i>
	Small (<i>n</i> = 17) 18–35	Medium (<i>n</i> = 17) 40–55	Large (<i>n</i> = 17) 56–110	Large (<i>n</i> = 14)
Chironomidae	0.0	0.0	0.0	27.3
Chaoboridae	84.4	98.6	91.1	0.0
Odonata	0.0	0.0	8.8	0.0
Other insect	5.7	0.2	0.0	2.6
Cladocera	9.6	1.1	0.1	11.7
Copepoda	<0.5	<0.5	<0.5	23.4
Rotifera	<0.5	<0.5	<0.5	0.0
Mollusca	0.0	0.0	0.0	10.4
Oligochaeta	0.0	0.0	0.0	23.4
Algae	<0.5	<0.5	<0.5	0.0
Detritus	0.0	<0.5	0.0	1.3

detected and females from both putative species demonstrated a significant tendency to mate with males from their own population: 14 out of the 15 trials (binomial test: $p < 0.001$) for female *L. sp.* ‘chilingali’ and all 7 trials ($p < 0.001$) for female *L. lethrinus* (Table 3; Fig. 5a).

Male Aggression Experiments

Although *L. sp.* ‘chilingali’ fish were significantly more aggressive overall than *L. lethrinus* (mean 89 and 54 aggressive behaviours per trial respectively; ANOVA; $F_{1,20} = 7.12$, $p = 0.01$), the use of different types of behaviour (inferred from the display:attack ratio) did not differ between populations (ANOVA; $F_{1,20} = 0.18$, $p = 0.67$). Both *L. lethrinus* (paired *t* tests: total aggressive behaviour $t_{10} = 3.114$, $p = 0.011$; displays $t_{10} = 3.10$, $p = 0.01$; attacks $t_{10} = 2.57$, $p = 0.03$) and *L. sp.* ‘chilingali’ (total aggressive behaviour $t_{10} = -3.691$, $p = 0.004$; displays $t_{10} = -3.55$, $p = 0.01$; attacks $t_{10} = -2.91$, $p = 0.02$) males showed a significant tendency to bias aggression towards males from their own species in laboratory based dichotomous simulated intruder choice tests (Table 4; Fig. 5b).

Discussion

Here we report a previously undocumented satellite lake cichlid fish population which demonstrates significant genetic, ecological, morphological, and behavioural differentiation from the most similar form found in the Lake Malawi system. Our findings support the hypothesis that peripatric speciation has occurred after divergence of a population during peripheral isolation from the main lake system. Analysis of mtDNA demonstrated significant genetic divergence among the three populations sampled and a greater degree of isolation and greater reduction in gene-flow is indicated by greater genetic differentiation of the isolated satellite lake (Chilingali) population compared to the populations from the connected lakes (Malawi and Malombe) which form part of the main Lake Malawi system (Fig. 4). Ecological differentiation was demonstrated through a comparison of stomach contents of populations from the main lake system and the isolated satellite lake (Table 1). In conjunction with significant morphological differentiation, particularly in the head area and position of the mouth (Fig. 3), this is suggestive of trophic adaptation due to divergent ecological selection in

Table 2 Summary of mtDNA characteristics (*n* = number of haplotypes, ‘unique’ = number of haplotypes unique to population, ‘Hd’ = haplotype diversity) and statistical comparison of genetic differentiation between putative species and populations

Species/pop.	mtDNA	Haplotype			F _{ST} matrix			
	<i>n</i>	<i>n</i>	Unique	Hd	<i>L. sp</i> ‘chilingali’	<i>L. lethrinus</i>	Malawi	Malombe
<i>L. sp.</i> ‘chilingali’	22	6	6	0.775	–	<0.001	<0.001	<0.001
<i>L. lethrinus</i> (total)	39	8	8	0.409	0.779/0	–	n/a	n/a
Malawi	24	5	4	0.435	0.741/0	n/a	–	0.045
Malombe	15	4	3	0.371	0.837/0	n/a	0.135/1	–

F_{ST} matrix showing F_{ST}/*n* shared haplotypes below diagonal and F_{ST} *p* values above

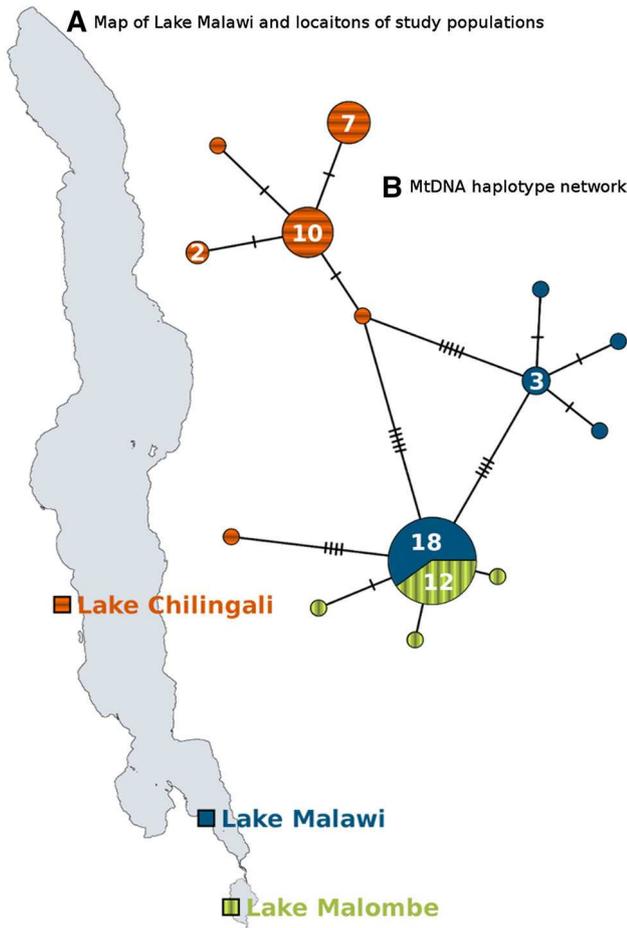


Fig. 4 **a** Map of Lake Malawi with locations of *Lethrinops* study populations. **b** Mitochondrial DNA minimum spanning haplotype network of the 61 *Lethrinops* mtDNA control region sequences indicates clear differentiation of the Chilingali population, and pairwise (F_{ST}) analysis found a significant difference between *L. sp.* ‘chilingali’ and *L. lethrinus* (Table 2 for more details)

the peripheral habitat. Behavioural experiments found that males biased aggression towards, and females preferentially mated with, males from their own population (Fig. 5). The high degree of assortative mating and overall preference of these fish to interact with other fish from their own population suggests that rather than loss of distinctness through homogenisation, secondary sympatry would result in the co-existence of these closely related, genetically and ecologically divergent, allopatric species.

Table 3 Summary of results from pairwise laboratory based female mate choice experiment with *L. lethrinus* and *L. sp.* ‘chilingali’

Focal species	n broods analysed	Mean brood size	Mean % brood genotyped	n Spawnings		p value (binomial)
				Malawi male	Chilingali male	
<i>L. lethrinus</i>	7	39	37	7	0	<0.001
<i>L. sp.</i> ‘chilingali’	15	46	39	1	14	<0.001

As far as we are aware, the only other tests of assortative mating involving satellite lake cichlid populations are two previously conducted with populations from Lake Chilingali. Genner et al. (2007) demonstrated significant assortative mating when females were given a choice of size-matched male *Rhamphochromis*. Although a large size discrepancy in male size led to biased mating in favour of the larger males, it is possible that this may have been a laboratory artifact as the experimental design did not prevent behaviour dominance among males in the confines of aquaria from over-riding female preferences. Thus, it seems likely that the satellite lake *Rhamphochromis* has evolved significant behavioural isolation from the main lake species. By contrast, mate choice experiments did not reveal significant assortative mating between *Astatotilapia calliptera* from the shores of Lake Malawi and the population from Lake Chilingali (Tyers and Turner 2013). *Rhamphochromis* and *Lethrinops* are both genera endemic to Lake Malawi, specialised respectively for midwater predation and sediment-sifting. Conversely, *Astatotilapia* is a widely distributed genus, found as far as Tunisia, Egypt and Israel and populations recognised as similar to or belonging to *A. calliptera* are found in many rivers, pools and smaller lakes in the Lake Malawi catchment and further afield in the Rovuma and Zambezi River systems, among others (Joyce et al. 2011). Thus, there are indications that specialised lake endemics may be more likely to undergo peripheral isolate speciation than are generalised species. Clearly, more studies of other satellite lakes are needed to discern if this is a significant relationship. If any such pattern exists, it may indicate either the greater tendency for diversification in the more specialised lacustrine endemic species or the greater potential for continuing gene flow in the taxa that are able to persist in riverine systems, such as *A. calliptera*. Although the river systems feeding Lake Chilingali have yet to be surveyed in detail, it seems likely that they would be inhabited by substantial populations of *A. calliptera*, but would not contain breeding stocks of lacustrine specialists, such as *Rhamphochromis* or *Lethrinops*.

Despite the potential for population bottlenecks and genetic drift to aid genetic divergence and the evolution of reproductive isolation of geographically isolated populations, evidence from both laboratory experiments

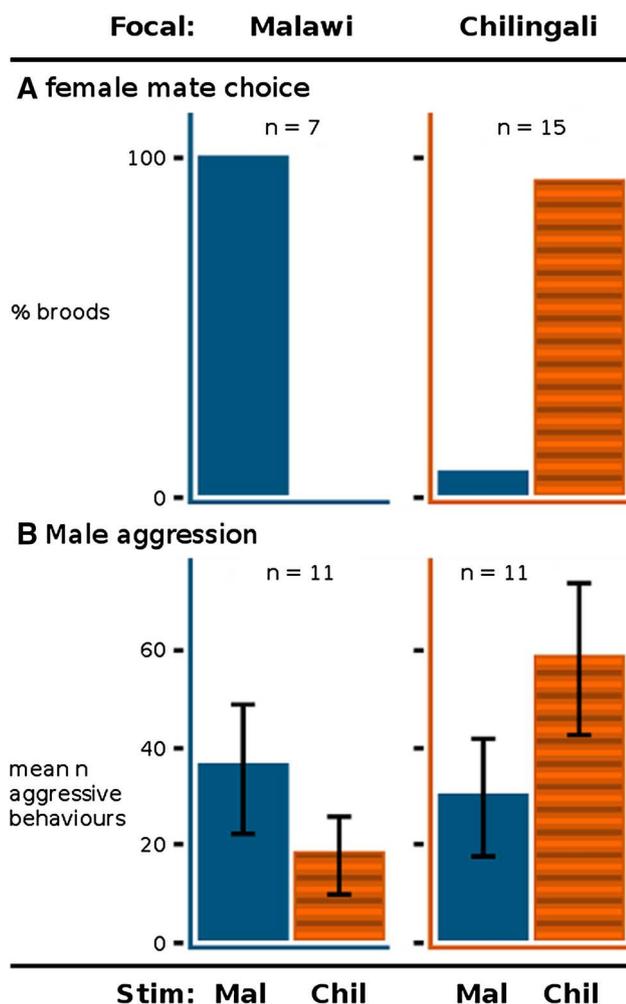


Fig. 5 a When given the choice between *L. lethrinus* from Lake Malawi and *L. sp.* ‘chilingali’ males, females from both taxa demonstrated a significant preference to mate with males from their own population (binomial test), charts show % of broods spawned with each type of male by each type of female (Table 3 for more details). **b** Both *L. lethrinus* and *L. sp.* ‘chilingali’ males showed a significant tendency to preferentially attack males from their own population (*t* tests, Table 4), charts show mean number of aggressive behaviours per 10-min trial and error bars show 95 % confidence intervals

(*Drosophila* spp.; Dodd 1989; Rice and Hostert 1993) and natural model systems (threespine sticklebacks; McKinnon and Rundel 2002) suggests a greater role for selection and pleiotropy/genetic hitchhiking (Rice and Hostert 1993). Therefore, the importance of geographical separation in geographical models of speciation may not necessarily be the complete prevention of gene flow, but rather the restriction of gene flow along with divergent ecological selection (Rice and Hostert 1993). Although genetic divergence has been demonstrated between main lake and satellite lake populations, of both *Rhamphochromis* (Genner et al. 2007) and *Lethrinops* (present study), in both cases analyses were based on selectively neutral (mtDNA) markers. If species that have not diverged under the same conditions (e.g. *Astatotilapia*, Tyers and Turner 2013) were also included in population genetic analysis, based on markers from areas of the genome that are known to be under selection, rather than neutral markers, a better insight might be gained as to whether morphological and behavioural divergence and the evolution of reproductive isolation are the result of selection or neutral variation/genetic drift. Some satellites of Lake Victoria also contain both generalist haplochromines and divergent forms of lacustrine habitat specialists (Odhiambo et al. 2011, 2012) and may provide a suitable system with which to further investigate reproductive isolation among main lake and satellite lake populations.

Within Lake Malawi, pelagic cichlids such as *Rhamphochromis* appear to encounter minimal barriers to gene flow (Genner et al. 2007), in marked contrast to the very fine-scale genetic structuring seen in rocky shore specialists (Genner and Turner 2005). We are unaware of any previous reports of tests of genetic structuring in *Lethrinops*, but relatively weak structuring was found in the ecologically similar *Protomelas similis* (Pereyra et al. 2004). It appears probable that different modes of speciation play greater or lesser roles in divergence of different types of habitat specialists. Whereas those that show strong intralacustrine population structuring (species associated

Table 4 Summary of results of laboratory male aggression experiments from dichotomous simulated intruder choice trials between *L. lethrinus* and *L. sp.* ‘chilingali’

Focal species	n Males tested	Mean n aggressive behaviours	Mean n aggressive behaviours		p value (<i>t</i> test)
			Malawi male	Chilingali male	
<i>L. lethrinus</i>	11	54 Total	36	18	0.011
		16 Displays	12	4	0.010
		39 Attacks	26	14	0.030
<i>L. sp.</i> ‘chilingali’	11	89 Total	30	59	0.004
		31 Displays	7	24	0.010
		57 Attacks	23	34	0.020

with the rocky-shore) may frequently undergo allopatric divergence within the main lake, those that show weaker population structuring (sandy-shore and pelagic species) may be more likely to undergo allopatric divergence in scenarios involving peripatric isolation. Perhaps it is due to the strong habitat preference of rocky-shore specialists that they have never been reported from satellite lakes, which probably form from muddy bays and tend to be inhabited by generalists and species with a preference for softer muddy/sandy substrates (generalist *Astatotilapia* Tyers and Turner 2013; Odhiambo et al. 2011, 2012: sandy/muddy/vegetated-benthic *Astatoreochromis*, *Xystichromis*, *Pseudocrenilabrus* Odhiambo et al. 2011, 2012; *Lethrinops* this study: pelagic *Rhamphochromis* Genner et al. 2007).

Across the three East African Great Lakes, experimental tests of mate preferences among allopatric populations of rocky shore specialists have generally shown incomplete assortative mating, even between forms showing marked differences in signal traits such as male courtship colour that have been proposed as significant in assortative mating (Seehausen 1997; Knight and Turner 2004; Egger et al. 2008, 2010; Blais et al. 2009; Pauers et al. 2010). This raises the possibility that allopatric isolation and signal divergence may be insufficient to cause complete reproductive isolation. Perhaps it is necessary for there to be simultaneous divergence in ecological adaptation, as suggested by ecological speciation models and meta-analysis (e.g. Doebeli and Dieckmann 2003; Funk et al. 2006). Isolation among patches of preferred habitat within a large lake may not always lead to adaptive divergence, because populations are able to maintain their habitat preferences. In contrast, populations isolated in small peripheral water bodies may have to adapt to local conditions which differ significantly from the conditions of their main lake ancestors. In this regard, it is interesting that the Lake Chilingali populations of both *Rhamphochromis* and *Lethrinops* showed significant divergence in body shape in relation to their main lake relatives. In *Lethrinops*, we have also shown divergence in diets. The Lake Malawi form has a longer snout and a more ventrally placed mouth, traits normally associated with a strategy of foraging for benthic invertebrates by plunging the snout deep into soft sediments, which is consistent with the stomach contents data collected by Darwall (2003) as well as more qualitative observations made in previous studies (Eccles and Lewis 1978; Turner 1996; Ngatunga 2000; Konings 2007). The Lake Chilingali form has a shorter snout and less ventrally-placed mouth, and it appears to feed largely on midwater invertebrates, such as chaoborid larvae and pupae and cladocerans, while occasionally taking large active insect larvae such as dragonfly nymphs that might be captured from the bottom or plucked individually from waterplants. No differentiation of diets has yet been noted for the Lake

Chilingali *Rhamphochromis*, but it has been shown to mature at and attain a much smaller size than known main lake populations, a life history trait also demonstrated by laboratory-bred individuals (Genner et al. 2007). Thus, it seems possible that there is heritable adaptive divergence between satellite lake populations of lacustrine specialists and their likely sister populations from Lake Malawi.

The present study has not investigated the sensory modalities of assortative mating, but the male aggression experiments, where ‘intruder’ males were confined in solid-walled chambers, suggest visual cues might be important in population recognition. Other studies have documented reduced aggression towards males of sympatric cichlid species or morphs that differ markedly in colour (e.g. Dijkstra et al. 2009) and there is now also evidence to suggest that divergence in the structure of the ‘bowers’ built by sand-dwelling species reduce interspecific aggression (Genner et al. 2008a, b; Magalhaes et al. 2013). It has been proposed that such divergent male aggression biases may facilitate the co-existence of populations in sympatry and even aid sympatric speciation (van Doorn et al. 2004; Mikami et al. 2004; Seehausen and Schluter 2004; Genner et al. 2008a, b; Magalhaes et al. 2013), although theoretical considerations indicate that reproductive character divergence can drive divergence in competitor recognition traits but not vice versa (Okamoto and Grether 2013). Our findings suggest the divergence of signals triggering male territorial aggression might also facilitate co-existence of allopatric species following secondary contact. It should be noted, however, that during these lab based aggression experiments, stimulus males did not have sufficient time to build bowers and therefore this potentially species specific traits was not available as a cue for the focal males.

Conclusions

It seems likely that cichlid speciation may be the result of many different geographic scenarios and may involve a varied mix of ecological and sexual selection and perhaps neutral processes. Very large freshwater lakes can be comparable to continental and marine systems with peripheral isolation occurring in satellite lakes in much the same way as terrestrial organisms become confined to oceanic islands and marine organisms to marine lakes. If the formation and loss of satellite lakes has been a feature of the rift valley throughout its geological history, they may play a greater role in isolation and diversification of non-rocky shore specialists than is often considered. Recent theoretical studies suggest that such dynamic habitat fragmentation is particularly conducive to adaptive radiation and generation of biological diversity (Aguilée et al. 2013).

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