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Divergence and speciation of East African haplochromine cichlid fish

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**Divergence and speciation of East African
haplochromine cichlid fish**

Thesis submitted for the degree of
Doctor of Philosophy
from Bangor University

by
Alexandra Morton Tyers

November 2013

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Abstract

In the Great Lakes of the East African Rift Valley, cichlid fishes have diversified into hundreds of species with great variety of ecomorphological, secondary sexual and behavioural characteristics. A strong role for sexual selection in their speciation is indicated by the presence of many closely related ecologically-similar sympatric species which differ in male secondary sexual characteristics. A review of previous research finds that reproductive isolation by direct mate choice may be a common isolating barrier among sympatric species. Observations of partial assortative mating among divergent geographic populations have led to theories of intralacustrine allopatric speciation of habitat specialists by divergence of signal traits involved in conspecific recognition.

Here I demonstrate that signal and preference divergence among populations is not limited to patchily distributed lacustrine endemics, but can also occur in the widely distributed riverine generalist lineage that is phylogenetically basal to the large lacustrine radiations, suggesting a role for divergence of secondary sexual traits in allopatry throughout adaptive radiation. This thesis also adds to the evidence for ecological divergence and peripatric speciation of lacustrine habitat specialists in the absence of significant colour differentiation. In simulated intruder choice tests, males tend to bias aggression towards males of their own species or populations, which may aid in the co-existence of allopatrically diverged populations under secondary sympatry and help to facilitate speciation, or even drive divergence of male traits that are involved in signalling during both courtship and territorial interactions.

Results from preliminary investigations into individual variation in mate preference suggest that partial assortative mating among allopatric populations may be due to variation within, rather than among, individuals in their choice of mate. Within-individual variation may be unlikely to cause divergence, but has the potential to aid in colonisation of new areas by a relaxation of directional selection allowing for divergence of male traits by environmental or other social selection pressures. To draw any firm conclusions about the impact of variation in mate choice on speciation, much more data from a wider variety of lineages is needed. Mate choice experiments, carried out as part of a larger ongoing project into sympatric divergence of haplochromine cichlids in isolated crater lakes, found little consistency of female mate choice despite morphological and genetic differentiation of forms. This indicates that, in contrast to previously tested sympatric species of cichlids, at the beginnings of adaptive radiation variety may not be maintained solely through reproductive isolation by direct mate choice.

Observations of interactions among animals may frequently reveal unexpected results with regards to what is and is not a species - for example, strong assortative mating among phenotypically similar allopatric populations and that assortative mating alone may not maintain diversity in sympatric populations. Behavioural experiments can complement more modern genetic and genomic techniques and therefore continue to be a valuable tool in the study of speciation and the mechanisms involved in divergence and the maintenance of diversity.

Contents

Acknowledgements		15
Chapter 1	General introduction	19
	Species and speciation	19
	What is a species?	19
	Geography of speciation	21
	Selective forces driving divergence	22
	The cichlid fish model	25
	Great Lakes and cichlid tribes	26
	Modern haplochromine cichlids: speciation and diversity	27
	Study taxa	34
	Summary	35
	Main aims and overview of experimental chapters	36
Chapter 2	Peripheral isolate speciation of a Lake Malawi cichlid fish from shallow muddy habitats	47
Chapter 3	Signal and preference divergence among allopatric populations of the non-endemic lake Malawi cichlid fish <i>Astatotilapia calliptera</i> (Perciformes: Cichlidae)	71
Chapter 4	Individual variation in female mate preference in the Lake Malawi cichlid fish <i>Astatotilapia calliptera</i>	88
Chapter 5	Diversification and speciation of haplochromine cichlids in small isolated crater lakes in Tanzania, East Africa	103
Chapter 6	General discussion	123
	Summary of main findings	123
	Synthesis	124
	Allopatric divergence of specialists and generalists	124
	Sympatric divergence from a generalist ancestor	126
	Individual variation in mate preference	127
	Concluding remarks	129

Appendices	Appendix I: Co-authors and contributions	137
	Appendix II: Extended phylogenetic analysis for Chapter 2	138
	Appendix III: Data from behavioural experiments	142
	Appendix IV: Lake Chilingali – potential loss of endemics	149

Tables & figures

- Chapter 1 **Table 1.** Female mate choice experiments investigating reproductive isolation in haplochromine cichlids. Including studies of assortative mating among sympatric species, allopatric populations and studies designed to investigate the cues involved in assortative mating. Grey text indicates studies based on non-spawning measures of preference (females association time or reaction to male displays), which can indicate recognition of own males. But, without observations of spawning these can not be compared directly to studies that show the degree of reproductive isolation by measuring spawning. To give a relative idea of the strength of assortative mating results have been categorised: complete = 100%, very strong = >90%, strong = 70-90%, weak = 50-70% (if significant). * = asymmetric assortative mating among populations or variation in strength where assortative mating is dependent on the degree of difference between populations in pairwise comparisons. 32
- Chapter 2 **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 southern Lake Malawi. 59
- Table 2.** Summary of mtDNA characteristics and statistical comparison of genetic differentiation between putative species and populations. F_{ST} matrix showing F_{ST} / n shared haplotypes below diagonal and F_{ST} p-values above. 60
- Table 3.** Summary of results from pairwise laboratory based female mate choice experiment with *L. lethrinus* and *L. sp.* 'chilingali'. 60
- Table 4.** Summary of results of laboratory male aggression experiments from dichotomous simulated intruder choice trials between *L. lethrinus* and *L. sp.* 'chilingali'. 62
- Figure 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 caudle 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 & 21 show the slope of the head). 53
- Figure 2.** Plan view of mate choice experimental tank set up. Three offset baffles in

the middle of the 2m 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.

56

Figure 3. Phenotypic differences between *L. lethrinus* and *L. sp. 'chilingali'*. **A)** Males and females. **B)** Deformation grids from geometric morphometric analysis (see Fig. 1 for landmarks) 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.

58

Figure 4. **A)** Map of Lake Malawi with locations of *Lethrinops* study populations. **B)** 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).

61

Figure 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-minute trial and error bars show 95% confidence intervals.

62

Chapter 3 **Figure 1.** Map of Lake Malawi with locations of *A. calliptera* study populations and results from female mate choice and male aggression experiments. Chart 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 to other-population males, there was also no significant preference between the Salima and Chizumulu populations (paired t-tests). But, Fisher's combined probabilities test records 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-minute trial and error bars show 95% confidence intervals.

80

Chapter 4 **Table 1.** Population level preferences from three mate choice experiments to test *A. calliptera* 'salima' female preference among yellow males from Salima (and Lake Chilingali) and blue males from Chizumulu Island. Exps 1 & 2 were re-analysed from Chapter 3. Exp 3 refers to the present experiment.

96

Table 2. Overview of the results from an experiment to test consistency of individual female mate choice. Letters A-D refer to the four pairs of males used for these experiments. Each trial (T) measured the spawning preference of a female with a pair of males and each female underwent three trials: the first two (T1 & T2) with the same pair of males and the third trial (T3) with a different pair.

97

Chapter 5 **Table 1.** Summary of the characteristics of the three lakes: elevation above sea level and above Lake Malawi; the characteristics of the bottom and secchi disk reading for water clarity; the fish fauna. All three lakes contain some macrophytes and bivalve molluscs. Lake Itamba is overhung by trees and also contains crabs and many sponges. * indicates introduced species.

108

Table 2. Summaries of female mate choices from two experiments to test assortative mating of populations of *Astatotilapia* from different lakes in the Lake Malawi catchment. Number of females tested, proportion of broods genotyped, number of offspring assigned to con- and heterotypic males, and p-values from Wilcoxon Signed Rank tests. Parentheses display mean values. 1) *A. calliptera* (Lake Malawi) vs *A. sp.* 'ilamba'. 2) *A. sp.* 'ilamba' vs *A. sp.* 'itamba'.

114

Table 3. Summary of the success (percentage of whole broods sired and parts of broods sired) of the different male types across the whole experiment, where more than one male of the same type was present, number of offspring was combined for the male type.

115

Table 4. Consistency of choice by the 46 females whose offspring were sired by more than one male 116

Figure 1. Location of crater lakes in relation to each other and Lake Malawi, with photographs of males from the *Astatotilapia* species and populations used in these mate choice experiments. *A. calliptera* from Lake Malawi, *A. sp.* 'ilamba' from Lake Ilamba, *A. sp.* 'itamba dark' from Lake Itamba and *A. sp.* 'massoko big calliptera' (yellow) and *A. sp.* 'massoko big predator' from Lake Massoko. 110

Chapter 6 **Table 1.** Female mate choice experiments investigating reproductive isolation in haplochromine cichlids. Including studies of assortative mating among sympatric species, allopatric populations, studies designed to investigate the cues involved in assortative mating and the experiments conducted for this thesis (grey cells). Grey text indicates studies based on non-spawning measures of preference (females association time or reaction to male displays), which can indicate recognition of own males. But, without observations of spawning these can not be compared directly to studies that show the degree of reproductive isolation by measuring spawning. To give a relative idea of the strength of assortative mating results have been categorised: complete = 100%, very strong = >90%, strong = 70-90%, weak = 50-70% (if significant). * = asymmetric assortative mating among populations or variation in strength where assortative mating is dependent on the degree of difference between populations in pairwise comparisons. 128

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Chapter 1

Species and speciation

Speciation, the evolutionary process by which new species arise, has always been central to the study of evolutionary biology. Not only does taxonomic classification ensure an understanding among biologists as to what organism is being discussed, agreement on what constitutes a species is a necessary starting point before embarking on investigations into how new species form. Although in some groups of organisms identification of distinct species may not seem to be a difficult problem, since the first formulation of an evolutionary theory for the origin of species (see Darwin 1859) it has been recognised that the variety of life is not as discontinuous as the idea of species can make it appear. Darwin considered the possibility that a species may be an artificial classification for well differentiated varieties within continuous variation (Darwin 1859; Coyne & Orr 2004). Despite the possible continuum of biological diversity, it is generally accepted that species do exist (Dobzhansky 1940; Coyne & Orr 2004) and it can be argued that there is a general consensus that species are 'independent evolutionary lineages' (General Lineage Species Concept, de Queiroz 1998). The exact point of divergence, however, at which a lineage becomes a species is still debated and the answer to the often posed question 'what is a species?' may frequently depend on the area of research in which it is being asked (de Queiroz 1998; Coyne & Orr 2004). This introduction provides an overview of the most widely used criteria for delimiting species, the geography of speciation and the selective forces that drive divergence. Prior to outlining the aims of this thesis, the model system used for the experimental chapters (haplochromine cichlid fish) will be introduced.

What is a species?

The criteria used to define a species relates to the point during divergence at which separation of lineages is considered complete. Species 'concepts' (or definitions) broadly fall into two categories: 'Prospective concepts' (which includes the 'Biological', 'Recognition' and 'Cohesion' Species Concepts) and 'retrospective concepts' (for example the 'Phylogenetic', 'Genealogical', 'Evolutionary' and 'Genotypic Cluster' Species Concepts). Prospective concepts delimit species by intrinsic biological isolation and are often favoured among researchers studying the processes of speciation (Turelli *et al.* 2001) as they allow for predictions about future patterns of variation, such as whether distinct divergent groups can persist in sympatry. Retrospective concepts, on the other hand, are often applied where the focus of research is current patterns of variation and the history of lineages (de Queiroz 1998; Coyne & Orr 2004).

As the aim of this thesis is to investigate 'divergence and speciation', the Biological Species Concept (BSC), which equates speciation to the point of divergence at which gene exchange between lineages is prevented by intrinsic reproductive isolating mechanisms, shall be discussed in further detail. In sexually

reproducing organisms, recombination produces new combinations of alleles in each generation, providing greater genetic variation (compared to asexually reproducing organisms) on which selection can act to produce evolutionary change within species. Divergence and speciation is therefore limited until there is isolation between portions of a gene pool, allowing for evolution along different trajectories (Coyne & Orr 2004). With an acknowledgement of its limitation to sexually reproducing organisms, we therefore often consider species to be '*groups of interbreeding natural populations that are reproductively isolated from other such groups*' (Mayr 1996). Species may consist of multiple geographically isolated populations that would interbreed if contact was possible (Stauffer *et al.* 1995) and the BSC highlights the importance of biological divergence between, rather than geographical separation of, populations in the development of pre-mating barriers to gene flow. Although this means that a species is defined by its biological isolation from other species, rather than possibly transient abiotic factors, it does make dealing with allopatric races problematical. There is no clear and reliable way of identifying allopatric species in the wild. However, despite this drawback of the BSC, it does allow for testing of the specific status of allopatric populations through laboratory based experiments, which can test for mating preferences and post-mating barriers to gene flow (Stauffer *et al.* 1995; Turner 1999; Turner *et al.* 2001). The stage of reproductive isolation (absent, partial or complete) can therefore be estimated and reasons for its evolution hypothesised, regardless of past, current or future geographical distribution.

It is perhaps not surprising that identification of species is more straightforward in some groups of organisms than others. From our 'snapshot' view of life, we are faced with both well differentiated forms and the continuous variation of taxa that are in the process of diversification and speciation (Coyne & Orr 2004). It is in the latter groups, those made up of closely related sister species, incipient species and divergent allopatric populations, that defining species can be problematical (Stauffer *et al.* 1995). Groups of organisms that are undergoing divergence are, however, of great interest as they may allow for identification of the differences between species that contribute to reproductive isolation, before the accumulation of other genetic differences after the development of isolating barriers (Seehausen *et al.* 1998). Comparative studies of taxa at different stages of divergence, with a variety of geographical distributions and ecologically and sexually selected traits (for example 15 species of finches on the Galapagos Islands, Darwin 1859; Sato *et al.* 2001; 40 species of birds of paradise centred around New Guinea in Australasia, Irestedt *et al.* 2009; ca. 500 species of *Drosophila* on the Hawaiian Islands, Kaneshiro 1988; hundreds of species of cichlid fishes in each of the East African Great Lakes, Turner *et al.* 2001; Salzburger *et al.* 2005), can aid in identifying barriers that are present throughout the transition from a species to species and in understanding the mechanisms that are involved in the process of speciation (Turelli *et al.* 2001; Coyne & Orr 2004). In many cases, behavioural isolation among closely related taxa (*e.g.* invertebrates, Dodd 1989; fish, Knight *et al.* 1998; Seehausen *et al.* 1998; Kodric-Brown & Strecker 2001; McLennan 2003; Hankinson & Morris 2003; amphibians, Blair 1964; Dawley 1986) is thought to evolve before post-zygotic isolation and is the predominant mechanism preventing gene exchange among recently diverged species (Blair 1964; Coyne & Orr 2004).

Geography of speciation

Three main geographical modes of speciation are based on the extent to which diverging taxa are isolated from each other. Models of allopatric (vicariant and peripatric) speciation stress the importance of geographical isolation preventing gene flow between populations during divergence. In sympatric and parapatric theories, on the other hand, it is argued that speciation is possible even when there is full or partial geographical overlap and the potential for gene flow between diverging lineages. Theoretically, all modes are possible and although allopatric speciation is often considered to be the predominant route to speciation in animals, sympatric speciation may be important in some taxa and it seems likely that parapatric speciation has been common in the marine environment (Ingram 2011).

Without gene flow

Theories of allopatric speciation are split into two modes, differing only in the relative sizes of the populations involved. In models of 'vicariant speciation', initially developed by Mayr in 1963, a species is split into populations by the appearance of new geological barriers within its range. Geography prevents gene flow and as there is no selection acting to maintain reproductive compatibility (Mayr 1963; Turelli *et al.* 2001), reproductive isolation may evolve as a by product of any evolutionary force. Populations often occupy ecologically different habitats that impose different forms of selection (Coyne & Orr 2004) and variation in morphology may arise through the actions of divergent natural and/or sexual selection acting on ecologically important or sexually selected traits (Turner 1999; Turelli *et al.* 2001; Coyne & Orr 2004; Baker 2005; van Doorn *et al.* 2009). Even if the habitats of allopatric populations are effectively identical, selectively neutral processes may result in divergence (Turelli *et al.* 2001; Coyne & Orr 2004): Different mutations are likely to arise in different populations and genetic drift can fix different genes (Coyne & Orr 2004). There is little doubt, therefore, that geographical separation of populations is a major driver of divergence and speciation (Mayr 1963; Rice & Hostert 1993; Barraclough & Vogler 2000; Turelli *et al.* 2001; Dawson & Hamner 2008).

Rather than the splitting of a single population, peripatric (or 'peripheral isolate') speciation considers the case when new species form in small range fragments around a widely distributed ancestral species (Barraclough & Vogler 2000; Genner *et al.* 2007a; Stuart *et al.* 2012). Although phenotypic variation is often found among continental populations, there may be weaker genetic differentiation due to continued gene flow. In contrast, continental and island populations, among which gene flow is much lower or may have ceased completely, may often show differentiation of the same magnitude observed between different species (*e.g.* Atlantic island chaffinches, Baker *et al.* 1990; pelagic Lake Malawi cichlids, Genner *et al.* 2007a). Theories of island speciation include 'habitat islands' such as mountain tops and isolated water bodies (Dawson & Hamner 2005; Genner *et al.* 2007a; Dawson & Hamner 2008) as well as offshore terrestrial islands, and are well established in evolutionary biology. Due to strong selection for local adaptation, higher ecological opportunity and the influence of founder effects, the probability of

speciation occurring is thought to be increased after invasion of novel habitats and isolation of colonising populations (Barraclough & Vogler 2000; Coyne & Orr 2004; Dawson & Hamner 2008; Stuart *et al.* 2012).

With gene-flow

Compared to the theoretical ease with which divergence may occur in isolated populations, the numerous conditional constraints required by theoretical models of sympatric speciation have meant that it is frequently doubted as a significant contributor to diversification in most groups of organisms. However, numerous theoretical models do suggest possible mechanisms for divergence without geographic separation – by disruptive natural or sexual selection (Dieckmann & Doebeli 1999; Higashi *et al.* 1999; Seehausen & van Alphen 1999) or divergent mate preferences driving divergence of male secondary sexual traits resulting in reproductive isolation (Turner & Burrows 1995). Although theory suggests that divergence is possible by disruptive sexual selection, convincing empirical evidence for sympatric speciation comes from studies in which divergence is primarily due to disruptive natural selection and ecological diversification: most notably from endemic fish species flocks isolated in extremely small crater lakes (*e.g.* cichlids, Schlieven *et al.* 1994; Barluenga *et al.* 2006; pupfish, Strecker & Kodric-Brown 2000). More recent considerations suggest that divergence is most likely with a combination of natural and sexual selection (Arnegard & Kondrashov 2004; van Doorn *et al.* 2009; Maan & Seehausen 2011). There are now a multitude of different theoretical models demonstrating the possibility of sympatric speciation but further empirical research is needed to identify the most common and likely mechanisms. Many theories rely on divergent mate preferences yet there is relatively little empirical evidence for such variation in preference within populations (Arnegard & Kondrashov 2004) and according to theories of sexual selection, mate preferences should be directional with females selecting for extreme male traits or traits that signal male quality (Andersson 1994; Arnegard & Kondrashov 2004).

Selective forces driving divergence

Natural selection, resulting from variance in individual survival and reproductive output, and sexual selection, resulting from variance in individual mating success (Darwin 1871) are recognised as the two main selective forces driving the evolution and divergence of species. In addition to debate over the relative importance of different geographical scenarios (sympatry/allopatry) in the generation of biological diversity, there is also often disagreement over the relative importance of these different selection pressures and the contribution of selectively neutral processes. Much of the time, it is likely that a combination of these processes will be involved in speciation – even early models of evolution by sexual selection recognised that all selection takes place in an ecological context and that natural selection will eventually check trait evolution in processes of runaway sexual selection (Fisher 1930; Maan & Seehausen 2011). However, each major selective force will first be considered alone, followed by discussion of theories of combined selection.

Natural selection

Divergence by natural selection is fairly intuitive; animals in different habitats may evolve differences due to different environmental selection pressures (Mayr 1963; Turelli *et al.* 2001; Coyne & Orr 2004). As previously discussed, the initial division of a population into areas with different ecological conditions may be due to the splitting of a species range by geological processes (vicariant speciation, Mayr 1963), or by the founding of a new environment after dispersal (peripatric speciation, Barraclough & Vogler 2000; Stuart *et al.* 2012). After colonisation events, the founding population may not only experience different environmental conditions as compared to the source population, but also, cases such as the colonisation of new oceanic islands or lakes, the potential to expand into many new habitats (Coyne & Orr 2004; Baker 2005; Turner 2007). In such cases of adaptive radiation, divergent ecological adaptation that occurs during specialisation to new habitats can reduce the likelihood of mating through spatial or temporal separation of populations (Maan & Seehausen 2011). In any scenarios involving divergent natural selection and ecological adaptation, sexual traits and preferences may diverge as a by product, increasing the likelihood of assortative mating and speciation (Dodd 1989; Turelli *et al.* 2001; Coyne & Orr 2004; Maan & Seehausen 2011).

Sexual selection

Introduced by Darwin in 1871, for a long time sexual selection was only applied to the evolution of male traits within species and it is only relatively recently (*e.g.* Lande 1981; West-Eberhard 1983) that it has been considered as an evolutionary force with potential for significant involvement in the divergence of species. As with natural selection, theoretically, sexual selection may contribute to divergence in allopatry or drive divergence in sympatry. It is now generally accepted that sexual selection has had a major role in speciation of some taxa and that divergent sexual selection can cause speciation (Turelli *et al.* 2001; Coyne & Orr 2004; Maan & Seehausen 2011). In highly speciose adaptive radiations (for example Hawaiian *Drosophila*, Kanishiro 1988; New Guinea birds of paradise, Irestedt *et al.* 2009; lacustrine cichlid fish, Turner 2007) there is often not only great diversity of eco-morphological and behavioural traits, but also spectacular diversity of male secondary sexual traits (Turelli *et al.* 2001).

Speciation by divergent sexual selection may be particularly likely in taxa where asymmetrical parental investment drives the evolution of secondary sexual characteristics within species. In many animal taxa, there is differential investment in offspring by males and females. A mating system in which choosy females (which have greater investment in offspring) select a mate from among many competing males will often lead to sexual dimorphism (Andersson 1994) through either intrasexual competition or intersexual preference (Darwin 1871). Mate selection in such systems is not a random process, but involves the assessment of traits that are differentially expressed among individuals, to select the most suitable individual with which to reproduce. An important part of the process of mate selection, particularly where closely related species exist in sympatry, is therefore the identification of conspecifics

(Stauffer *et al.* 1995). Under classic runaway and good-genes models of sexual selection (Zahavi 1975; Andersson 1994), correlated additive genetic variation is necessary for female preference to drive evolution of elaborate male ornamentation (Brooks & Endler 2001) and should lead to strong consistent mate preferences and agreement among females on the most desirable male (Forstmeier & Birkhead 2004). However, variation in choice of mate, among individuals, is fairly common (Bell *et al.* 2009) and where there is variation in preference coupled with a reduction in directional selection, there is potential for divergence by sexual selection (Boake 1989; Jennions & Petrie 1997; Brooks & Endler 2001; Turner & Burrows 1995; Haesler & Seehausen 2005).

Divergence of male secondary sexual traits among closely related species may not only result from intersexual selection through divergence of female preference, but also by intrasexual selection through male competition (Coyne & Orr 2004). If the traits involved in species recognition by females during mate choice are also used in signalling among males during intrasexual encounters then divergence of such traits in allopatry is likely to increase the likelihood of co-existence of population upon secondary contact. Female mate preference for homotypic males, those from their own population, may reduce gene flow between divergent forms, while at the same time reduced male territorial aggression among the different forms (Andersson 1994) aids in their co-existence by reducing potential energy expenditure and risk of being attacked in interactions among individuals that are not in direct competition for mates (Seehausen *et al.* 1998; Seehausen & Schluter 2004; Kaneshiro 2006).

Selection for recognition

Speciation by reinforcement requires both allopatric and sympatric stages during divergence. If physical barriers to gene flow break down before the evolution of complete reproductive isolation, and mating among individuals from allopatrically diverged populations results a reduction in fitness due to selection against hybrid offspring, there may be selection for greater mate discrimination and the possibility of completion of speciation under secondary sympatry (Dobzhansky 1940). Reinforcement is often inferred from comparisons of the degree of difference between sympatric and allopatric taxa in secondary sexual characteristics and assortative mating (Coyne & Orr 1989; Rice & Hostert 1993). Greater phenotypic differentiation and a higher degree of reproductive isolation in sympatry indicates selection for recognition (Blair 1964). However, the collapse of divergent lineages back into a single interbreeding population may make it difficult to determine the likelihood or frequency of reinforcement occurring (Coyne & Orr 2004).

Combined forces: environmental differences impact sexual selection

Where sexual selection drives the evolution of male secondary sexual traits within species, environmental differences among populations may result in variation in the equilibrium point between directional sexual selection and natural selection (Fisher, 1930). The result of which may be divergence of mating preferences and traits (Maan & Seehausen 2011) and the evolution of reproductive isolation by pre-

zygotic mechanisms before secondary sympatry (Turelli *et al.* 2001). Differential natural selection on mate choice may also result in divergence if, for example, a relaxation of sexual selection on trait evolution occurs due to limitations on choosiness – perhaps due to greater risk of predation in some populations (Maan & Seehausen 2011).

Divergent sexual selection by sensory drive, which emphasises the role of the environment as a selective force that can influence the evolution of sensory systems and communication signals, including courtship displays (Rice & Hostert 1993; Seehausen & van Alphen 1999; Coyne & Orr 2004; Maan & Seehausen 2011; Smith *et al.* 2012), may be important in animal speciation and has been proposed to be involved in the evolution of reproductive isolation in fish taxa that inhabit heterogeneous light environments (*e.g.* Boughman 2001; Seehausen *et al.* 2008; Smith *et al.* 2012). For example, in sticklebacks, female perceptual sensitivity to red light varies with the extent of red-shift in the environment and contributes to divergent preferences. Male nuptial colour varies with environment and is tuned to female perceptual sensitivity. The extent of divergence among populations (in red colouration and preference for red) is correlated with the extent of reproductive isolation (Boughman 2001).

More recent alternative models suggest that speciation by combined natural and sexual selection can be achieved without divergent sexual selection, resulting in environment dependent reproductive isolation without any intrinsic assortative mating (van Doorn *et al.* 2009): If mating success is based on condition dependent signals (Andersson 1994) and the best displays are produced by locally adapted males, sexual selection may promote divergent ecological adaptation and preference for locally adapted males may strengthen disruptive selection between habitats. However, this mechanism relies on strong habitat fidelity as although males in the 'wrong environment' would not be as successful in mating, females in the wrong environment would accept locally adapted males (van Doorn *et al.* 2009).

The cichlid fish model

Fish in the family Cichlidae are currently found throughout the tropics (Genner *et al.* 2007c; Friedman *et al.* 2013). In Africa, cichlids are widespread in the river systems and lakes and this continent is home to the greatest proportion of species, *ca.* 60% (Turner 2007). Several hundred species are found in rivers and smaller lakes (Salzburger *et al.* 2005; Turner 2007), but the majority, somewhere in the region of 1500, are confined to the three biggest lakes (Tanganyika, Malawi and Victoria) in the East African Rift Valley (Turner *et al.* 2001; Genner *et al.* 2004).

Radiation into the hundreds of ecologically, morphologically and behaviourally diverse species found today began shortly after the formation of the lake-basins (Turner 2007; Genner *et al.* 2007c) making the species within each of the East African Great Lakes more closely related to each other than they are to the cichlids of the other lakes – the great similarities in morphological diversity between lakes being a result of parallel evolution rather than evolutionary relationships (Salzburger *et al.* 2005; Turner 2007). Recent phylogenetic analyses have brought into question the acceptance that the cichlids within each lake are

monophyletic (e.g. Meyer *et al.* 1990; Moran *et al.* 1994; Salzburger *et al.* 2005). It appears more likely that the assemblages and species flocks of each lake are the result of multiple invasions by divergent lineages of riverine cichlids from the surrounding catchments, which have resulted in the formation of new adaptive radiations alongside existing cichlid tribes (Salzburger *et al.* 2005; Turner 2007; Genner *et al.* 2007c), or the production of new specialist groups through hybridisation with existing habitat specialists (Joyce *et al.* 2011). Different cichlid tribes, lineages and groups speciate more or less readily and at different rates and there is little doubt that their evolution has been shaped by both natural and sexual selection, possibly under a variety of geographical scenarios (Stauffer *et al.* 1995; Turner *et al.* 2001). These fish therefore provide a great model system with which to test theories of diversification and speciation.

Great Lakes and cichlid tribes

Despite its age of several million years and the great phenotypic, behavioural and genetic diversity of cichlid species within its assemblage of 12 morphologically and phylogenetically distinct tribes, the species richness of Lake Tanganyika is relatively poor (probably around 250 species) compared to the other East African Great Lakes (Salzburger *et al.* 2005). Lake Victoria contains about twice as many species, but all are from a single tribe, the haplochromines (Salzburger *et al.* 2005; Turner 2007). There is still debate as to whether the endemic fauna arose in the 15,000 years since the lake last dried out (Johnson *et al.* 1996; Stager & Johnson 2008), or whether the endemic species flock is older, as suggested by molecular dating (100,000 years, Verheyen *et al.* 2003). However, Lake Victoria is estimated to be 0.4 – 0.75 MY old (Salzburger *et al.* 2005; Turner 2007) and it is likely that most of its cichlid diversity has arisen within 0.25 MY at the very most (Joyce *et al.* 2005), making it by far the youngest of the Great Lake cichlid radiations. With the exception of a couple of Lake Tanganyikan tribes, adaptive radiations of non-haplochromine cichlids rarely attain more than a dozen species (Salzburger *et al.* 2005). This makes the haplochromines one of the most interesting, and therefore extensively studied, groups. Of the four haplochromine lineages, three are widely distributed in Africa but contain only a few species. It is the 'modern haplochromines', in which the characteristic maternal mouthbrooding of offspring and 'true egg-spots' arose, that has repeatedly given rise to species flocks in East African lakes, including those of Lakes Malawi and Victoria (Salzburger *et al.* 2005).

Lake Malawi is intermediate in terms of the age of the lake and cichlid radiation. It contains only two cichlid tribes: a few species of tilapines (Won *et al.* 2006) alongside 500 – 1000 species of haplochromines (Salzburger *et al.* 2005; Konings 2007; Joyce *et al.* 2011). Six distinct mitochondrial clades have been identified in the modern haplochromines (excluding *Serranochromis* and *Pseudocrenilabrus*) of Lake Malawi (Joyce *et al.* 2011), five of which represent the major endemic multi-species groups of habitat specialists (Genner & Turner 2012), the other representing the non-endemic generalist riverine species *Astatotilapia calliptera*, found widely distributed in the Lake Malawi catchment, and beyond (Joyce *et al.* 2011). This flock is not only the most speciose and diverse radiation

of haplochromine cichlids, but also forms the largest rapid (0.7 – 4.6 MY old, Won *et al.* 2006; Genner *et al.* 2007c) adaptive radiation of vertebrates known (Turner *et al.* 2001).

Modern haplochromine cichlids: speciation and diversity

Although thought to be the source of the haplochromines, which derived from a specialist lacustrine endemic after its colonisation of rivers, Lake Tanganyika only contains a single group of about about two dozen species (Salzburger *et al.* 2005). In contrast, the haplochromine radiations of Lakes Malawi and Victoria are vast, made up of hundreds of ecologically diverse species. In Lake Malawi, discordance between the phylogenies reconstructed from mitochondrial and nuclear markers are suggestive of hybridisation among invading riverine lineages and lacustrine endemics (Joyce *et al.* 2011) and between distinct groups of endemic habitat specialists (Genner & Turner 2012) – both of which may have contributed significantly to the production of novel phenotypes and new specialist lineages in the course of this adaptive radiation (Joyce *et al.* 2011; Genner & Turner 2012).

It is clear that natural selection has acted in the evolution of all cichlid species flocks. It also seems likely, however, that sexual selection has contributed the production of the larger cichlid radiations (Andersson 1994; Salzburger *et al.* 2005; Wagner *et al.* 2012). The presence of many closely related species that differ mostly in male courtship traits suggest that divergence in female preference may have driven divergence in male colour cues in some of the most species rich lineages (Turner 1999; Maan *et al.* 2004; Maan & Seehausen 2011). Sensory drive may have been important for the divergence of female preferences and male secondary sexual traits in shallow water Lake Victoria cichlids (Seehausen *et al.* 2008). However, this mechanism of divergence relies on habitat heterogeneity and in the cases of fish this is often related to the light environment (*e.g.* Boughman 2001; Seehausen *et al.* 2008). Although the dim narrow-spectrum light in Lake Victoria constrains visual communication which can impose selection on visual system and male nuptial displays, the light environment in the species rich shallow-waters of Lake Malawi is broad spectrum and intense with gradual changes between habitats and depth. This contrast between lakes means that the environmental constraints on signal perception and evolution that are found in Lake Victoria do not act on divergence in Lake Malawi cichlids from the same shallow habitats (Smith *et al.* 2012). Recent research suggests that two of the main factors that contribute to the largest cichlid radiations are depth of lake, which provides both different habitats and different light environments, and potential for sexual selection in colonising lineages (Wagner *et al.* 2012). There is not only great diversity of shallow-water Lake Malawi cichlids, but also many deep/dark adapted species that, in contrast to the brightly coloured shallow-water species, possess monochromatic male visual courtship traits that differ among species (*e.g.* Genner *et al.* 2007b). Additionally, species of *Astatotilapia*, a genus basal to both the Lake Malawi and Lake Victoria haplochromine radiations, are generalists and are found in both turbid and clear water environments in and around Lake Malawi. It therefore seems possible that sensory drive could have been important during the first stages of adaptive radiation of Lake Malawi cichlids, on axes such as murky-clear shallow water and shallow-deep clear water. However, the possibility of sensory

drive has been little investigated in these groups.

The importance of colour differentiation in cichlid speciation is not limited to female mate choice. Colour differences may not only maintain reproductive isolation between sympatric species (Table 1), but may also aid in the co-existence of ecologically similar species by reducing aggression among heterospecifics (van Doorn *et al.* 2004; Mikami *et al.* 2004; Dijkstra *et al.* 2005), adding to species richness without ecological diversification. It has been proposed that African lacustrine cichlid radiations may evolve in three major phases, initially diversifying on the basis of gross habitat preferences after the invasion of a new lake by the ancestral species, then by fine-scale trophic adaptation as all available niches within each habitat are occupied, and only later on through divergence in sexual signals and preferences among closely related taxa that share habitat preferences and trophic adaptations (Danley & Kocher 2001).

Ecologically driven sympatric speciation has been proposed in non-haplochromine cichlids (Schliewen *et al.* 1994; Barluenga *et al.* 2006). Theories of sympatric divergence and speciation of haplochromines, on the other hand, often rely on divergence of female mate preferences to drive divergence of male secondary sexual traits with minimal ecological divergence (*e.g.* Seehausen & van Alphen 1999). Although such mechanisms may help to explain the high diversity of ecologically similar species, there is as yet little convincing evidence. However, a lack of genetic population structuring in pelagic species with lake-wide distributions and little restriction to gene flow, makes the possibility of intralacustrine allopatric speciation seem unlikely and it has been suggested that sympatric speciation may have contributed to the high species richness of these monophyletic groups with pelagic ancestors (Shaw *et al.* 2000).

Various theories and scenarios of allopatric speciation, often involving climatic and geologically driven changes in lake water level (see Sturmbauer *et al.* 2001), have been suggested to explain the diversity of different groups of habitats specialists that contain multiple currently sympatric species. At the extremes of water level fluctuations, for example during the last ice age, major falls may have split deep lakes into multiple smaller basins, allowing for vicariant speciation before water levels rose again re-joining the basins into a single connected water body (Genner *et al.* 2007c). Smaller fluctuations have been more frequent, influenced by rainfall, temperature and evaporation, and it would appear that intralacustrine divergence is common after the breaking apart of littoral habitats (rocky areas and possibly other shallow benthic habitats) and their cichlid populations (van Oppen *et al.* 1997; Koblmüller *et al.* 2012). Isolation and re-connection of satellite lakes is another likely outcome of lake level changes and may result in divergence of peripherally isolated populations (Genner *et al.* 2007a). If full reproductive isolation evolves during divergence in allopatry, any of the suggested methods of geographic isolation (vicariant, peripatric and intralacustrine habitat patchiness) may result in an increase in alpha diversity, *i.e.* the number of sympatric species, each time populations are brought back into secondary contact (van Oppen *et al.* 1997; Genner *et al.* 2007a,c; Genner *et al.* 2010). The African lake cichlids are no exception to the difficulty of assigning species status to allopatric populations and estimates of species number may

therefore vary because of the differential assignation of species status to allopatric variants in different lineages and lakes (Turner *et al.* 2001; Genner *et al.* 2004; Genner & Turner 2005; Salzburger *et al.* 2006), partly due to how extensively they have been studied.

Mating systems

In haplochromine cichlids, female limited parental care by mouthbrooding (protection of vulnerable eggs and developing fry in the buccal cavity for several weeks after fertilisation) and in some species guarding of free-swimming offspring after release, has resulted in a skew in parental investment between the sexes (Parker & Kornfield, 1996; Amcoff *et al.* 2013). Sexual dichromatism and larger, highly aggressive and territorial, 'showy' males are characteristics of the majority of species (with the notable exception of the maternally mouthbrooding sexually monomorphic haplochromine *Tropheus* from Lake Tanganyika). Parentage analysis of broods from wild caught females and laboratory based mate choice experiments, indicate that multiple paternity is common and females may often not be limited to one sire per clutch of eggs (Kellogg *et al.* 1995; Parker & Kornfield, 1996; Kellogg *et al.* 1998; Tyers & Turner 2013). Alternatively, females may chose a single male per clutch (see Genner *et al.* 2007a; Egger *et al.* 2008; Chapter 2 this thesis), but spawn with multiple males over the course of their reproductive life. In both cases, males will mate with multiple females. The assessment of multiple territory holding males before spawning is common and the lek-like mating systems of many species allow for the comparison, by females, of males in close proximity. In reflection of the ecological diversity of haplochromine cichlids, there is great variety in the locations in which spawning takes place: In rocky-shore species, males defend caves throughout the year, which provide shelter from predators and a safe place to spawn (Genner & Turner 2005). Benthic species associated with soft substrates (sand/mud), and open water species that migrate inshore to breed, often form more temporary seasonal leks where males construct (or obtain) and defend 'bowers'. These, often elaborate structures, which are made out of sand/mud act as extended phenotypic traits attracting females and providing a place for spawning (McKaye 1990). In some open water species, breeding may take place in the water column, in which case rather than being laid onto the substrate, eggs are released a few at a time in short strings (pers. obs.) which allows the female time to turn and collect them before they disperse.

The well studied rocky-shore cichlids

Lineages of specialist rocky-shore cichlids are among the most speciose and account for a large proportion of the endemic diversity of each lake (Genner *et al.*, 2004). Not only did the generalist riverine ancestor that emerged in the Lake Tanganyika catchment spread through the river systems of East Africa, seeding younger lakes along its path with modern haplochromine cichlids, it also re-invaded Lake Tanganyika where, in the presence of pre-existing radiations of other tribes, it produced *ca.* 25 species – the rocky-shore Tropheini (Salzburger *et al.* 2005). In Lake Malawi there are about 13 genera of rocky-shore (or 'mbuna') cichlids, containing about a third (approximately 327) of the lakes haplochromine

species (Konings 2007; Genner *et al.* 2012). Recent research suggests that the mbuna may have also originated after re-invasion of the lake by a riverine generalist: Discordance between mitochondrial and nuclear DNA indicates that this group of highly specialised lacustrine species may have arisen after hybridisation between lacustrine endemics and a re-invading riverine lineage (Joyce *et al.* 2011).

In contrast to the sexually monomorphic Tropheini, most rocky-shore cichlids, like most other haplochromines, are sexually dimorphic. It is therefore likely that female choice plays a major role in pair formation (Andersson 1994). Hybrids are easily produced under no choice conditions and reproductive isolation is maintained by female mate preference for conspecific males (Table 1). Because of the potential for evolution by sexual selection provided by this mating system (Andersson 1994), along with the great colour diversity and overlap in basic ecology among many species (Genner & Turner 2005), much of the research into the role of mate preferences and secondary sexual trait diversification in speciation has focused on these taxa (Table 1). Recognition of conspecifics (during both mate selection and territorial defence / aggressive competition) is often associated with distinctive male courtship colours (Table 1; Genner *et al.* 1999; Pauers *et al.* 2008; Young *et al.* 2009). However, it should be noted that among some species, female preference does not appear to be linked to male colour despite this being one of the most obvious phenotypic difference. There is evidence to suggest that a variety of other cues (melanin pattern, behaviour, shape, smell, sound; Table 1; Amorim *et al.* 2004; Simões *et al.* 2008) may play an important role in species recognition.

Despite the relative paucity of rocky-shore species in Lake Tanganyika, intraspecific geographic colour diversity is just as high (*e.g.* Salzburger *et al.* 2006) as in rocky-shore species from the other lakes. Preference to remain in a habitat that is patchily distributed along the shoreline, as well as a lack of dispersal phase, has led to strong population genetic differentiation (Sturmbauer & Meyer 1992; Verheyen *et al.* 1996; van Oppen *et al.* 1997; Arnegard *et al.* 1999; Markert *et al.* 1999; Rico & Turner 2002; Egger *et al.* 2007; Sefc *et al.* 2007) and with the obvious differences in male colour among populations, a role for intralacustrine allopatric speciation by colour diversification seems likely. Laboratory based mate choice experiments between some local endemic forms show a general trend of divergent mate preference (Table 1). However, despite differences in cues associated with assortative mating between sympatric species, *i.e.* male courtship colour, complete assortative mating is rarely observed. Mechanisms allowing for continued divergence under secondary contact, for example by relaxed competition between males of different colours in combination with divergent female mate preferences, or reinforcement or reproductive character displacement, have often been invoked for the completion of speciation and co-existence of so many different colour species in sympatry (van Doorn *et al.* 2004; Mikami *et al.* 2004; Seehausen & Schluter 2004).

Offshore species of Lake Malawi

There is little restriction to gene flow among populations in the offshore habitats of lakes, limiting the potential for intralacustrine allopatric speciation. The high diversity of species in open-water habitats of

Lake Malawi may therefore seem surprising. Included in this group, which forms three distinct mtDNA clades, are the predatory pelagic genera (ca. 15 species of *Rhamphochromis*, Genner *et al.* 2007a; Konings 2007; and 19 species of deeper-water *Diplotaxodon* / *Pallidochromis*, Konings 2007) and the shoaling zooplanktivores (or 'utaka' – dominated by the genus *Copadichromis*, Genner *et al.* 2012). Peripatric speciation of a pelagic species in Lake Malawi has been convincingly demonstrated, but relies on species becoming trapped in satellite lakes which are likely to result from the isolation of inshore lagoons (not the usual haunt of offshore species) during lake level drops (Shaw *et al.* 2000; Genner *et al.* 2007a). For pelagic species that do not migrate inshore to breed, vicariant speciation after the splitting of a population by large scale geological/climatic activity, such as the division of larger lakes into multiple smaller lake basins for extended periods, may be more likely (Shaw *et al.* 2000; Genner *et al.* 2007b). However, these mechanisms do not exclude the possibility of sympatric speciation in these groups (Shaw *et al.* 2000) and the recent discovery of what appears to be a pair of *Rhamphochromis* species isolated in a small crater lake in Tanzania may add to the evidence for sympatric divergence (Turner pers. comm.).

Table 1. Female mate choice experiments testing reproductive isolation in haplochromine cichlids. Including studies of assortative mating among sympatric species, allopatric populations and studies designed to investigate the cues involved in assortative mating. Grey text indicates studies based on non-spawning measures of preference (females association time or reaction to male displays), which can indicate recognition of own males. But, without observations of spawning these can not be compared directly to studies that show the degree of reproductive isolation by measuring spawning. To give a relative idea of the strength of assortative mating results have been categorised: complete = 100%, very strong = >90%, strong = 70-90%, weak = 50-70% (if significant). * = asymmetric assortative mating among populations or variation in strength where assortative mating is dependent on the degree of difference between populations in pairwise comparisons.

Study	Habitat	Associated Great Lake (catchment)	Experiment type		<i>n</i> studies	Male differences	Assortative	Cues implicated	Refs.	
			Assay	Cues available						
Sympatric	Shallow-rocky	Malawi	Spawning	Full contact	2	Colour/melanin pattern	Complete	Visual (colour/pattern)	1,3	
				Visual only	1	Melanin pattern	Complete	Visual (pattern)	4	
			Observation	Visual only	1	Colour/melanin pattern	Yes	Visual (colour/pattern)	2	
		Victoria	Spawning	Full contact	1	Colour/melanin pattern	Complete	Visual (colour/pattern)	5	
			Observation	Visual only	2	Colour/melanin pattern	Yes	Visual (colour/pattern)	5,6	
Allopatric	Shallow-rocky	Malawi	Spawning	Full contact	2	Carotenoid/melanin pattern	Random – strong	Visual (colour/pattern)	7,8	
			Observation	Visual only	2	Body/dorsal fin colour	Yes	Visual (colour)	9,10	
		Tanganyika	Spawning	Full contact	2	Body colour	Random – very strong *	Visual (colour)	11,12	
	Pelagic	Malawi	Spawning	Full contact	1	Size/ventral body-fin colour	Complete (size matched)	Visual possible	13	
Cues	Shallow-rocky	Malawi (mix?)	Observation	Visual only	2	Colour/melanin pattern	Yes	Visual (colour/pattern)	14,15	
			Malawi (symp)	Spawning	Full contract	1	Dorsal fin colour	Complete	Visual (colour)	3
				Visual/olfactory	1	Dorsal fin colour	Very strong	Visual (colour)	3	
		Visual only		1	Dorsal fin colour	Random	Olfactory/auditory	3		
		Malawi (symp)	Observation	Visual only	1	Colour/melanin pattern	Yes	Visual (colour/pattern)	2	
			Monochromatic	1	(Colour)/melanin pattern	Yes	Visual (pattern)	2		
			Olfactory/auditory	1	(Colour/melanin pattern)	No interaction	Visual necessary	2		
			Olfactory only	1	(Colour/melanin pattern)	No interaction	Visual necessary	2		
		Victoria (symp)	Observation	Visual/olfactory	1	Body colour/melanin pattern	Yes	Visual (colour/pattern)	16	
			Monochromatic/olfactory	1	(Colour)/melanin pattern	No	Visual (colour)	16		
Malawi (allo)	Spawning	Full contact	1	Dorsal fin colour	Weak – complete *	Visual (dorsal fin colour)	8			
	Monochromatic	1	None	Weak – very strong *	Visual (non-colour)	8				
	Visual only	1	Dorsal fin colour	Random	Non-visual	8				

References: 1) Knight & Turner 1998; 2) Jordan *et al.* 2003; 3) Plenderleith *et al.* 2005; 4) Kidd *et al.* 2006; 5) Seehausen 1997; 6) van der Sluijs *et al.* 2008; 7) Knight & Turner 2004; 8) Blais *et al.* 2009; 9) Pauers *et al.* 2010; 10) Pauers & McKinnon 2012; 11) Egger *et al.* 2008; 12) Egger *et al.* 2010; 13) Genner *et al.* 2007a; 14) Couldridge & Alexander 2002; 15) Jordan 2008; 16) Seehausen & van Alphen 1998.

Study taxa

Non rock-restricted benthic habitat specialists of Lake Malawi

Perhaps the most ecologically diverse group of Lake Malawi cichlids are found in the non-rocky shore benthic habitats and include specialists adapted for many environments – from sandy / muddy substrates and the rock-sand interface in both the shallow- and deep-benthic zones to macrophyte-dominated habitats (Anseeuw *et al.* 2008; Genner *et al.* 2012). These fish account for approximately half (474 species in 38 genera) of the Lake Malawi haplochromines (Genner *et al.* 2012) and comprise two mtDNA clades: the dark-adapted 'deep-benthic' (part of the mbuna-dominated clade) and the 'shallow-benthic'. However, these groups are generally morphologically similar and are closely related based on nuclear markers (Loh *et al.* 2008; Genner *et al.* 2012) – again mito-nuclear discordance suggests a role for hybridisation in the evolution of new lineages of habitat specialists (Genner & Turner 2012). Further to this, greater morphological diversity of the deep-benthics, as compared to the mbuna and shallow-benthic groups, has been demonstrated. This supports the hypothesis that radiation into the darker and deeper habitats of the lake occurred after hybridisation of mbuna and the non-rocky-shore shallow-benthic cichlids.

Interspersal of rocky areas, sandy beaches, muddy river mouths and reedy areas along the shore line, provides the potential for intralacustrine allopatric divergence of non-rock-restricted benthic habitat specialists within Lake Malawi. However, with regards to genetic population structuring and geographic phenotypic variation, sandy-shore species appear to be more similar to open-water species (*e.g.* Shaw *et al.* 2000; Taylor & Verheyen 2001; Genner *et al.* 2007a; Anseeuw *et al.* 2008) than the mbuna – showing little differentiation over large geographic distances (Pereyra *et al.* 2004; Anseeuw *et al.* 2008). It has been suggested that population structuring may be more the result of habitat preference than lineage. Whereas non-mbuna that inhabit sandy-shores, or the rock-sand interface may show little population structuring, higher levels of structuring have been found in species of the same genus that inhabit the rocky-shores (Pererya *et al.* 2004). Therefore, although the model of allopatric speciation based on habitat fragmentation may be equally applicable to rocky-shore non-mbuna as to mbuna, population genetic evidence from these more mobile taxa suggests that it may not be as common (Pererya *et al.* 2004). The potential for peripatric speciation, as suggested for pelagic species, remains to be investigated.

The basal generalist

Generalist riverine species from the genus *Astatotilapia* are likely to represent the body plan and lifestyle of the ancestors of both the Lake Victoria and Lake Malawi haplochromine species flocks. *A. burtoni* (Günther 1894), found in and around Lake Tanganyika, appears to be the closest living relative to the Lake Victoria super-flock and *A. calliptera* (Günther 1894), widely distributed in the Lake Malawi catchment (Konings 2007; Joyce *et al.* 2011), as well as other rivers in East Africa, is widely believed to represent the sister group of the Lake Malawi species flock (Meyer 1993; Moran *et al.* 1994; Turner 2007;

Joyce *et al.* 2011). This genus may therefore be a suitable model for investigating the beginnings of adaptive radiation and speciation of the haplochromine cichlids. Throughout its range in the Lake Malawi catchment, male *A. calliptera* are predominantly yellow, although a population with blue/grey males is known from a single offshore island in the middle of the lake. Additionally, populations from the Eastern-flowing Rovuma river system differ slightly, males tend to be a little more drab and possess a more greenish hue with more intense orange egg spots, rather than the bright yellow of Lake Malawi males. Therefore, although not as pronounced or as common, it is possible for populations of this species to differ in male colour, in similar ways to that found among allopatric populations of lacustrine habitat specialists. This may suggest that the potential for allopatric speciation by male colour divergence was passed on to the lacustrine radiations, despite being restricted in basal lineages because of their generalist life style, wide distribution in river systems and resulting potential for gene flow among populations.

Recently discovered populations of *Astatotilapia* in isolated crater lakes in Tanzania, to the North of Lake Malawi (see Chapter 5, this thesis, for more detail of these lakes and their fish), may provide a new model system with which to investigate the processes of speciation in haplochromine cichlids. These lakes contain previously unknown *Astatotilapia* colour variants (completely black with a red submarginal dorsal fin stripe being one of the most striking) allowing for further investigation of allopatric divergence and speciation of *Astatotilapia*. However, more interestingly, some lakes also contain putative species flocks with individuals differing from *A. calliptera* in size, shape and colour. Therefore, these systems may provide replicated cases of the beginnings of adaptive radiation by sympatric speciation in a basal lineage with strong potential for sexually selected divergence, that has produced some of the largest and most rapid adaptive radiations.

Summary

Speciation equates to reproductive isolation, the evolution of which may be driven by a variety of abiotic and biotic selective forces under different geographical distributions. To test whether certain theoretical scenarios of divergence are plausible, and to attempt to identify routes to speciation, biologists often turn to recently diverged groups of organisms. In many such groups, sexual selection has been invoked to account for the speed of diversification and the observed variety of male secondary sexual characteristics between species.

Several major theories of cichlid evolution have derived from studies of population divergence and behavioural interactions in the habitat specialist lacustrine endemics. Intralacustrine speciation resulting from allopatric divergence of male courtship traits may be likely, although it has been suggested that such divergence may only be possible in the most philopatric of shallow benthic habitat specialists. Due to the focus on the highly colour-diverse rocky shore species, less attention has been granted to the many other lineages, habitat generalists or non-rocky shore habitat specialists, particularly when it comes to testing for population based divergent mate preferences and assortative mating. A few studies have investigated

speciation in pelagic Lake Malawi taxa in which, due to a lack of potential barriers to gene flow in open water habitats, intralacustrine allopatric speciation seems unlikely. Vicariant and peripatric divergence may have contributed to the diversity of these groups, and sympatric speciation may also be acting. This leaves shallow benthic species relatively little investigated. Because of the patchiness of their preferred habitat around the lake shore, it is possible that similar mechanisms driving intralacustrine divergence of rocky-shore species may apply. However, non-rocky shore benthic habitat specialist tend to be more mobile and barriers to gene flow and population structuring may be more akin to pelagic species. Additionally, unlike the rocky shore species, the sandy/muddy shore species may more frequently become isolated in satellite lakes formed from shallow lagoons and muddy bays. Therefore, it is possible that part of the diversity of such species is also the result of peripheral isolation rather than intralacustrine allopatric speciation. Although allopatric colour divergence may be most striking in the rocky-shore fish, it is found in other lineages, including those phylogenetically basal to the Lake Malawi radiation. Although it has been suggested that signal and preference divergence may be limited to a third stage of speciation among the habitat specialist endemics, the potential for such divergence may have been passed on by colonising lineages.

Main aims and overview of experimental chapters

The first experiments presented here test the application of theories of allopatric cichlid divergence and speciation, developed from empirical investigations of habitat specialist, to little-investigated lineages:

- Peripheral isolation of populations in satellite lakes has occurred in the catchments of both lakes Malawi and Victoria and although divergence of such populations is often apparent, tests of assortative mating are rare. We aim to test mate preferences of populations of a specialist Lake Malawi species from shallow muddy habitats. Further evidence of reproductive isolation among main lake and satellite lake forms will add to the evidence for peripheral isolate speciation having added to the diversity of large lacustrine radiations by allowing for allopatric divergence of mobile taxa which tend to experience weak geographic isolation within the large lakes.
- To date, tests of reproductive isolation among colour divergent allopatric populations have been biased towards highly colour-diverse derived rocky-shore specialists. We aim to test the mate preferences of populations of a basal Lake Malawi cichlid which also show some geographic variation in male colour. Evidence for an association between signal and preference divergence in this lineage would indicate that preference divergence related to signal divergence in allopatry may have operated throughout the adaptive radiation of the Malawian cichlids.

The majority of cichlid mate choice experiments have focused on population/species level preference to test for assortative mating and reproductive isolation. The second aim of this thesis is to test individual consistency of mate choice to identify whether choices are consistent within individuals and preferences

divergent among them, or whether variation within populations reflects the variation within individuals. By focusing on the preferences of individuals we aim to increase our knowledge of the patterns of variation in taxa which have demonstrated only partial assortative mating among divergent populations.

Finally, sympatric divergence has been proposed as a mechanism for speciation of cichlids, however, there is little convincing evidence from studies of haplochromines. We aim to test mate preferences in morphologically and genetically diverse putative young species flocks from isolated crater lakes in Tanzania:

- Firstly, we test whether a crater lake *Astatotilapia* is reproductively isolated from *A. calliptera* from the Lake Malawi catchment. This will allow us to predict whether geographic isolation and phenotypic divergence is likely to result in reproductive isolation in this lineage.
- Where crater lakes are inhabited by multiple divergent forms, we aim to test whether these represent reproductively isolated biological species, morphs or incipient species in the process of sympatric divergence and speciation. These recently discovered cichlids may provide a new model system with which to investigate sympatric divergence of haplochromine cichlids.

Chapter 2

'Peripheral isolate speciation of a Lake Malawi cichlid fish from muddy habitats'

Tyers AM, Bavin D, Cooke GM, Griggs C & Turner GF. 2014. *Evolutionary Biology*. DOI: 10.1007/s11692-014-9277-4

Non rocky-shore benthic habitat specialist taxa account for a large proportion of the species richness of Lake Malawi. Many species experience patchiness of preferred habitat comparable to that of the rock-restricted species, suggesting the potential for intralacustrine allopatric speciation. However, they also tend to show much lower levels of genetic population structuring, more akin to that found in the more mobile specialist pelagic species. Shallow-benthic species that inhabit lagoons and the mouths of inflowing rivers may also be among the most likely to become trapped in satellite lakes. Here we investigate peripatric divergence of a specialist Lake Malawi species from shallow muddy habitats. The satellite lake form showed significant morphological differentiation associated with a shift in diet, substantially reduced mitochondrial DNA diversity and no haplotype sharing with populations from the main lake system. Additionally, we found a high degree of assortative mating and preference for territorial males to attack other males of their own population. This study adds to the evidence for high levels of reproductive isolation among main lake and peripherally isolated populations, suggesting that peripatric speciation may have added to the diversity of large lacustrine radiations.

Chapter 3

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

Tyers AM & Turner GF. 2013. *Biological Journal of the Linnean Society*. 110: 180-188.

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 therefore providing a good model for the ancestor of the lacustrine radiation, *Astatotilapia calliptera* also shows allopatric variation in colour. Here, we demonstrate that this signal divergence is associated with tendencies of females to mate with males of their own population and also for males to 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, becoming more important in, rather than being limited to, speciation of lacustrine habitat specialists.

Chapter 4

'Individual variation in female mate preference in the Lake Malawi cichlid fish *Astatotilapia calliptera*'

A preference to mate with similar types of individuals over different types, assortative mating, is a step towards the evolution of reproductive isolation. Therefore, mate preferences may be a major force in the process of speciation – individual variation in preference within a population has the potential to drive divergence. There is evidence of variation in mate preference among female cichlid fish. However, little is known about the nature of such variation. In this study, we test consistency of female mate choice to try to identify whether variation within a single population is best explained by variation within or among individuals and find little evidence for consistent individual variation in preference, suggesting that variation within populations may be mostly due to variation within individuals.

Chapter 5

'Diversification and speciation of haplochromine cichlids in small isolated crater lakes in Tanzania'

The small, ecologically homogeneous, crater lakes North of Lake Malawi, have recently been discovered to contain populations of previously unknown *Astatotilapia*. These putative species flocks may provide a model for investigating both allopatric and sympatric divergence of the generalist riverine fish that are sister groups to the largest lacustrine radiations of cichlids in East Africa - the haplochromine flocks of Lakes Malawi and Victoria. First we demonstrate very high levels of reproductive isolation between *A. calliptera* from Lake Malawi and one of the common colour variants from these small lakes. However, most interestingly, tests of female mate preference for different types of males from a single crater lake found little consistency of female choice, despite significant differentiation of types by morphology, ecology and neutral genetic markers. One potential explanation, which requires further investigation, is that these fish are not yet reproductively isolated by female mate choice but may be in the process of sympatric divergence with assortative mating due to strong habitat preference and greater success of locally adapted males.

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Chapter 2



Lethrinops sp. 'chilingali' male

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.

Key words: Aggression, Allopatric, Cichlid, Lake Malawi, Mate choice, Peripheral isolate, Speciation

Introduction

Geographical separation of populations is important for divergence and speciation (Mayr 1963; Rice & Hostert 1993; Barraclough & Vogler 2000; Coyne & Orr 2004; Dawson & 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 & Hostert 1993; Coyne & Orr 2004). In Lakes Malawi, Tanganyika and Victoria, cichlid fish have diversified into over 1000 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 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 & 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 & 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 & 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 & 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 & 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 & 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; Odhiambo *et al.* 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 & 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 & 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 & 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 & 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 & 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 & Trewavas 1989). Thus, unusually for a Lake Malawi haplochromine cichlid fish, *L. lethrinus* is a relatively distinctive species (Appendix II). 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 34m in some areas (Eccles & 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 160mm 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 & 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 x 1km with a maximum depth of 5.1m) satellite lake located 11.5km from the western shore of, and approximately 30m 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 & 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 & 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.

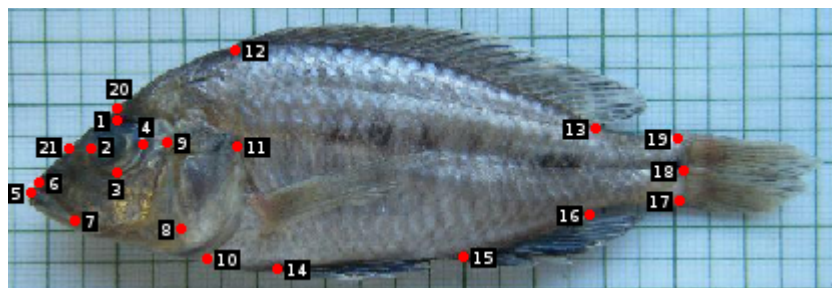


Figure 1. Landmarks used for geometric morphometric analysis: 1-4 = upper, anterior, lower and posterior point of eye; 5 = tip of rostrum; 6 = tip of upper lip; 7 = posterior tip of upper jaw; 8 = most posterior and ventral extent of gill cover; 9 = origin of gill cover; 10 = base of isthmus; 11 = most 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 & 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 (18-35mm SL), medium (40-55mm SL) and large (56-110mm 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 KJ496145 - KJ496205) 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.

An extended phylogenetic analysis, including extended haplotype networks with additional sequences from other *Lethrinops* species (obtained from GenBank), and phenotypic comparisons of the species which appear most closely related to our study taxa, can be found in Appendix II.

DNA extraction and sequencing

DNA was isolated from ethanol preserved fin tissue by salt extraction (Aljanabi & Martinez 1997). An approximately 1000 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 25µl reactions containing 1µl DNA, 2.5µl 5x PCR buffer, 2.5µl dNTPs (1mM), 1µl each primer (10µM), 1.6µl MgCl (25mM), 1 unit Taq, 15.2µl SDW H₂O. PCR conditions were: 1 min at 95°C, followed by 34 cycles of 30 sec at 95°C, 30 sec at 50°C, 60 sec 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 & Xie 2001). DnaSP v.5.10.01 (Librado & 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 2m 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 three 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 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 9mm/10.5g (7.5/24.9% of mean size), while the smaller lab-bred fish were matched to within 4mm/1.2g (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 hetero-spawning 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 9-111 (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.

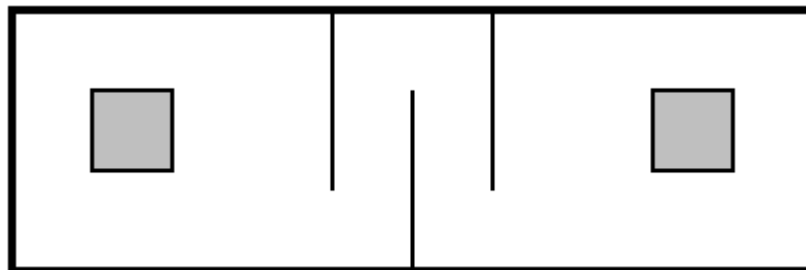


Figure 2. Plan view of mate choice experimental tank set up. Three offset baffles in the middle of the 2m long tank separated the tank into two distinct areas to encourage co-existence of two males by reducing visual contact once territoriality had been established. Platforms at each end provided a focal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

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 0.9m 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 0.3m x 0.2m) 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 hours 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 minute 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).

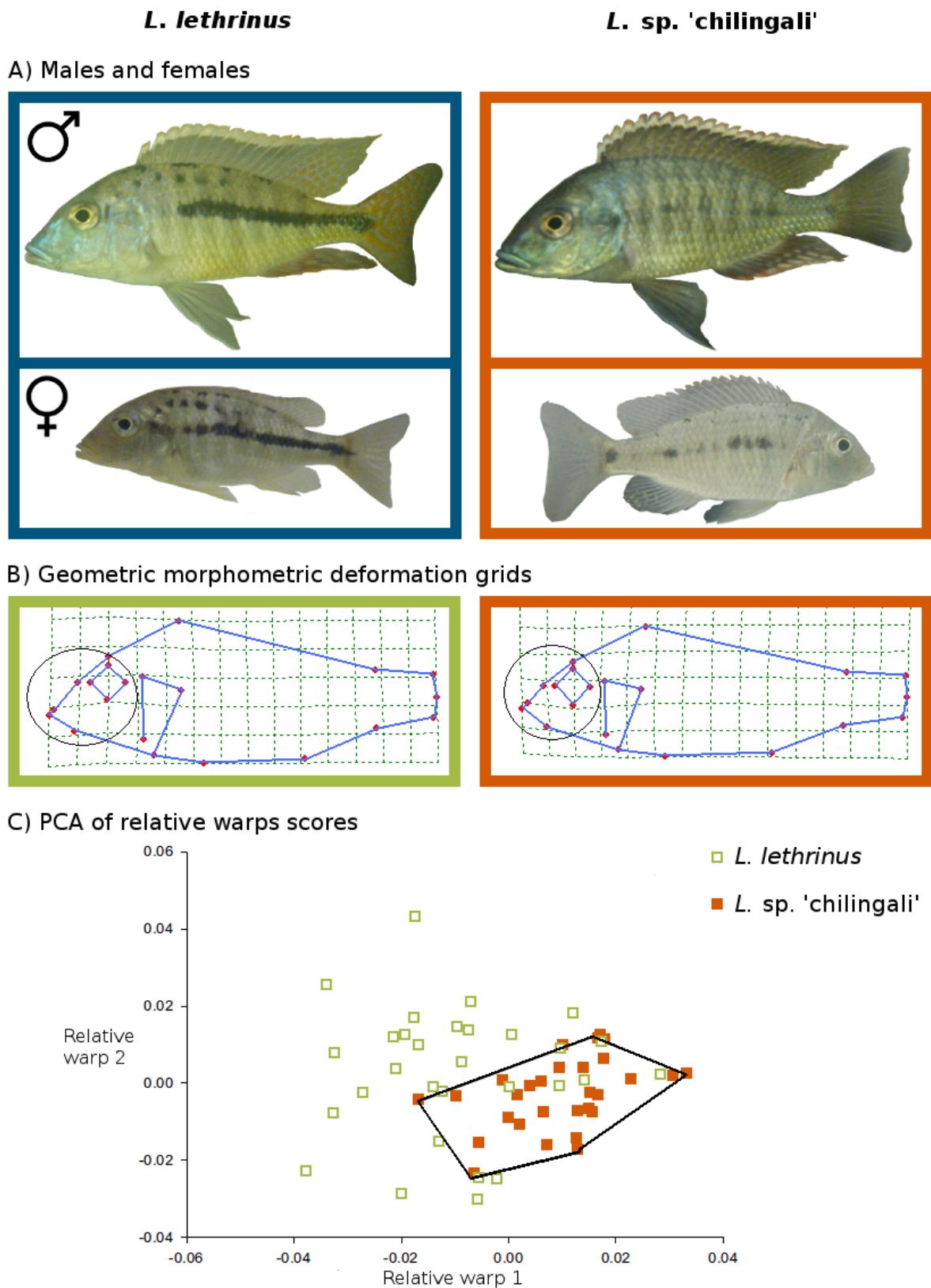


Figure 3. Phenotypic differences between study species, *L. lethrinus* and *L. sp. 'chilingali'*. **A)** Males and females. **B)** Deformation grids from geometric morphometric analysis (see Fig. 1) show that the main difference among the populations from the main Lake Malawi system and Lake Chilingali 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.

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.

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 & <i>n</i> Standard Length (cm)	<i>L. sp.</i> 'chilingali'			<i>L. lethrinus</i>
	Small (<i>n</i> = 17) 1.8 – 3.5	Medium (<i>n</i> = 17) 4.0 – 5.5	Large (<i>n</i> = 17) 5.6 – 11.0	Large (<i>n</i> = 14)
Chironomidae	00.0	00.0	00.0	027.3
Chaboridae	84.4	98.6	91.1	00.0
Odonata	00.0	00.0	08.8	00.0
Other insect	05.7	00.2	00.0	02.6
Cladocera	09.6	01.1	00.1	11.7
Copepoda	<0.5	<0.5	<0.5	23.4
Rotifera	<0.5	<0.5	<0.5	00.0
Mollusca	00.0	00.0	00.0	10.4
Oligochaeta	00.0	00.0	00.0	23.4
Algae	<0.5	<0.5	<0.5	00.0
Detritus	00.0	<0.5	00.0	01.3

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 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).

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

Species / pop.	<i>n</i> mtDNA sequences	Haplotype			F_{ST} matrix			
		<i>n</i>	unique	Hd	<i>L. sp. 'chilingali'</i>	<i>L. lethrinus</i>	Malawi	Malombe
<i>L. sp. 'chilingali'</i>	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	-

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 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, Appendix III).

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

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

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, Appendix III).

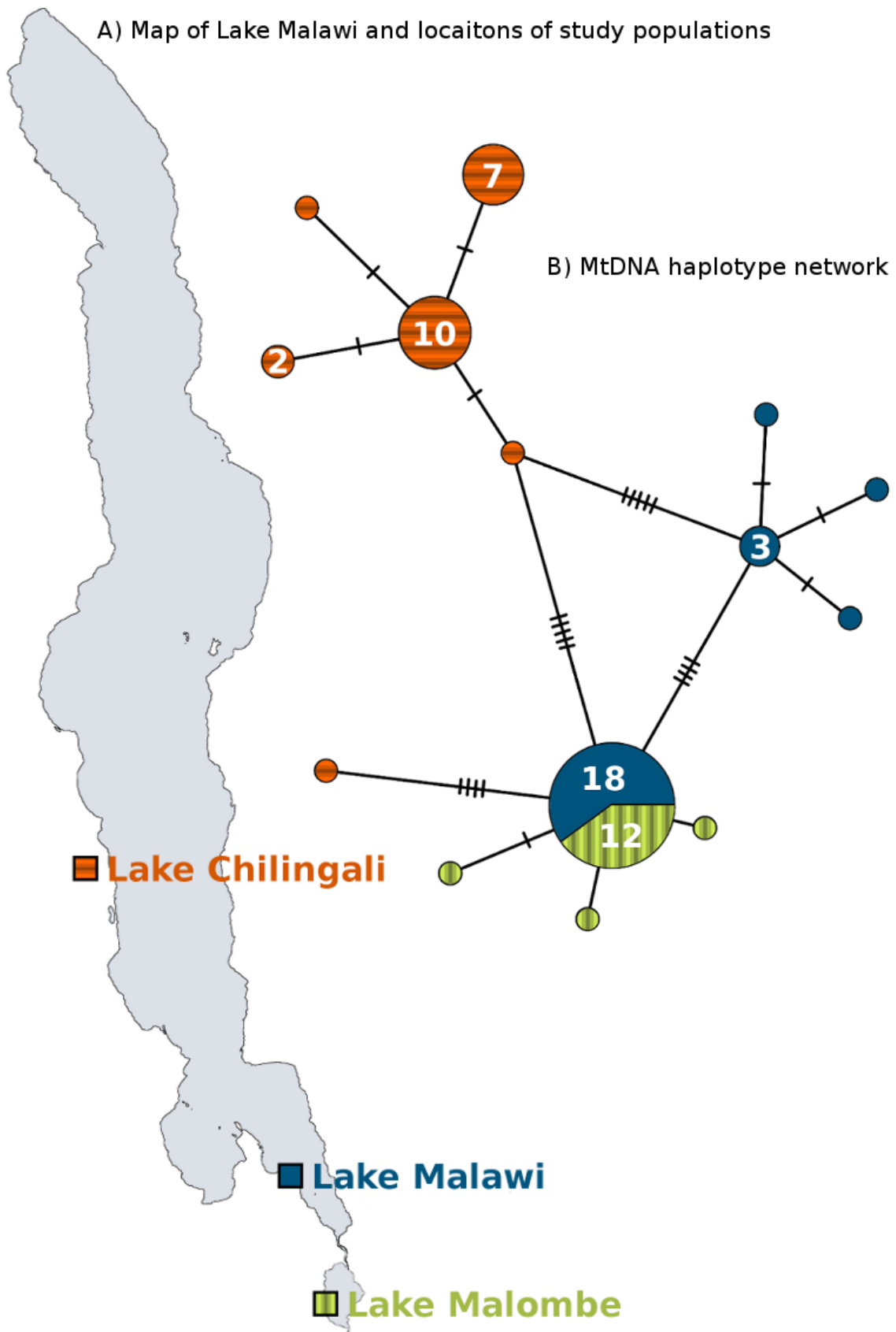


Figure 4. A) Map of Lake Malawi with locations of *Lethrinops* study populations. B) Minimum spanning haplotype network of the 61 *Lethrinops* mtDNA control region sequences indicates clear differentiation of the Chilingali population, and pairwise (F_{ST}) analysis found a significant difference between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

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 (t-test)
			Malawi male	Chilingali male	
<i>L. lethrinus</i>	11	54 total	36	18	0.011
		16 displays	12	04	0.010
		39 attacks	26	14	0.030
<i>L. sp. 'chilingali'</i>	11	89 total	30	59	0.004
		31 displays	07	24	0.010
		57 attacks	23	34	0.020

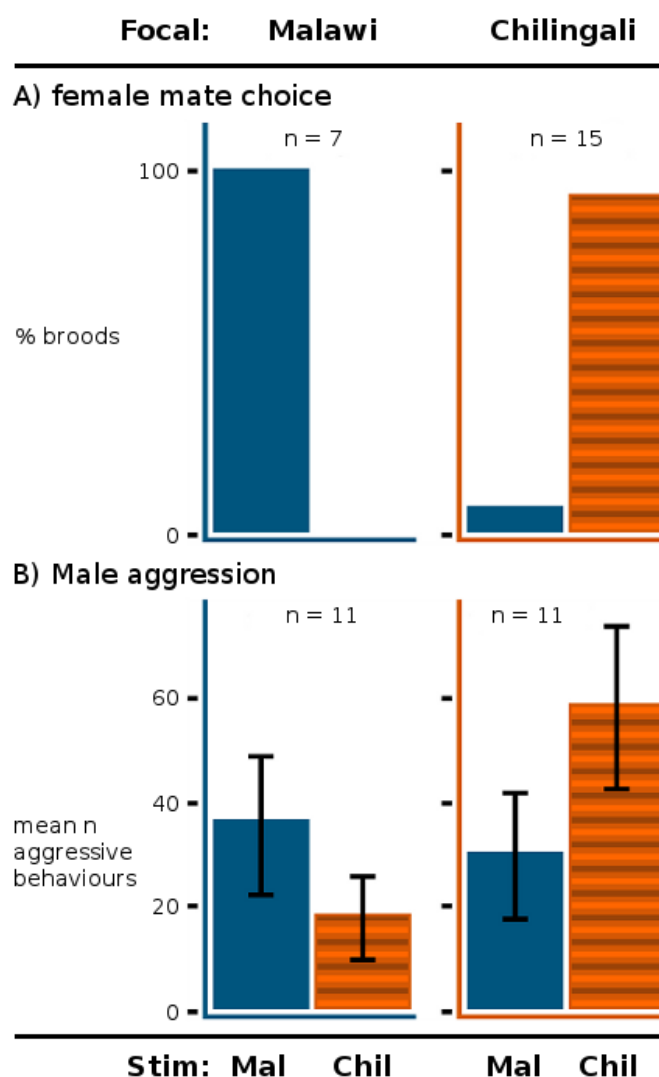


Figure 5. A) When given the choice between *L. lethrinus* from Lake Malawi and *L. sp. 'chilingali'* males, females from both taxa demonstrated a significant preference to mate with males from their own population (binomial test), charts show % of broods spawned with each type of male by each type of 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-minute trial and error bars show 95% confidence intervals.

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 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.

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 & 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 (*Drosophila* spp.; Dodd 1989; Rice & Hostert 1993) and natural model systems (threespine sticklebacks; McKinnon & Rundle 2002) suggests a greater role for selection and pleiotropy/genetic hitchhiking (Rice & 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 & 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 & 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; Odhiambo *et al.* 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 & 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 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 & Turner 2013; Odhiambo *et al.* 2011; Odhiambo *et al.* 2012; sandy/muddy/vegetated-benthic *Astatoreochromis*, *Xystichromis*, *Pseudocrenilabrus* Odhiambo *et al.* 2011; Odhiambo *et al.* 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 & Turner 2004; Egger *et al.* 2008; Blais *et al.* 2009; Egger *et al.* 2010; 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 & 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 & 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 many sand-dwelling species reduce interspecific aggression (Genner *et al.* 2008; 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 & Schluter 2004; Genner *et al.* 2008; Magalhaes *et al.* 2013), although theoretical considerations indicate that reproductive character divergence can drive divergence in competitor recognition traits but not vice versa (Okamoto & 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 (Aguilee *et al.* 2013).

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Chapter 3



Astatotilapia calliptera male

Signal and preference divergence among populations of the non-endemic basal
Lake Malawi cichlid fish *Astatotilapia calliptera*

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Abstract

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 co-existence of divergent colour morphs or species. Behavioural mate choice and aggression biases of species and allopatric populations of specialised 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.

Key words: Aggression – Allopatric divergence – Cichlid – 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 co-existence 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; Seehausen *et al.* 1998; van Oppen *et al.* 1998; Knight *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 comprised of numerous, often distinctive, geographic 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 & 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 pre-requisite 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 co-existence or even sympatric speciation (van Doorn, Dieckmann & Weissing 2004; Mikami, Kohda & Kawata 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 co-evolved 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 co-existence 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 specialised 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 specialised forms. Indeed, it has been proposed that African cichlid radiations follow a 3-phase model, initially diversifying on the basis of gross habitat preferences, then on fine-scale trophic adaptation and only later on 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 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 lab bred fish.

General mate choice experimental methods

Within 2m-long aquaria connected to a recirculating system maintained at 25C, male territories of 300 x 300mm 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 x 10mm), 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 & 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.

3-population 3-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 seven 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 105mm SL. We attempted to size match them within sets, generally keeping differences to within 5mm and 5g, but with a maximum difference of 26mm and 13.48g between males. Each time the males were changed each population occupied a different compartment.

2-population 6-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, a total of 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 spawning by Chizumulu females (individual males re-used 0-5 times). For the choice between same-coloured males, a total of 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 test were carried out in two replicate sets of aquaria, each consisted of a central 30 litre tank (490x430x140mm) with a refuge (plastic pipe) for the focal fish, with adjacent tanks (200x430x140mm) for the stimulus fish. All tanks were part of a large re-circulating system, but each with it's own inflow and outflow, preventing direct water exchange between the focal and stimulus. Focal and stimulus fish were separated by a 5mm 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 minute 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, comprised of 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, transformation were attempted, if these failed to rectify the problem, non-parametric equivalents were used. For the 3-way female mate choice trials, preference by females from each population was tested using a 3-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 2-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 to the two stimulus types by males of each focal population.

Results

3-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: $N = 8$, $\text{ChiSq} = 7.200$, $\text{d.f.} = 2$, $p = 0.027$), 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: $N = 8$, $\text{ChiSq} = 3.769$, $\text{d.f.} = 2$, $p = 0.152$) and Chilingali ($N = 8$, $\text{ChiSq} = 1.500$, $\text{d.f.} = 2$, $p = 0.472$) did not show a significant preference, although they laid more eggs with males from their own populations (Fig. 1a, Appendix III).

2-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: $t_7 = -0.359$, $p = 0.730$) or Salima females ($t_7 = -1.148$, $p = 0.289$; Fig. 1b, Appendix III).

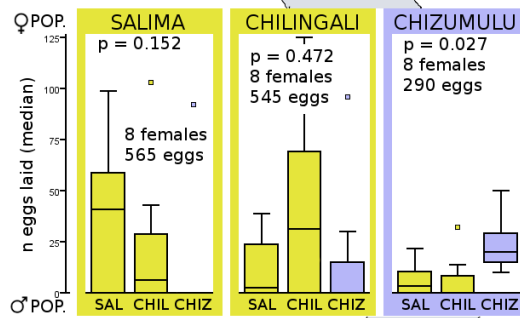
Salima females still showed no significant preference when given the choice between three Chizumulu and three own-population males ($t_5 = 0.208$, $p = 0.843$). Although they did lay many more eggs with own-population males, the preference by Chizumulu females was not significant ($t_{12} = -1.673$, $p = 0.120$; 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: $t_{11} = 3.622$, $p = 0.004$) and Chizumulu ($t_{11} = -2.453$, $p = 0.032$) showed a significant tendency to bias aggressive behaviour towards males from their own population in dyadic simulated intruder choice tests (Fig. 1b, Appendix III).

A) 3-population female mate choice



Lake Malawi
(ca. 570 km)

B) 2-population female mate choice and male aggression

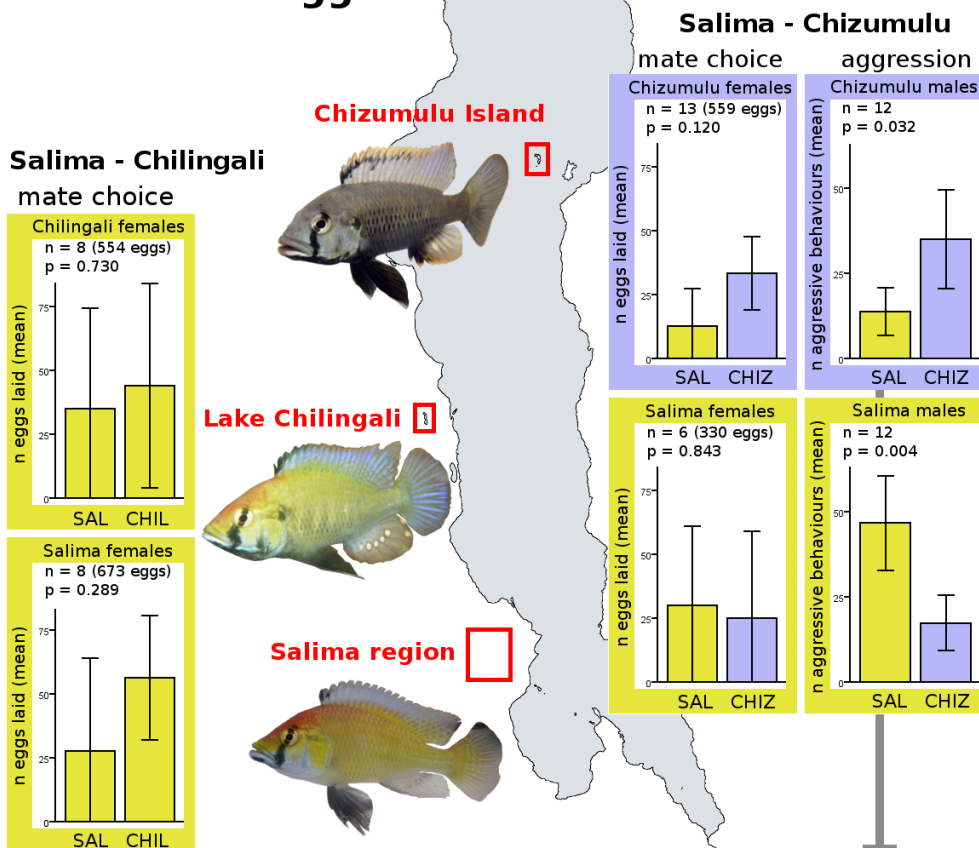


Figure 1. Map of Lake Malawi with locations of *A. calliptera* study populations and results from female mate choice and male aggression experiments. Chart Y-axes show either number of eggs laid with males in the female mate choice experiments or number of aggressive behaviours towards stimulus males 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 to other-population males, there was also no significant preference between the Salima and Chizumulu populations (paired t-tests). But, Fisher's combined probabilities test records 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-minute trial and error bars show 95% confidence intervals.

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 generalised riverine fishes that are believed to represent the sister group of the specialised 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, Kornfield & Reinthal 1994). The situation has been complicated by later molecular studies (Shaw *et al.* 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 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 but also much more widely in the South East of 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 specialised 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 likely 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 can not claim that these populations represent the unchanged ancestors that originally colonised 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 3-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 specialised 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 will 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 regards 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 pattern 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 (for example 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 Chizumulu population.

Females from the population with males of the likely 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 likely 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 specialisation to patchy lacustrine habitats prevent gene flow between populations distributed around a lake. However, generalist species may not realise this potential as easily as their specialist endemic descendants which more readily become isolated due to specialisation 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 specialisation, as proposed in an influential model.

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Chapter 4



Astatotilapia calliptera female

Individual variation in female mate preference in the Lake Malawi cichlid fish *Astatotilapia calliptera*

Abstract

Mate preferences are a major force in the process of speciation. A preference to mate with similar types of individuals over different types, assortative mating, is a step towards the evolution of reproductive isolation. Individual variation in preference within a population has the potential to influence the direction and strength of evolution by sexual selection and to drive divergence. In cichlid fish, there is evidence of variation in female mate preference, not only among, but also within populations. However, few investigations have focused on the nature of variation at the level of the individual. In this study, we investigate consistency of female mate choice to try to identify whether variation at the population level is mainly due to variation within individuals or consistent difference in preference among individuals. The results suggest that variation within females in their choice of males, rather than consistent individual differences in preference are most likely to explain the partial assortative mating often documented in tests of mate preference among allopatric populations of cichlids.

Key words: Malawi, cichlid, mate choice, mate preference, individual variation, repeatability sexual selection

Introduction

Mate preferences are a major force in the process of speciation. The development of assortative mating (preference to mate with individuals of the same or similar type) reduces gene flow between diverging taxa (Andersson 1994; Turner & Burrows 1995; Widemo & Sæther 1999; Coyne & Orr 2004) and can provide pre-zygotic reproductive isolation between closely related species without the need for geographical or ecological separation (Crapon de Caprona 1986; Seehausen *et al.* 1998; Knight *et al.* 1998). Mate preferences influence the direction and strength of evolution by sexual selection (Boake 1989; Jennions & Petrie 1997; Brooks & Endler 2001) and it may be possible for variation in mate preferences to drive divergence and speciation (Turner & Burrows 1995; Haesler & Seehausen 2005). Investigations of within- and between-female variation in preference and consistency of choice may aid our understanding of sexually selected divergence (Boake 1989; Wagner 1998) and help to explain intra-specific variation in sexually selected male traits (Brooks & Endler 2001) and also inter-specific differences in the evolution of secondary sexual characteristics (Jennions & Petrie 1997).

Studies of individual variation in behavioural traits and consistency of mate choice have been conducted over a range of organisms (*e.g.* insects, Moore 1989; fish, Bakker 1993; Godin & Dugatkin 1995; Cummings & Mollaghan 2006; birds, Møller 1994; Forstmeier & Birkhead 2004; for a comprehensive overview, Bell *et al.* 2009). However, due to the need for independent observations in population level studies, the nature of variation at the individual level and the potential impact of such variation on the processes of speciation is still often overlooked (Jennions & Petrie 1997; Widemo & Sæther 1999). It is possible that the preference of each female could match that of the population as a whole, and variation around the 'mean choice' could be 'noise' due to 'errors' in mate assessment or other external influences (such as limited availability of partners, contest and interference competition, threat of predation or poor observational or experimental methods) which prevent the expression of preference during mate choice (Jennions & Petrie 1997; Wagner 1998; Widemo & Sæther 1999). It is also possible, however, that individual females differ in their preference for certain male traits (Boake 1989) and strong yet divergent preferences may result in observations of random mating or a weak preference at the population or species level (Jennions & Petrie 1997; Widemo & Sæther 1999). Whereas a one off observation may suggest a certainty of mating with only one type of male, 'preference functions' calculated from repeated observations of individuals can give a probability, for each female, of mating with any male encountered (Boake 1989; van der Sluijs *et al.* 2008a), providing a better estimate of between-female variation and within-female repeatability. It has therefore been suggested that random mating should be defined on an individual basis, rather than as a population trait (Widemo & Sæther 1999) in order to correctly interpret within-population variation and draw conclusion about the impact of such patterns of preference on evolutionary processes such as speciation (Jennions & Petrie 1997; Widemo & Sæther 1999; Brooks & Endler 2001).

Divergence of male breeding colour (often yellow/orange/red vs blue) is common among closely

related haplochromine cichlids from both Lake Victoria and Lake Malawi (Seehausen 1997; Allender *et al.* 2003; Konings 2007). These differences in male colour are important in complete reproductive isolation among sympatric species (Seehausen 1997; Seehausen & van Alphen 1998; Knight *et al.* 1998; Seehausen *et al.* 1998; Couldrige & Alexander 2002; Jordan 2008) and partial assortative mating among allopatric races (Knight & Turner 2004; Egger *et al.* 2010; Tyers & Turner 2013 (chapter 3 this thesis)), suggesting that divergent mate preferences have played a strong role in the rapid speciation of haplochromines within the East African Great Lakes. Heritable differences in mate preference have been demonstrated between closely related species of cichlids (Haesler & Seehausen 2005). However, genetic variation between species only shows that variation was present in the past and that it has likely contributed to divergence. Although interspecific/hybrid studies may be important in mapping preference genes, intraspecific studies may be important to assess the potential for such variation to drive divergence (Bakker & Pomiankowski 1995).

A recent meta-analysis of studies on repeatability of behaviour suggests that mate preference may be among the least consistent behaviours (Bell *et al.* 2009) and to date, studies of consistency of mate preference have had mixed results: Some indicate high repeatability (*e.g.* Moore 1989; Bakker 1993; Møller 1994; Godin & Dugatkin 1995) and others show inconsistency in female choice between observations (Boake 1989; Bakker & Pomiankowski 1995; Bell *et al.* 2009). It should be noted, however, that there is some discrepancy between 'repeatability' (r) and individual consistency of behaviour. Repeatability expresses the proportion of variation in the behaviour of a population that is due to differences among individuals and differences within individuals. If the average individual is consistent, the average within individual variation will be low, making the ratio of among individual variation to within individual variation (the repeatability) high. In the case of mate choice, repeatability will be high whether all females consistently agree (no variation within the population) or whether females consistently disagree (variation within the population but not within individuals) and will be low when individuals are inconsistent (variation within the population and within individuals). Where some individuals are consistent and others are inconsistent, however, it may be more interesting or appropriate to look at the behaviour of individuals rather than relying on a statistical measure of consistency in the population: If there is a much higher proportion of consistent individuals to inconsistent individuals, then on average consistency, and therefore repeatability, could still be high.

Variation in mate preference among female cichlids is known from a phenotypically variable population of *Pundamilia* spp., a rocky-shore cichlid from Lake Victoria. Males in this population may be red, blue or intermediate and when given the choice of only distinct males (red vs blue) some females show strong divergent preferences whereas others show no preference (van der Sluijs *et al.* 2008a,b). This system has been used to model the possibility that variation in mate preferences and male traits in a population in turbid water, may lead to divergence driven by disruptive selection via female mate choice in clear water, resulting in reproductively isolated species (Seehausen 1997; van der Sluijs *et al.* 2010). However, it seems as likely that the variable population is the result of hybridisation, in turbid water,

among species which remain distinct at clear water locations (van der Sluijs *et al.* 2008a). Variation in mate preference is also indicated by studies that show partial assortative mating among populations of recently diverged rocky-shore cichlids from Lakes Malawi and Tanganyika (Knight & Turner 2004; Egger *et al.* 2010). However, variation in female preference is not the focus of these investigations, and in most cases the evolutionary history of colour diversification among populations is unknown and the ancestral state can not be inferred. Species with more limited geographic variation in male colour may provide better models for investigating variation in mate preference within a single species which is monomorphic in each population but has the potential for diversification of male colour traits. Is variation in mate choice within a population accounted for by random variation within individuals or by divergent preference among individuals, which has the potential to drive divergence of male secondary sexual traits (particularly in allopatry where they may be more likely to diverge as a by-product of natural selection due to ecological differences, but potentially also in sympatry)?

Methods

Study Species

The sexually dimorphic cichlid fish *Astatotilapia calliptera* is widely distributed, not only within Lake Malawi but also in surrounding water bodies (Konings 2007) – streams, swamps, ponds, and along the margins of rivers and smaller lakes. Territorial males of most riverine and lacustrine populations are predominantly yellow, but a population with blue males is known from Chizumulu Island in the middle of Lake Malawi. Due to the limited distribution of this blue-male population it is likely that it derived from the more common and widely distributed yellow form. Recent laboratory-based mate choice experiments demonstrated a significant tendency of females from this population to preferentially mate with males of their own population. This was in contrast to females from a population with yellow males, which appeared to show random mating with regards to male colour/population (chapter 3). In the present study, blue males from Chizumulu Island and yellow males from Salima (a swampy area to the south-west of Lake Malawi) were used to investigate individual variation in mate preference by females from the population with yellow males.

Mate choice experiment

Eight laboratory bred males, ranging from 61-65mm and 5.99-7.17g were used to make four pairs (size matched to within 2mm and 0.87g and labelled A-D in Table 2), each containing one yellow Salima and one blue Chizumulu male. Mate choice trials were run in four replicate aquaria, one for each pair of males, which remained *in-situ* throughout the experiment. Males were separated by a mesh grid, which allowed for the transmission of all cues (visual, olfactory and auditory) and aggressive displays – promoting territorial behaviour while preventing attacks and injury. Although of a small enough aperture (10x10mm) to prevent males from crossing into each other's territories, the mesh partial partition allowed

the smaller females to swim freely between males. Before the trials began, males were left to settle until both showed full courtship colours. Tanks contained a mixed substrate of sand and gravel and each male was provided with a terracotta plant pot and some artificial plants, positioned in the far back corners, to provide a focal point for territorial and courtship behaviour. Each tank also contained an air-driven box filter, an internal heater (maintaining water temperature between 22-25°C) and a collection of small plastic pipes, positioned next to the central partition, to provide refuge for the focal female. All of the females used were not previously mated before their first trial, and had been kept in single-population stock tanks since release from their mother, so they were naive to both breeding and to the blue males from the Chizumulu Island population. All females spent several days investigating and courting with both males before spawning. In all trials both males courted the female vigorously. Limitations on choosiness (*e.g.* time spent sampling and interference competition) were not limited by the experimental conditions, allowing preferences to be expressed without constraints.

Protocol

The experimental protocol combined two methods of measuring consistency of female preference outlined by Forstmeier & Birkhead (2004). Firstly, the spawning preference of each focal female was tested with the same stimulus pair on two separate occasions, to test for consistency of choice when presented with the same males and to test between-female agreement by using each male pair to test the preferences of more than one female. Secondly, consistency of choice of male colour (rather than individual) was tested with a different pair of males in a third trial. Eleven females were used in total (2-3 with each pair of males), tested one at a time. To allow females to be tracked through the experiment, when they were not being used in trials, they were physically isolated, but maintained visual contact with conspecifics through glass partitions.

DNA extraction and genotyping

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. A total of 1120 offspring were produced (mean = 35 per brood, range = 4 - 64). With the exception of broods that contained less than 10 offspring, 10 fry from each brood (a total of 309 offspring from 32 broods) were genotyped for parentage analysis and mothers and potential fathers were used for assigning paternity 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 PeakScanner v.2.

Data analysis

A Fisher's exact test was used to compare population level assortative mating between this experiment

(referred to as 'Experiment 3') and two previous experimental tests of assortative mating among the same populations (from chapter 3 of this thesis, here referred to as 'Experiments 1 & 2'). Data from the previous experiments (chapter 3) were re-analysed to allow for a direct comparison with the data from this experiment. Exp. 1 involved an additional population, from Lake Chilingali, with yellow males. As this is the common colour of *A. calliptera* males in the Lake Malawi catchment, and there was no indication of divergent mate preferences among this and the Salima population with yellow males, they are considered to be conspecific. Analysis is therefore based on paternity by 'own-colour' vs 'other-colour' males. Multiple paternity of broods is not uncommon in cichlids and was found frequently in Exps. 1 & 2. Therefore, as all females in all experiments had the opportunity to mate with more than one male, each male that was mated with was considered to be a different spawning decision. A choice of more than one type of male may therefore be either expressed as multiple paternity within one brood, or different paternity of different broods. Females were divided into two groups – those that mated only with 'own-colour' males and the rest (which mated either solely or partially with allopatric, 'other-colour', males).

Results

Population level preference

Based on calculated ratios of females that only mated with own-colour males to female that mated with other-colour males (Table 1), there is no evidence that the results of Experiment 3 (individual female preference experiment from this chapter) differ from the results of Experiments 1 & 2 (allopatric mate choice experiments from chapter 3) (Fisher's exact test: d.f. = 1; $p = 0.407$).

Table 1. Population preferences from three mate choice experiments to test *A. calliptera* 'salima' female preference among yellow males from Salima (and Lake Chilingali) and blue males from Chizumulu Island. Exps 1 & 2 were re-analysed from chapter 3. Exp 3 refers to the present experiment.

Exp	Number of			Broods sired by (1&2) or sires per female (3)			Ratio yellow:other	% yellow only	
	Pops.	Males/rep.	Females	Broods	Yellow only	Blue only			Mixed
1	3	3	8	8	7	1	0	7:1	88
2	2	6	6	6	2	1	3	2:4	33
1&2			14	14	9	2	3	9:5	64
3	2	2	11	32	9	0	2	9:2	82
All								18:7	72

Individual variation in preference

There was no indication of multiple paternity in any of the broods produced during this experiment. Multiple paternity usually involves one primary male, which sires the majority of offspring in a brood, and one or two secondary males which fertilise a much smaller proportion of eggs. In Exps. 1 & 2, which used egg catchers to determine mate preference, where multiple paternity was found, on average 77% of

eggs were laid with the primary male, leaving 23% with the other male(s). In this experiment, on average, 38% of each brood was genotyped. With this proportion and a total of 32 broods, it seems unlikely that we would have, by chance, missed a strong signal of multiple paternity.

Most females (9 of the 11) spawned with males from their own population in all trials. Females that did spawn with males from the other population (2 of the 11) were not consistent in their choice. A summary of individual consistency/variation in choice is given in Table 2.

Table 2. Overview of the results from an experiment to test consistency of individual female mate choice. Letters A-D refer to the four pairs of males used for these experiments. Each trial (T) measured the spawning preference of a female with a pair of males and each female underwent three trials: the first two (T1 & T2) with the same pair of males and the third trial (T3) with a different pair.

Fem.	Male pair		Trial 1			Trial 2			Trial 3			Weeks between		
	T1&2	T3	Brood size	% analysed	Chosen male	Brood size	% analysed	Chosen male	Brood size	% analysed	Chosen male	T1&2	T2&3	
01	A	B	19	53	Sal	16	63	Sal	45	22	Sal	<23	<14	
02	A	D	36	28	Chiz	44	23	Chiz	64	16	Sal	<9	<16	
03	B	C	12	83	Sal	61	16	Sal	51	19	Sal	<50	<9	
04	B	A	4	100	Sal	20	50	Sal	36	28	Sal	<13	<31	
05	B	D	31	32	Sal	30	33	Sal	55	18	Sal	<16	<16	
06	C	B	26	38	Sal	64	16	Sal	5	100	Sal	<40	<6	
07	C	B	39	26	Sal	18	56	Sal	47	21	Sal	<13	<20	
08	C	D	31	32	Sal	23	43	Sal	51	20	Sal	<7	<7	
09	D	C	29	34	Sal	51	20	Sal	55	18	Sal	<7	<20	
10	D	n/a	25	40	Sal	34	29	Sal	n/a	n/a	n/a	<9	n/a	
11	D	B	19	53	Chiz	61	16	Sal	18	56	Sal	<8	<28	
Broods from own-pop male (%):					81.8						90.9	100		

Discussion

Most females chose to spawn with males from their own population in all of their mate choice trials. However, some females did choose to mate with males from the allopatric population, which supports the previous findings that despite considerable colour differences between males, complete reproductive isolation has not developed between these allopatric colour races (chapter 3). A comparison of three different experimental tests of assortative mating (two from chapter 3 of this thesis, and one from the present chapter) suggest that at as measures of population level preferences, the results do not differ significantly.

From these three experiments with *Astatotilapia calliptera* (this chapter and chapter 3), we have

identified variation in mate choice not only within a population but also within individuals of that population. There is no indication of consistent divergent mate preferences, individual females either consistently mated with males from their own population or spawning was split between males from both populations. Because of this lack of consistency of choice by some females, the apparent consistency of choice by the other females should be interpreted with caution, it is possible that with further trials, each individual would mate with an allopatric male at some point. However, despite the limited scope of this study, the results do suggest the possibility that some females have a weaker preference than others, *i.e.* are less choosy with regards to traits differing among populations. Investigating difference in fussiness among females may be an interesting avenue of future research. If they do actually exist, and are not just an artefact of this small scale investigation, individual differences in preference among female cichlids from a single population, could be due to individual differences in physiology (Widemo & Sæther 1999). Differences in female sense organs, for example visual colour sensitivity, could possibly limit the ability of some females to distinguish between males on the basis of colour, leaving these females to base their choice of male on other cues or traits. In cichlids, females showing less discrimination among males of different populations may give greater weight to traits that vary mostly within populations, such as courtship vigour, rather than those that vary between populations, such as colour. Preference to mate with rare males has been documented in some guppy females, and it has been suggested that a rare male phenotype may be advantageous if it is strongly preferred by some females. Mating success of rare males may actually be greater, than 'normal' males which are moderately attractive to more females, but also suffer greater competition for those females (Brooks & Endler 2001). A difference in female mate preference may not be the only way for a novel male colour to become established. Male cichlids are highly aggressive and observations and experiments have found that there is a tendency of territorial males to bias aggression towards similar looking fish, *i.e.* conspecifics or those from the same population (Genner *et al.* 1999; Pauers *et al.* 2008; Tyers & Turner 2013). It has been suggested that such aggression biases may aid in the co-existence of ecologically-similar species in sympatry or aid in the co-existence of populations that have undergone divergence of sexually selected traits in allopatry, under secondary contact (Mikami *et al.* 2004; Young *et al.* 2009). Intraspecific aggression biases have also been demonstrated in species with rare morphs (Dijkstra *et al.* 2005) and whether biases are for own-morph or common-morph individuals, the result is a reduction in aggression towards the rarer morph(s). So if we assume, for example, that a founding population invades a new area with a similar resident fish species. Any move away from similarity between species, *i.e.* novel male phenotypes arising in the founding population, may aid in the establishment of a species in a new area. If novel males do experience reduced aggression, from both the similar resident, and also other conspecifics of the founding population, females that are less fussy about colour may be more likely to mate with them due to their increased courtship vigour when compared to 'normal males' which must spend more time and energy on territorial defence.

Environmental differences may also drive divergence in male traits and female preferences among

populations. Even small differences between habitats may influence the detectability of male signals triggering changes in female preference (Schluter & Price 1993). Relaxation of female choosiness for species-specific male traits may be common in founding populations, and it has been suggested that such a relaxation on selection may aid in colonisation of new areas (*e.g.* Kaneshiro 1976; Tinghitella & Zuk 2009). If such a relaxation of preference for specific male traits during a founder event was to coincide with different environmental conditions in the new area, which favour an alternative male traits, then rapid allopatric divergence of male secondary sexual characteristics may be likely.

All mating with males of the allopatric population occurred before the tested female had mated with a male from her own population: if a switch in chosen male did occur between trials it was always from other population to own population. This is an interesting point to note, as it has been suggested that multiple paternity in cichlids (within species) could be due to bet-hedging - an attempt to reduce the potential impact that errors in mate assessment could have on fitness by selecting more than one male to sire each brood (see Kellogg *et al.* 1995; Parker & Kornfield 1996). It is therefore possible that in mate choice trials where only one male of each type is available to the female, multiple paternity involving heterospecific or allopatric males may be due to the expression of this mating strategy despite the limitation on the number of conspecific males present. However, previous experiments demonstrated that even if multiple males of the same population are present in the experiment, multi-population paternity will still occur (Tyers & Turner 2013). When considered together, these observations (multiple paternity of broods involving allopatric males when multiple own-population males are present, and spawning with allopatric males in trials prior to spawning with own-population males in later trials), suggest that a lack of choice coupled with the desire for multiple paternity of broods is unlikely to be the reason for heterospecific/allopatric spawning in cichlid mate choice experiments. Further studies with more females, different taxa, and more trials per female will help to clarify the patterns of mating among and within females.

It has been suggested that repeatability might decrease with the interval between measurements on the same individual, because the 'same' phenotypic trait may be influenced by different sets of genes at different ages (Bell *et al.* 2009). In this study, time between repeat trials was inconsistent due to differences in the amount of recovery time needed by each female until breeding condition was reached again. However, there was no indication that inconsistent choices were due to greater waiting periods between trials (many of the consistent choices were much further apart than the inconsistent choices).

Conclusions

This study serves as an initial investigation into variation among females and consistency of mate choice in female cichlid fish from a monomorphic population of a species that shows geographic variation in male colour. Although perhaps raising more questions than they answers, these preliminary results suggest that there is not complete agreement among females as to which male is most attractive. Females seem to differ in their choice of males and population studies of mate preferences should perhaps be

cautious when assuming that observed mean population level preferences are applicable to all individuals of a population. Although for many animals, including many species of cichlid, repeated measures of mate choice may be logistically difficult and time consuming, some species of cichlid, for example those in the genus *Astatotilapia*, breed rapidly under laboratory conditions allowing repeat trials within reasonable time frames, a factor that has perhaps retarded the study of consistent variation in mate preference in vertebrates. Investigations into individual variation in female mate preferences in cichlids may therefore not only aid studies of speciation through sexual selection, but also provide a good model for studies interested in investigating variation in mate preferences.

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Chapter 5



Astatotilapia sp. 'massoko predator' male

Diversification and speciation of haplochromine cichlids in small isolated crater lakes in Tanzania, East Africa

Abstract

We tested mate preferences of haplochromine cichlid fish from small isolated crater lakes in Tanzania, which may be a suitable new model system for studying the beginnings of adaptive radiation and sympatric speciation in taxa closely related to the ancestors of the large haplochromine radiations in Lakes Malawi and Victoria. Mate choice experiments were carried out to test for reproductive isolation among putative allopatric and sympatric species. We demonstrated a high degree of assortative mating among allopatric species that differ considerably in male colour, and no assortative mating between allopatric populations that do not appear to differ greatly. Tests of female mate preference for different types of males from a single crater lake found variation in the choices of individuals, despite significant differentiation of types by morphology, ecology and neutral genetic markers and high repeatability of mate choice at the population level. This suggests that, in contrast to other studies of sympatric species of cichlid fish that differ in male colour, there is no reproductive isolation by direct female mate choice. It is suggested that models of environment dependent assortative mating due to strong habitat preference and greater success of locally adapted males in their own habitat, may explain the maintenance of distinctness among these sympatric forms, which may represent the beginnings of adaptive radiation in haplochromine cichlids.

Introduction

Theoretically, speciation is possible with or without the potential for gene-flow between diverging taxa, and divergence under any geographical distribution may be due to environmental selection pressures (adaptation to different habitats/niches) or the actions of sexual selection (Lande 1981; West-Eberhard 1983; Turelli *et al.* 2001; Baker 2005; van Doorn *et al.* 2009; Maan & Seehausen 2011). Although divergence may frequently occur when geographic isolation restricts gene flow among portions of a species gene pool, homogenisation through interbreeding may also frequently result in a loss of distinctness under secondary sympatry (Coyne & Orr 2004). Under the Biological Species Concept speciation is therefore considered to be the evolution of reproductive isolation – a biological mechanisms for the prevention of gene-flow (Mayr 1996; Turelli *et al.* 2001; Coyne & Orr 2004). However, speciation is not an end point of evolution and continued divergence after cessation of gene flow may often result in differences among species that are unrelated to the processes of divergence and the evolution of reproductive isolation (Dobzhansky 1940; Seehausen *et al.* 1998). The need to make inferences about past processes (are observations of partial assortative mating are due to ongoing sympatric divergence or introgression of previously allopatric populations?), and predictions about future species status (will incipient species develop full reproductive isolation?) can be reduced by studying adaptive radiations of recently diverged and diverging taxa. In these systems, isolating mechanisms that are actually involved in the processes of speciation, rather than those which may have evolved subsequently, are more likely to be identified (Seehausen *et al.* 1998; Turelli *et al.* 2001; Coyne & Orr 2004).

Arguably one of the best available models for studying the relative contribution of different geographical scenarios (allopatry/sympatry) and selective processes (natural/sexual/neutral) in the production of new species is the haplochromine cichlid flock of Lake Malawi. Younger than the lake Tanganyikan cichlid assemblage which includes haplochromines among it's several tribes (*ca.* 250 species arisen within several million years, Salzburger *et al.* 2005), and with a greater diversity than the Lake Victoria haplochromine super-flock (*ca.* 500 species in 15,000 – 100,000 years, Johnson *et al.* 1996; Verheyen *et al.* 2003; Stager & Johnson 2008), the haplochromines species flock in Lake Malawi is thought to be the largest (possibly >1000 species, Salzburger *et al.* 2005; Konings 2007; Joyce *et al.* 2011) rapid (*ca.* 2 million years old, Won *et al.* 2006; Genner *et al.* 2007) adaptive radiation of vertebrates (Turner *et al.* 2001) with great eco-morphological and sexual trait diversity among very closely related, recently diverged species. Intralacustrine allopatric speciation is likely to have contributed to the evolution of so many species in larger lakes, and sympatric speciation has also been proposed as contributing to the diversity of cichlids (Seehausen & van Alphen 1999; Shaw *et al.* 2000; Schliewen *et al.* 1994; Barluenga *et al.* 2006; Elmer *et al.* 2010). Although evidence from small species flocks isolated in small crater lakes (tilapines in Cameroon, Schliewen *et al.* 1994; Midas in Nicaragua, Barluenga *et al.* 2006; Elmer *et al.* 2010) does strongly support sympatric speciation, there is little convincing evidence from the haplochromines. However, haplochromine cichlids, which are thought to have originated in the

Lake Tanganyika catchment and spread out to colonise the other Great Lakes, are also wide spread throughout the river systems and smaller lakes in East Africa (Salzburger *et al.*, 2005; Joyce *et al.*, 2011) and recent sampling of isolated volcanic crater lakes in Tanzania, to the North of Lake Malawi (Fig. 1), has found what appear to be several young species flocks of *Astatotilapia*, a genus basal to the radiations of both Lakes Victoria and Malawi (Salzburger *et al.* 2005; Joyce *et al.* 2011). These lakes and their cichlids may therefore provide a potential new study system for investigations of the processes of allopatric and sympatric speciation in a cichlid lineage in which sexually selected divergence is thought to have contributed to rapid and extensive diversification (Andersson 1994; Salzburger *et al.* 2005; Wagner *et al.* 2012). These lakes are a few hundred meters in diameter at most and relatively shallow, suggesting sympatric diversification.

Two of the main factors that contribute to the large lacustrine cichlid radiations are thought to be the potential for sexual selection in colonising lineages and the depth of the lake that they enter (Wagner *et al.* 2012). Depth in some of the deeper crater lakes may provide an environmental gradient, from shallow/light to deep/dark, often associated with divergence by sensory drive, in otherwise ecologically homogenous environments. Although the possibility of sensory drive has been dismissed for shallow water Lake Malawi habitat specialists (Smith *et al.* 2012), it may have acted in the evolution of reproductive isolation in other fish taxa, including Lake Victoria cichlids, which inhabit heterogenous light environments (Boughman 2001; Seehausen *et al.* 2008; Smith *et al.* 2012). The potential for sensory drive, or other environment driven models of male trait divergence (*e.g.* van Doorn *et al.* 2009) to act at the beginning of adaptive radiation after the invasion of a lake by a riverine ancestor, remains to be tested.

The behavioural experiments presented here contribute to an ongoing project which includes investigation of the ecology and genetics of these fish. Firstly, we tested assortative mating among allopatric populations to establish whether *Astatotilapia calliptera* from Lake Malawi and a divergent putative crater lake species are reproductively isolated. We also tested assortative mating among phenotypically similar inhabitants of two different crater lakes (which have been identified as being reciprocally monophyletic based on SNPs identified by RAD sequencing, R. Challis pers. comm.). The third experiment tested mate preferences among phenotypically, ecologically and genetically divergent (M. Genner pers. comm.) inhabitants of a single lake, to test for assortative mating by direct mate choice, a common isolating mechanism among sympatric cichlid species (Knight & Turner 1998; Seehausen *et al.* 1998). An absence of consistent choice of the same type of male by individual females, over multiple spawning tests, would indicate incomplete speciation and suggest polymorphism or incipient speciation based on ecological rather than behavioural isolation of forms.

Methods

Study system

During an expedition in 2011, several putative species flocks of haplochromine cichlid fish of the genus

Astatotilapia, were discovered in small isolated crater lakes in Tanzania, to the North of Lake Malawi (Fig. 1). The fish within these lakes are reciprocally monophyletic based on SNPs identified by RAD sequencing (R. Challis pers. comm.), suggesting sympatric diversification among the inhabitants after colonisation by a common ancestor. Fish from three lakes (Ilamba, Itamba, Massoko) were shipped to the UK for aquaria based experiments. *Astatotilapia calliptera* from Lake Malawi was also used as an 'outgroup' for initial behavioural experiments, to test for allopatric species status of these newly discovered putative species. *A. calliptera* is one of the few species found in Lake Malawi that is also found in the surrounding water bodies and is closely related and phylogenetically basal to the Lake Malawi haplochromine radiation (Joyce *et al.* 2011). Molecular studies in progress appear to show it is also the sister group to the crater lake fish (G. Turner pers. comm.).

Geology and ecology of the lakes

The volcanic crater lakes in the Rungwe volcanic area in southern Tanzania, to the north of Lake Malawi, were formed by the flooding of shallow maar craters which result from explosions caused by contact between ground water and hot magma under the Earth's surface. Due to the method of formation, crater lakes are usually fairly similar with a circular shape and shallow depth (Garcin *et al.* 2006). The ecology and fauna of the lakes sampled for this set of experiments is summarised in Table 1.

Table 1. Summary of the characteristics of the three lakes: elevation above sea level and above Lake Malawi; the characteristics of the bottom and secchi disk reading for water clarity; the fish fauna. All three lakes contain some macrophytes and bivalve molluscs. Lake Itamba is overhung by trees and also contains crabs and many sponges. * indicates introduced species.

Lake	Age (yrs)	Elevation (m above)		Diameter (m)	Depth (m)	Bottom		Secchi depth (m)	Fish fauna	
		sea	LM			slope	substrate		cichlids	other
Ilamba		563	83	613-839	19	Steep	Muddy	1.5	<i>Astatotilapia</i> sp. <i>Otopharynx</i> <i>Oreochromis shiranus</i> <i>O. squamipinnis</i>	<i>Clarias</i> <i>Mesobola</i> spp. <i>Barbus</i> spp.
Itamba		831	350	424-473	17	Steep		4.5	<i>Astatotilapia</i> (flock?) <i>Oreochromis shiranus</i> <i>O. karongae</i> <i>O. niloticus</i> *	
Massoko	<50000	840	387	670-700	38	Gentle	Sandy, some rocks	5.5	<i>Astatotilapia</i> (flock) <i>Oreochromis squamipinnis</i> <i>Tilapia rendalii</i>	<i>Clarias</i>

Astatotilapia fauna

The *Astatotilapia* sp. from Lake Ilamba is similar in size and shape to *A. calliptera*, and appears to differ mainly in male courtship colour: black body and fins with a red dorsal fin margin (Fig. 1). Females are silver-grey.

Lake Itamba fish are also similar to *A. calliptera* in size and shape, but may come in a variety of male colours. There is at least one black morph comparable to the Lake Itamba fish (other putative 'dark' forms include brownish and blueish), and a pale morph (yellow-green) that look more like the Lake Malawi yellow *A. calliptera*. Females are small and drab, having the appearance of females from most *Astatotilapia* spp. For these mate choice experiments, only the black and very dark-blue/black and dark-brown/black males were used (Fig. 1).

Lake Massoko was chosen as the starting point for investigations of sympatric divergence and speciation. The *Astatotilapia* from this lake are more varied, all fish appear to differ from *A. calliptera* in some morphological traits, such as body size, shape and colour. There are 3 (possibly 4) distinct male forms differing in a combination of ecomorphological and colour traits. Male colour may be shared between ecomorphological types and there is also colour variation within one of the ecomorphological types. The most distinctive types are big dark blue males with a distinctive head shape (although it is currently unknown whether this type is actually predatory, it is referred to as 'big predator') and big yellow males with a more *Astatotilapia calliptera* like morphology (although bigger, with a less 'delicate' head morphology, here they are referred to as 'big calliptera') (Fig. 1), which also seem to occur in a brownish and greenish hue and a blue colour, similar to the *A. calliptera* found at Chizumulu Island in Lake Malawi. There is also a 'small slim' form, with a body shape more similar to the 'bullet like' mbuna such as *Melanochromis* spp.. This form is blue in colour and may also occur in yellow (although possibly absent from our currently held lab stocks). During the mate choice experiments the small slim males grew rapidly. They did not, however, reach the size of the big morphs and did maintained their distinctive slim form. Preliminary investigations have grouped these four morphs based on microsatellite and dietary differentiation of wild caught males (M. Genner pers. comm.). Further observation and sampling is necessary to gain a more accurate picture of depth distribution and life-style of the different morphs within Lake Massoko. However, from observations during preliminary sampling it appears that 'big predator' with the darker blue male colour are found in deeper water, whereas 'big calliptera' possessing the lighter yellow colour are found in the shallower water (G. Turner pers. comm.). For shipping to the UK, fish were split by lake and upon arrival were transferred to single-lake stock tanks. As with many cichlids, it appears that interspecific differences are less pronounced in females than males and therefore we were initially unable to assign females to 'type'. Based on the behaviour of females from previous cichlid mate choice experiments (*e.g.* Knight *et al.* 1998; Seehausen *et al.* 1998), however, we predict that if these sympatric forms do represent reproductively isolated species, we should be able to identify females and assign them to type based on their consistent preference for spawning with one male type or another (*i.e.* conspecifics).

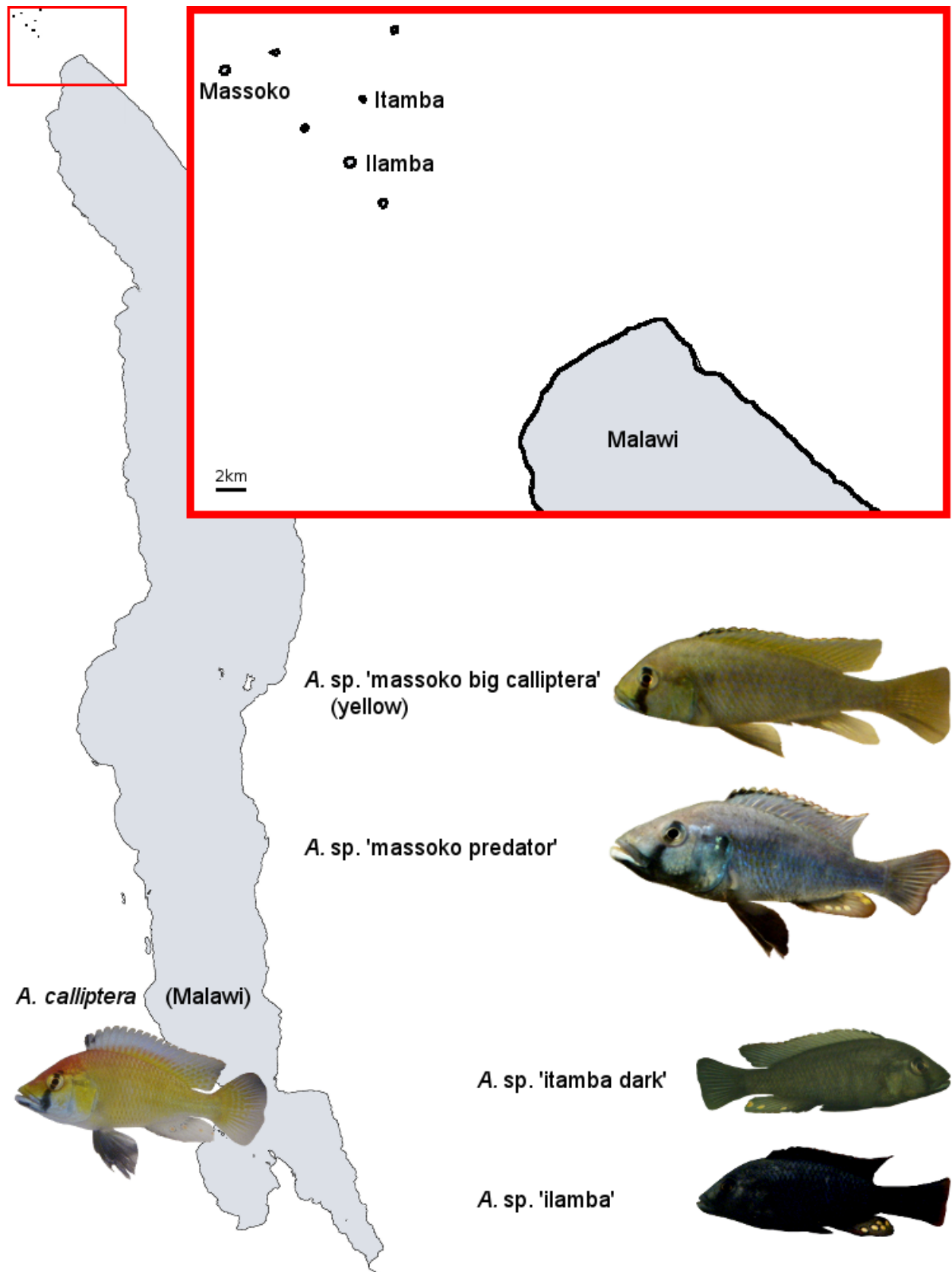


Figure 1. Location of crater lakes in relation to each other and Lake Malawi, with photographs of males from the *Astatotilapia* species and populations used in these mate choice experiments. *A. calliptera* from Lake Malawi, *A. sp. 'ilamba'* from Lake Ilamba, *A. sp. 'itamba dark'* from Lake Itamba and *A. sp. 'massoko big calliptera' (yellow)* and *A. sp. 'massoko big predator'* from Lake Massoko.

General mate choice experimental methods

In all experiments, male territories were enclosed 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. As with many haplochromine cichlids, these fish are maternal mouthbrooders, females pick up eggs immediately after spawning and brood the offspring in their mouths for approximately 3 weeks. Experimental tanks contained terracotta plant pots and plastic plants to act as a focal point for the males' territories. Water temperature was maintained at *ca.* 26°C. Fish were fed daily on algae flake and 2-3 times weekly on frozen bloodworm. 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 either returned to mixed sex, single population stock tanks, or isolated in individual tanks for identification of individuals.

Three separate mate choice experiments were carried out. Those involving more than two males, and multiple females at a time, were conducted in large tanks on a recirculating system with external filtration and heating (experiments 1 and 3). Experiment 2 was carried out in free standing tanks with internal filtration and heaters. Experiment 1 tested for mate preferences between Lake Malawi *Astatotilapia calliptera* (yellow males from the Salima population to the south west of the lake) and *A. sp.* 'ilamba' (black males). Females of the two putative species are very similar, although the Ilamba females appear slightly darker grey, as opposed to the brown-grey of *A. calliptera* females. Preference of females from both populations were tested. Experiment 2 tested for mate preferences between *A. sp.* 'ilamba' and *A. sp.* 'itamba (dark)'. Due to the variation of male colour phenotypes within Lake Itamba, and the inability to tell females apart based on colour differences, only Ilamba females, those with known black males, were used in this experiment. Experiment 3 was carried out using only fish from Lake Massoko to test for reproductive isolation among sympatric forms from within a single crater lake.

Apart from the second round of the Massoko experiment, in which females were allowed to brood offspring until release and five individual offspring from each brood were then taken for paternity analysis, broods were taken from females mouths approximately 10 days after spawning, euthanised and preserved in ethanol. Ten offspring from each female were tested for broods produced from the allopatric experiments (apart from 4 broods in which total offspring number was < 10). Due to the large number of broods produced in the Massoko experiment, only five offspring from each brood were used for paternity analysis. However, as most females spawned twice over the course of the experiment, this equated to 10 offspring per female.

Tissue samples were taken from the preserved fry and DNA obtained by salt extraction. DNA samples from offspring, mothers and potential fathers were used for assigning paternity 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 using Peak Scanner v.2.

Experiments 1 & 2: Allopatric mate choice

1) Malawi – Ilamba

A 4-way female mate choice experiment was carried out in two replicate 2m long aquaria, one for Malawi females and the other for Ilamba. All fish used in this experiment were the 1st generation laboratory bred offspring of wild caught parents. A total of 30 males (16 Ilamba and 14 Malawi) were used to make 10 sets of males, each consisting of two males from each population. Male sizes ranged from 58 – 74mm SL (difference of 16mm) and 5.61 – 11.25g (difference of 5.64). Within sets, all males were size matched to within 6 mm and 2.32g. However, within each set, the size difference between the two closely matched males from different populations was usually 0mm and < 0.5g (at most 2mm and 1.82g), ensuring that there was at least one pair of very closely size matched males within each replicate. Multiple females at a time were left in the experimental tanks, once a spawning occurred the males were changed with populations assigned to a new chamber. Eight Malawi and 10 Ilamba females were tested.

2) Ilamba – Itamba

Pairwise female choice trials were carried out in four replicate free-standing aquaria. Before the trials began, males were left to settle until both showed full courtship colours. Tanks contained a mixed substrate of sand and gravel and each male was provided with a terracotta plant pot and some artificial plants, positioned in the far back corners, to provide a focal point for territorial and courtship behaviour. Each tank also contained an air-driven box filter, an internal heater and a collection of small plastic pipes, positioned next to the central partial partition, to provide refuge for the focal female. 14 males from each population were used to make 14 unique pairs of males, to test the preference of 14 Ilamba females, one at a time. All fish used in this experiment were wild caught and has been kept in single population stock tanks prior to use. Once a female spawned, all fish were removed from the experiment and the male pair and female replaced with new fish. Each time a pair was replaced, populations occupied the opposite end of the tank. Males ranged from 68 – 82mm (difference of 14mm) and 9.12 – 16.70g (difference of 7.58g), with complete overlap in size between populations. For each pair, males were usually matched to within 2mm and < 3g (maximum 4mm difference).

Experiment 3: Sympatric mate choice (Lake Massoko)

For this 8-way female mate choice experiment, a single 4m long tank was divided into eight compartments. Two trials were carried out with two different sets of eight males, each set was composed of a selection of different males representing all of the putative Lake Massoko species and morphs. Set A for trial 1 consisted of three 'big calliptera' type (one light yellow, one brown/yellow and one blue), one 'big predator' type and four 'small slim' type (three blue and one yellow). Set B for trial 2 was made up of three 'big calliptera' type (one light yellow, one green/yellow and one blue), two 'big predator' type and

three 'small slim' type (all blue). Due to the size differences between the big and small ecomorphotypes, males could not be size matched (although all males of the big types were comparable in size) and a slightly modified partial partition design was required. Large males were placed in every-other compartment, leaving the territories in-between available to the smaller males which could fit through the partial partitions. Before the introduction of the females, males were left until the smaller males had settled into one of the 'empty' territories between two bigger males. This design not only allowed a partial partition experiment to be carried out with different size males (some of which were smaller than some of the females), but also ensured that 'same type' males did not inhabit adjacent territories. All fish were wild caught. All 50 available Massoko females were tested in the first trial, 45 of which also produced broods in the second trial (a couple of females were lost and others failed to spawn within a reasonable amount of time in the second round). Diversity at the microsatellite loci chosen for paternity testing was sufficiently high to allow for the identification of individual females based on their microsatellite profile. It was therefore possible to return all female to the experiment at the same time for their second test.

Data analysis

Allopatric experiments

For analysis of the Malawi/Ilamba 4-way female mate choice trials, due to the sharing of alleles between some males of the same population, it was not always possible to assign paternity to specific individuals. Therefore, multiple paternity was ignored if both males were from the same population, and preference assigned to population. Some data were not normally distributed or homoscedastic, transformations did not rectify the problems. Non-parametric Wilcoxon Signed Rank tests were used to test for significant differences in number of offspring tested that were the result of own population vs. other population paternity.

Sympatric experiment

Analysis was split into two parts: success of the different male types with a summary of consistency of female choices. Paired t-tests were used to test for a significant difference in the percentage of broods (all and part) sired by the different types of males in the two trials. To investigate consistency of female choice, female broods were combined from the two trials and each chosen male (rather than each brood produced) was counted as a separate spawning event. 46 of the 50 females spawned with more than one male over the course of the experiment. Multiple paternity of some broods (44%) resulted in 2-4 males per mother. Consistency of female choice for different male traits is summarised by categorisation of females into four different groups: those that showed consistent choice for male ecomorph and colour, those that showed consistent choice for male ecomorph; those that showed consistent choice for male colour; inconsistent females.

Results

Experiments 1 & 2: Allopatric mate choice

1) Malawi – Ilamba

When given the choice of two males from each of the two populations (2-population, 4-way choice), females of both populations showed a strong and significant preference for mating with males from their own population. Of the eight Malawi females tested, all spawned with males from their own population – only one of these was identified as having also spawned with allopatric males. 69 out of the 282 offspring produced (mean 53% of each brood) were tested for paternity, 96% were assigned to conspecific males (Wilcoxon Signed Rank test: $Z_8 = -2.555$, $p = 0.011$). All of the 10 Ilamba females also spawned with own-population males and two also spawned with allopatric males. 99 out of the 214 offspring produced (mean 55% of each brood) were paternity tested, 92% were assigned to conspecific males ($Z_{10} = -2.805$, $p = 0.005$) (Table 2, Appendix III).

2) Ilamba – Itamba

When given the pairwise choice between black males from their own lake and black/dark males from Lake Itamba, females from Lake Ilamba did not show any indication of assortative mating ($Z_{14} = -0.885$, $p = 0.376$). Of the 140 offspring genotyped, from 14 females/broods (mean of 39% of each brood), only 62 % were assigned to own population males (Table 2, Appendix III).

Table 2. Summary of mate choices from experiments testing assortative mating among *Astatotilapia* populations from lakes in the Lake Malawi catchment. Number of females tested, proportion of broods genotyped, number of offspring assigned to con- and heterotypic males, and p-values from Wilcoxon Signed Rank tests. Parentheses display mean values. 1) *A. calliptera* (Lake Malawi) vs *A. sp.* 'ilamba'. 2) *A. sp.* 'ilamba' vs *A. sp.* 'itamba'.

Experiment	Fem. pop.	n broods	n females spawned with			n fry genotyped	n fry assigned	Con-typic fry		Hetero-typic fry		W-S-R p-value
			own pop.	other pop.	both			n	%	n	%	
1) Mal-Ila	Malawi	8	8	1	1	69	69	66	96	3	4	<0.001
	Ilamba	10	10	2	2	99	96	88	92	8	8	<0.001
2) Ila-Ita	Ilamba	14	9	8	3	140	140	87	62	53	38	0.38

Experiment 3: Sympatric mate choice (Lake Massoko)

475 offspring, from 95 broods, produced over two replicates were genotyped for paternity analysis (250 from 50 broods in trial 1; 225 from 45 broods in trial 2). 447 (94 %) were successfully assigned to an individual male. Due to allele sharing among males from set B (trial 2), 19 offspring could only be assigned to male ecomorphological type, not colour and two could not be assigned unambiguously. Only two offspring (from one brood) could not be assigned at all due to allele sharing. Seven offspring (from

six different broods; 2 in trial 1 and 5 in trial 2) could not be assigned due to problems with amplification or disagreement between microsatellite loci (possibly due to cross-contamination of samples). Overall, 8 – 10 offspring from each female that produced more than one brood were unambiguously assigned to father.

Success of male types

With the exception of the 'small slim yellow' male, which struggled to maintain a territory after the introduction of the females in trial 1, sired no offspring and was therefore excluded from all analyses, all male types sired some offspring in both trials. Spawning success was greatest for the 'big calliptera' males, which sired at least part of 81 (85%) of the all the broods produced. Despite the good condition of, and vigorous courtship displays from, the 'big predator' males, they were only involved in siring 10 (11%) of the total broods. 'Small slim' males had similar success and were involved in siring 12 (13%) of broods (Table 3). Ad-hoc observations of the males during the experiment indicated that all were interested in courting passing females, males were filmed for quantitative analysis of behaviour, although footage has yet to be analysed. When categorised by male type, taking into account the different colours of the 'big males' ($n = 5$ types of male: 'big calliptera' light yellow, other yellow, blue; 'big predator'; 'small slim'), there was no significant difference in either the percentage of whole broods (paired t-test: $t_4 = 0.22$, $p = 0.84$), or the percentage of part broods ($t_4 = -0.34$, $p = 0.75$) sired by the different types of male in the two trials.

Table 3. Summary of the success (percentage of whole broods sired and parts of broods sired) of the different male types across the whole experiment, where more than one male of the same type was present, number of offspring was combined for the male type.

Male type	Trial 1 (50 females)		Trial 2 (45 females)		Combined (95 broods)	
	% broods sired		% broods sired		% broods sired	
	whole	part	whole	part	whole	part
Big calliptera	82	90	76	80	79	85
yellow	42	50	47	71	44	60
light	20	32	18	33		
other	12	28	22	44		
blue	34	42	09	27	22	35
Big predator	2	42	11	16	6	11
Small slim	6	14	7	11	6	13
blue	06	14	07	11		
yellow	00	00				

Consistency of individual female choices

Only 28% of females consistently chose males with the same ecomorphological and colour traits. 24% of

females were completely inconsistent, choosing to spawn with males of both different colours and shapes (Table 4).

Table 4. Consistency of choice by the 46 females whose offspring were sired by more than one male. Type = ecomorphological characteristics. Colour = male courtship colour.

Type / Colour	Same	Different	Total
Same	13 (28%)	17 (37%)	30 (65%)
Different	5 (11%)	11 (24%)	16 (35%)
Total	18 (39%)	28 (61%)	46

Discussion

Recently discovered *Astatotilapia* in isolated crater lakes in Tanzania show divergence of male colour and ecomorphological traits among allopatric and within sympatric populations. Using laboratory based mate choice experiments we found strong assortative mating between *A. calliptera* (with yellow males) from Lake Malawi and a putative crater lake species with black male courtship colour (from Lake Ilamba). Previous studies, of both habitat specialist species from within the Great Lake radiations (Knight & Turner 2004; Egger *et al.* 2008; Egger *et al.* 2010; Pauers *et al.* 2010; Pauers & McKinnon 2012) and a more widely distributed riverine generalist species from the genus *Astatotilapia* (Tyers & Turner 2013), have found similar results, that allopatric divergence of male colour is associated with divergence in mate preference (but see Blais *et al.* 2009). However, the degree of reproductive isolation demonstrated here is much greater than among colour divergent allopatric populations of *A. calliptera* from within Lake Malawi, in which assortative mate preferences were relatively weak and asymmetric (Tyers & Turner 2013). There is no indication of assortative mate preferences among dark forms from two different crater lakes, this finding is not surprising and supports previous work which found no assortative mating among allopatric populations of *A. calliptera* that share male courtship colour (Tyers & Turner 2013).

The surprising result from these experiments came from investigation of mate preferences of females from Lake Massoko, which contains a small putative species flock of *Astatotilapia*. Firstly, there was a significant difference in the success of the different male types – in both trials 'big calliptera' males sired many more offspring than any of the other male types present. It is possible that a sampling bias resulted in the over representation of one type of female in our lab stocks, which could explain the overall preference for calliptera type males. This, however, does not explain the variation that individual females showed in their choice of males over multiple spawnings: although there was no significant difference in the success of the different male types across trials, individual females frequently chose to spawn with a variety of males both within and between broods. In sympatric populations of cichlids, female mate preference for conspecific males is often sufficient to maintain reproductive isolation between closely

related species (*e.g.* Seehausen *et al.* 1998; Knight *et al.* 1998) and in the absence of consistent mate preferences it is unclear how phenotypic, ecological, and genetic differentiation is maintained in a sympatric population. There is a strong association between depth and cichlid diversification (Wagner *et al.* 2012), which is consistent with depth being an important axis of niche differentiation in speciation of fishes (Ingram 2011; Wagner *et al.* 2012). In the early stages of adaptive radiation after population expansion into deeper waters by colonising shallow water riverine generalist taxa, disruptive sexual selection on female preferences and male traits has the potential to drive divergence. However, many models of environment dependent secondary sexual trait divergence require that female preferences are linked to sensory sensitivity which may diverge in different environments, driving male trait divergence. Preferences for differences in male traits become heritable and are maintained independent of the environment (sensory drive, *e.g.* Seehausen *et al.* 2008). Therefore, a lack of consistency of individual female preference in a laboratory setting is suggestive of an alternative model of environment driven sexual trait diversification. If females choose males based on traits that are linked to quality there is potential for female choice to drive divergence of both ecological and male secondary sexual traits without divergence of mate preference. Locally adapted males will be better able to attract mates and therefore will have higher fitness while they remain in the environment to which they are adapted. Although dispersal may prevent divergence in such a model, selection against dispersal may result from both reduced reproductive success of males that disperse and a reduction in fitness when intermediate offspring are produced from matings between females that have dispersed and accepted locally adapted males as mates. The result of such a scenario would be environment dependent assortative mating among ecomorphologically divergent species that differ in male secondary sexual characteristics, but retain uniform female preferences based on traits important in intraspecific mate selection in the ancestral species, *e.g.* conspicuousness / courtship vigour (van Doorn *et al.* 2009).

Conclusions

Rather than diversification by sexual selection being limited to a third phase of lacustrine cichlid speciation, recent work involving basal riverine species suggests that sexual trait divergence has contributed to diversification throughout the adaptive radiations of lacustrine cichlids (this study; Tyers & Turner 2013). However, although sexual selection may be involved in diversification, there is little evidence from allopatric populations that colour divergence results in complete reproductive isolation and in this study, sympatric colour divergence also does not appear to result in reproductive isolation in a laboratory setting. Sympatric diversification in the haplochromine cichlids may be possible. Rather than being driven by divergent sexual selection for male colour, however, initial sympatric divergence along an environmental depth/light gradient may result in environment dependent reproductive isolation without the evolution of assortative mating based on actual differences in male traits.

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Chapter 6

This thesis is primarily concerned with the mechanisms of divergence and speciation in cichlids. In this final chapter I shall first give a summary of the main findings from my experiments with haplochromine cichlid fish, a model system with extraordinary diversity of both ecological and sexually selected traits, which is frequently used to investigate theories related to diversification and the maintenance of diversity. I discuss these findings in the context of the main theoretical ideas and themes – the geography of, and selective forces involved in, speciation.

Summary of main findings

Varying degrees of assortative mating were demonstrated among allopatric populations of non-rocky shore cichlids. Male aggression biases were also found among populations:

- Strong assortative mating was found among *Lethrinops* populations that have undergone ecological divergence after isolation in a satellite lake of Lake Malawi. Males biased aggression towards males of their own population (chapter 2).
- Among colour divergent populations of *Astatotilapia calliptera* from Lake Malawi, females showed partial assortative mating and an asymmetric preference to mate with males of their own population. Males biased aggression towards males of their own population. No assortative mating was found among phenotypically similar populations (chapter 3).
- Among *Astatotilapia calliptera* from Lake Malawi and a colour divergent *Astatotilapia* sp. isolated in a crater lake to the North of Lake Malawi there was strong assortative mating. Among two phenotypically similar populations from different crater lakes there was no assortative mating (chapter 5).

Repeat tests of female mate choice found variation within individuals in the types of male they chose to spawn with:

- Females from a single monomorphic population of *Astatotilapia calliptera* also showed variation in their choice of mate when given the choice of males from their own population and a population with different colour males (chapter 4).
- In a sympatric population of *Astatotilapia* isolated in a crater lake, individual females chose to spawn with a variety of males types during laboratory based mate choice experiments (chapter 5).

Synthesis

Morphological divergence may result from ecological and/or sexual selection and is often associated with behavioural divergence – females have a tendency to mate with males of their own species or population and males will often bias aggression towards males of their own species or population. Testing for mate preferences and assortative mating among putative species has been a common method for identifying the differences among recently diverged and diverging taxa which may contribute to reproductive isolation. The ecology and life history traits of different lineages are likely to have a great influence on the modes of speciation that dominate during their diversification. In this thesis I have tested theories of allopatric cichlid speciation with previously little studied taxa from Lake Malawi (Table 1; chapters 2, 3 & 5), carried out experiments into consistency of mate choice (chapter 4 & 5) and contributed to investigations into the possibility of sympatric speciation at the beginning of adaptive radiation of haplochromine cichlids (Table 1; chapter 5).

Allopatric divergence of specialists and generalists

One of the main themes that runs through theories of allopatric speciation in cichlids is the potential for cyclical lake level fluctuations to split apart populations of habitat specialists allowing for divergence during geographic isolation (van Oppen *et al.* 1997; Sturmbauer *et al.* 2001; Genner *et al.* 2007a,b; Koblmüller *et al.* 2012). If reproductive isolation evolves among isolated populations, the result of secondary sympatry as populations are reconnected, may be an increase in alpha diversity (the number of sympatric species). In recent years there has been a strong focus on shallow-water rocky-shore cichlids (Table 1), a fairly distinct group of habitat specialists which tend to have well differentiated colour patterns (compared to the more 'blended' body colours of many other lineages, Konings 2007; Maan & Sefc 2013) and which demonstrate strong genetic population structuring and allopatric colour variation. This research has led to suggestions that intersexual selection may drive divergence (Knight *et al.* 1998; Seehausen *et al.* 1998) and that intralacustrine allopatric speciation by male signal trait diversification may have played a strong role in speciation during the adaptive radiation of lacustrine habitat specialists (van Oppen *et al.* 1997). Tests of assortative mating among colour divergent populations of a generalist species basal to the Lake Malawi radiation, found similar patterns to those observed among rocky-shore fish from all of the great lakes – partial assortative mating associated with colour divergence (chapter 3). Suggesting that signal and preference divergence in allopatry is not limited to the recently diverged endemics that easily become isolated at patches of suitable habitat distributed around the lake. However, if female preference was a driving force of male trait divergence, would there not be a consistent pattern of strong assortative mate preference almost regardless of the degree of differentiation or lineage in which it was occurring? Signal divergence in allopatry may not always occur along with corresponding divergence in behavioural preference, relaxation of female choosiness for species-specific male traits may be common in founding populations (*e.g.* Kaneshiro 1976; Tinghitella & Zuk 2009) and it may therefore

be common for females of ancestral species/populations to be more discriminating than females from derived species/populations, which may readily accept 'ancestral type' males as mates (Ryan & Wagner 1987; Bradley *et al.* 1991; Kaneshiro 2006; Rafferty & Boughman 2006). Although geographic variation in male colour may suggest intralacustrine allopatric speciation by male signal trait divergence, asymmetries and variation in the strength of assortative mate preferences among populations (Table 1) brings into question the plausibility of female preference driven divergence (Koblmüller *et al.* 2012). Additionally, some studies suggest that even among sympatric species that differ in male colour, assortative mating may be reliant on the other cues (Plenderleith *et al.* 2005) and that allopatric populations that share male colour may be partially reproductively isolated (Blais *et al.* 2009). Strong selection on male colour from intersexual aggression is indicated by the ability of males to colour 'up' or 'down', or even change colour, to signal social status (Maan & Sefc 2013). In the high-density highly-aggressive social environment of the rocky shore, it would therefore seem possible that intrasexual selection has the potential to drive divergence of male signal traits with differential selection occurring among populations due to differences in community composition. A pattern of stronger assortative mate preferences among sympatric species than allopatric populations has frequently been used to infer a strong role for reinforcement in speciation of other taxa (*e.g.* Coyne & Orr 1989, but see Konijnendijk *et al.* 2012). Although suggested for cichlids (*e.g.* Blais *et al.* 2007) this mechanism of speciation has been little investigated, despite its potential to add to species richness following sympatric or allopatric divergence by either natural or sexual selection.

Although geographic isolation of populations of rocky-shore habitat specialists is the result of strong habitat preference, making it unlikely that ecological selection is involved in colour divergence in these shallow/clear-water taxa in Lake Malawi (Smith *et al.* 2012), environmental differences may commonly drive divergence among populations of other cichlids and other animals (Schluter & Price 1993). Colour divergence of cichlids in Lake Victoria has been associated with environmental conditions and sensory drive (Seehausen *et al.* 2008) and the East African Great Lakes (Victoria in particular) have numerous shoreline lagoons and inland satellite lakes, which may allow for periods of peripatric divergence under ecologically different conditions. Endemic cichlid fish, often differing in ecomorphological characteristics are known from these lakes and where tested against their main lake counterparts, populations demonstrate strong assortative mating (Genner *et al.* 2007a; chapter 2). Offshore species that are known to migrate inshore to breed and taxa which inhabit muddy bays have both been found in a single satellite of Lake Malawi. These and other similar species are perhaps likely candidates for peripheral isolation and peripatric divergence may account for some of the diversity of these taxa, which tend to show little geographic isolation and genetic population structuring within the main lake (Pereyra *et al.* 2004; Genner *et al.* 2007a; chapter 2). The role of satellite lakes in cichlid speciation requires more attention. Although evidence is currently limited, it appears likely that ecological divergence in peripheral isolation may frequently result in reproductive isolation of specialised lake endemics with minimal male colour divergence. A connection between natural selection / ecological adaptation and the evolution of pre-

zygotic reproductive isolation has been known for some time (Rice & Hostert 1993), however the reason for this connection remains relatively poorly understood. Recently diverged ecologically differentiated geographic species and a greater availability of molecular methods for investigating genetic divergence, may make satellite lakes species such as these a valuable model for research into the link between divergent ecological selection and the evolution of reproductive isolation.

Sympatric divergence from a generalist ancestor

Although much of the later stage speciation of cichlids may be due to allopatric divergence among habitat specialists, if we use the well established theory that, as lakes form, they are invaded (perhaps repeatedly) by generalist species from the surrounding river systems which then undergo intralacustrine adaptive divergence, it follows that initially (particularly within smaller lakes) divergence may frequently be sympatric. Broad scale ecological divergence of generalists first requires the evolution of strong habitat preference to reduce gene flow among populations as they spread out into the different lacustrine habitats. Fine scale trophic adaptation within each habitat type may also initially be driven by behavioural divergence within a sympatric population, with behavioural preference for the use of specific resources driving the morphological adaptation and specialisation of feeding methods. Theoretical scenarios suggest that sympatric speciation is possible (Turner & Burrows 1995; Dieckmann & Doebeli 1999; Higashi & Yamamura 1999; van Doorn *et al.* 2009) and convincing empirical evidence is gathering (Schliewen *et al.* 1994; Strecker & Kodric-Brown 2001; Barluenga *et al.* 2006; Elmer *et al.* 2010; chapter 5).

Evidence is pointing towards the possibility that a combination of ecological and secondary sexual trait divergence may act to produce and maintain variation at the beginnings of adaptive radiations of haplochromine cichlids after the invasion of a novel environment (chapter 5). A lack of strong consistent mate choice for different types of males, in a sympatric population that shows morphological, ecological and genetic differentiation suggests that, in contrast to well differentiated or older species, female mate preference alone is not sufficient for the maintenance of distinctness of sympatric forms. Models of environment dependent assortative mating due to strong habitat preference along a depth gradient and greater success of locally adapted males in their own habitat (*e.g.* van Doorn *et al.* 2009), may explain the variety of haplochromines isolated in small crater lakes. Depth is an important axis of divergence in many fish taxa (Wagner *et al.* 2012; Hulsey *et al.* 2013): Ecological divergence may occur along benthic to limnetic (sticklebacks, Schluter 1993; perch, Svanbäck & Eklöv 2004; cichlids, Hulsey *et al.* 2013) or shallow- to deep-benthic habitat axes (Boughman 2001; Seehausen *et al.* 2008; Ingram 2011). Recent research suggests that morphological divergence associated with ecological specialisation for benthic/limnetic lifestyles has probably occurred multiple times during the adaptive radiation of Lake Malawi cichlids, although it may not account for a great deal of the diversity of the Lake Malawi species flock (Hulsey *et al.* 2013). However, this study was limited to comparisons of divergence of functional morphology and the possibility that concurrent divergence of sexually selected phenotypic traits along the same habitat axis (due, for example, to differences in the signalling environment) has contributed greatly

to speciation during the adaptive radiation of haplochromines remains to be investigated fully. The depth/light gradient found in all but the shallowest of lakes, may be an important first axis of sympatric divergence at the beginnings of adaptive radiation, after colonisation of a lacustrine environment by shallow-water generalist riverine species (chapter 5).

Individual variation in mate preference

Although no firm conclusions can be drawn from this study, preliminary results suggest that there is not complete agreement among females as to which male is most attractive and that variation within females in their choice of males, rather than consistent individual differences in preference are most likely to explain the partial assortative mating often documented in tests of mate preference among allopatric populations of cichlids (chapter 4). Although such variation is unlikely to drive divergence by sexual selection in sympatric populations, it may aid in the establishment of founding populations by relaxation of directional sexual selection, allowing for divergence of male secondary sexual traits to suit new environmental conditions and selection pressures. Population studies of mate preferences should perhaps be cautious when assuming that observed mean population level preferences are applicable to all individuals of a population. Much more research is needed into the nature of variation within and among females especially in systems used to test theories about sexually selected divergence and speciation. The possibilities that females differ in their choosiness or perhaps even their (sensory) ability to distinguish between different types of male (Widemo & Sæther 1999) remain to be investigated in cichlids and may have strong implications for studies of speciation (chapter 4).

Table 1. Female mate choice experiments investigating reproductive isolation in haplochromine cichlids. Including studies of assortative mating among sympatric species, allopatric populations, studies designed to investigate the cues involved in assortative mating and the experiments conducted for this thesis (grey cells). Grey text indicates studies based on non-spawning measures of preference (females association time or reaction to male displays), which can indicate recognition of own males. But, without observations of spawning these can not be compared directly to studies that show the degree of reproductive isolation by measuring spawning. To give a relative idea of the strength of assortative mating results have been categorised: complete = 100%, very strong = >90%, strong = 70-90%, weak = 50-70% (if significant). * = asymmetric assortative mating among populations or variation in strength where assortative mating is dependent on the degree of difference between populations in pairwise comparisons.

Distribution	Habitat	Associated Great Lakes (catchments)	Experiment type		Studies (n)	Male differences	Assortative	Cues implicated	Refs.
			Assay	Cues available					
Sympatric (& unknown)	Shallow-rocky	Malawi & Victoria	Spawning	Full contact	3	Colour/melanin pattern	Complete	Visual (colour/pattern)	1,3,5
			Spawning	Visual / olfactory	1	Dorsal fin colour	Very strong	Visual (dorsal fin colour)	3
			Spawning	Visual only	2	Melanin pattern Dorsal fin colour	Complete Random	Visual (melanin pattern) Olfactory/auditory	4, 3
			Obs.	Visual / olfactory	1	Colour/melanin pattern	Yes	Visual (colour/pattern)	16
				Visual only	5	Colour/melanin pattern	Yes	Visual (colour/pattern)	2,5,6,14,15
				Monochromatic	3	Melanin pattern	No	Visual (colour)	2,16
				Olfactory / auditory	1		No interaction	Visual necessary	2
				Olfactory only	1		No interaction	Visual necessary	2
	Shallow (general)	Malawi	Spawning	Full contact	1	Size/shape/colour	No (no consistency)	Environment dependent?	17
Allopatric	Shallow-rocky	Malawi & Tanganyika	Spawning	Full contact	4	Colour/melanin pattern	Random – complete *	Visual (colour/pattern)	7,8,11,12
				Monochromatic	1		Random – complete *	Non-colour	8
				Visual only	1	Dorsal fin colour	Random	Non-visual	8
			Obs.	Visual only	2	Colour	Yes	Visual (colour)	9,10
	Pelagic	Malawi	Spawning	Full contact	1	Size/shape/colour	Complete (size matched)	Possibly visual	13
	Shallow-muddy	Malawi	Spawning	Full contact	1	Shape/possibly colour	Very strong	Possibly visual	18
	Shallow (general)	Malawi	Spawning	Full contact	2	None or Colour	Random – very strong *	Visual (colour)	19,17

References: 1) Knight *et al.* 1998; 2) Jordan *et al.* 2003; 3) Plenderleith *et al.* 2005; 4) Kidd *et al.* 2006; 5) Seehausen 1997; 6) van der Sluijs *et al.* 2008; 7) Knight & Turner 2004; 8) Blais *et al.* 2009; 9) Pauers *et al.* 2010; 10) Pauers & McKinnon 2012; 11) Egger *et al.* 2008; 12) Egger *et al.* 2010; 13) Genner *et al.* 2007a; 14) Couldridge & Alexander 2002; 15) Jordan 2008; 16) Seehausen & van Alphen 1998; 17) chapter 5 this thesis; 18) chapter 2 this thesis; 19) Tyers & Turner 2013.

Concluding remarks

Together, the combined findings from the experiments presented here and previous studies suggest a strong role for ecological selection in the evolution of reproductive isolation during adaptive radiation of haplochromine cichlids - colour and ecomorphological divergence in haplochromine crater lake cichlids may occur without assortative mating by direct mate choice; strong assortative mating with ecological divergence with minimal divergence of male colour of specialists has been found among main lake populations and those isolated in satellite lakes; partial or asymmetric assortative mating among allopatric populations that differ in male colour but not ecological traits is frequently observed. In a recent review of colour diversity in cichlid fish, Maan & Sefc (2013) highlighted the taxonomic bias in the mate choice literature and the need for more studies into the role of colour and other cues in mate choice among populations and species of a wider variety of cichlids. I hope to have added to our knowledge of patterns of reproductive isolation with these experiments (Table 1). However, my experiments have been limited to identifying the presence/absence/strength of assortative mating under full contact conditions and I reiterate the need for further investigation of the cues involved in mate choice in different taxa.

Although it is likely that divergence of secondary sexual traits has contributed to diversification throughout the adaptive radiations of lacustrine cichlids, different cues may be more or less important in assortative among different species pairs, depending on their shared history and the selection pressures that have acted in the evolution of reproductive isolation among them. Broad scale assumptions about the likelihood of assortative mating among allopatric populations may be unjustified if based on a single type of cue that has been suggested to be important in one lineage of cichlids. It is clear that within species, male colour trait divergence in allopatry is not only associated with female mate preference, but also male aggression biases. It has been suggested that divergence in female preferences in isolated population may drive such divergence in traits. However, rather than consistently finding that female preferences among allopatric populations are strong, we find variation in the strength of assortative mating, asymmetries in pre-zygotic behavioural isolation, and sometimes only weak associations. Perhaps male aggression has a greater role in allopatric colour divergence than it is often credited for. Populations regularly come in and out of contact as lake levels fluctuate and males interact with conspecifics and heterospecifics, often in tightly packed highly aggressive communities. Although conspecific aggression biases are frequently found this does not translate to a complete absence of aggression among heterospecifics, which although not in competition for mates, are still in competition for other resources. If there is variation in female mate preference or fussiness in a founding population, it is possible that female mate preferences may follow male trait divergence – a relaxation of directional sexual selection may aid in colonisation of new areas by allowing male traits to diverge due to different selection pressures such as predation pressure or aggression from similar heterospecifics.

Theoretical models suggest that sympatric speciation is more likely under the combined forces of natural and sexual selection, it seems probable that speciation along a depth gradient and the combined

potential for ecological and sexual selection that it provides may prove to be a very strong force in 'kick-starting' the rapid diversification of shallow-water riverine generalists into a diverse range of lacustrine specialists. We hope that the recently discovered *Astatotilapia* species flocks will provide a model system for comparative studies of populations at different points in the early stages of adaptive radiation, in a lineage basal to the great lacustrine radiations.

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Appendices

Appendix I: Co-authors and contributions

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Contributed to planning of many of the experiments and to manuscript preparation (chapters 2 and 3). Proof read and commented on other chapters.

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Organised the logistics and funding for the student field trip which resulted in the collection of the Lake Chilingali fish used in the published *A. calliptera* paper (chapter 3) and the *Lethrinops* manuscript (chapter 2). Also helped with maintenance of stock fish.

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Contributed dietary analysis for the *Lethrinops* manuscript (chapter 2)

Tara Donnelly, Hannah Bell and Holly-May Watson helped with setting up and running some of the behavioural experiments for chapters 2 and 3.

Appendix II: Extended phylogenetic analysis for Chapter 2

This appendix gives an extended explanation for our reasons for believing that *L. lethrinus* is the most likely sister taxon for *L. sp. 'chilingali'*, and for the reasoning behind our omitting other GenBank *Lethrinops* spp. sequences from the haplotype network presented in Chapter 2.

Figure 1 illustrates a minimum spanning network including 48 additional sequences from GenBank, representing putative 'shallow-water' and 'deep-water' *Lethrinops* species. It can be seen that the two specimens identified as *Lethrinops marginatus* appear to lie intermediate between *Lethrinops lethrinus* and the majority of *L. sp. 'chilingali'* haplotypes. A single *L. sp. 'chilingali'* lies intermediate between *L. lethrinus* and two specimens assigned to another taxon, *L. furcifer*.

Figure 2 is based on the same specimens, but with the removal of 31 specimens normally assigned to the 'deep-water' clade though to be distantly related to the shallow-water *Lethrinops* species. The position of *L. furcifer* and *L. marginatus* samples is substantially different, indicating that we probably should not have too much confidence in their relationship with our focal study taxa.

Furthermore, many previous studies of mtDNA of Lake Malawi cichlid fishes show very high levels of intraspecific polymorphism, extensive cross-species haplotype sharing and little indication of reciprocal monophyly (e.g. Meyer *et al.* 1996; Mims *et al.* 2010; Anseeuw *et al.* 2012). Thus, it is unwise to infer relationships from small sample sizes, such as $n = 2$ for *L. marginatus* and *L. furcifer*. With a larger sample size, relationships might look very different. Indeed, many studies have shown inconsistencies between mitochondrial and nuclear trees (e.g. Joyce *et al.* 2011; Anseeuw *et al.* 2012; Genner & Turner 2012), suggesting that even well-supported mtDNA trees can be fundamentally misleading.

Additionally, identification of *Lethrinops* species is not straightforward. Although *L. lethrinus* should be readily identified from the horizontal melanin pattern, this is not obvious in territorial males. The samples of *L. furcifer* and *L. marginatus* on GenBank come from an unpublished study and are not accompanied by voucher specimens or illustrations that would enable us to judge whether they were correctly identified. *L. lethrinus* and *L. sp. 'chilingali'* share the following external features: females and immature / non-territorial males with two horizontal stripes on the flanks, a series of spots at the base of the dorsal fin and a thin red/orange margin to the dorsal fin; relatively long snout and deep body (further comparisons of the species discussed here can be found in Fig. 3).

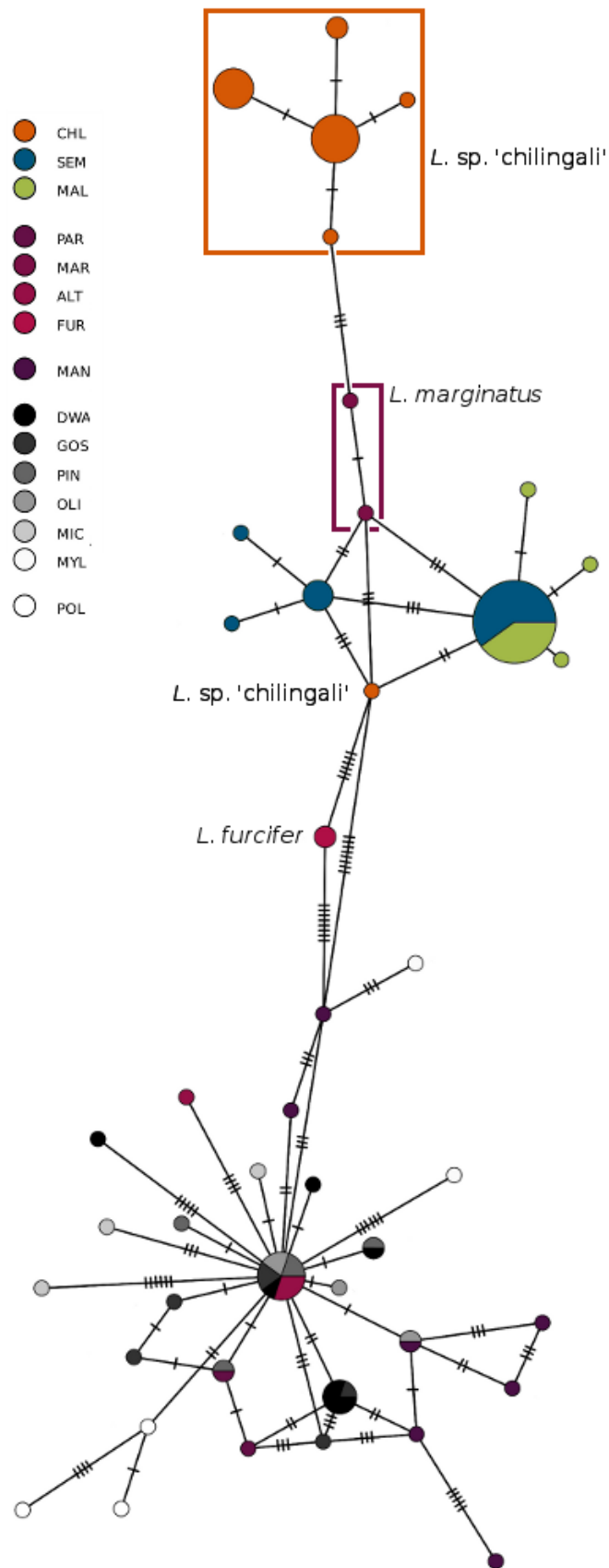


Figure 1. Minimum spanning haplotype network of 109 *Lethrinops* mtDNA sequences: From the study taxa used in this thesis ($n = 61$ from the three lakes CHL/SEM/MAL), species often considered to be associated with a 'shallow-water' depth distribution ($n = 17$ from 5 taxa, from GenBank and Lake Malawi trawl catches) and other species associated with a 'deep-water' distribution ($n = 31$ from 7 taxa). See Table 1 for key to species abbreviations and more sample details.

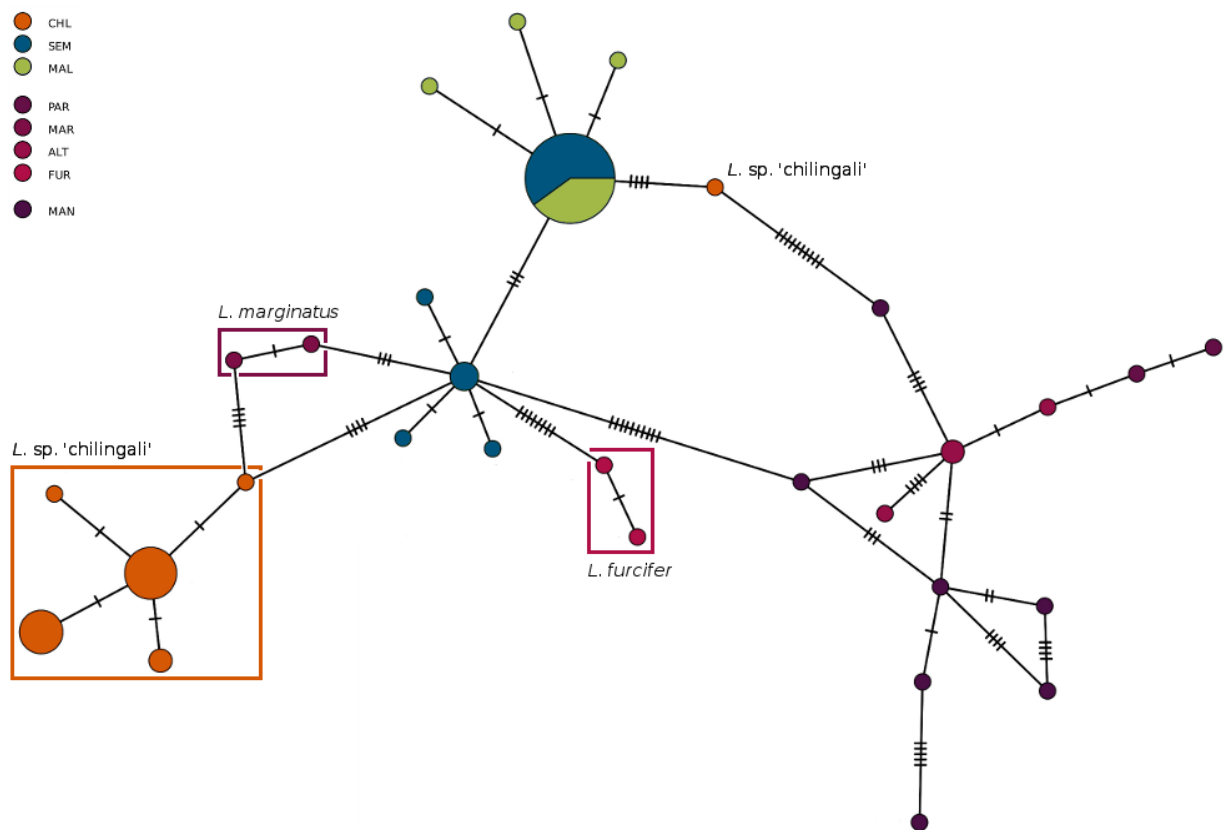


Figure 2. Minimum spanning haplotype network of 78 *Lethrinops* mtDNA control region sequences: From the study taxa used in this thesis ($n = 61$ from the three lakes CHL/SEM/MAL) and species often considered to be associated with a 'shallow-water' depth distribution ($n = 17$ from 5 taxa, from GenBank and Lake Malawi trawl catches). See Table 1 for key to species abbreviations and more sample details.

Table 1. Key to species/population abbreviations, number and origin (see Chapter 2 methods for more detailed information on the origin of taxa used in this study) of sequences. Where possible, information regarding depth preference, distribution and habitat (substrate) has been included (Konings 2007).

Taxa	n	Sample origin	Code	Depth / distribution / habitat
<i>L. sp. 'chilingali'</i>	22	Lake Chilingali	CHL	Shallow / Lake Chilingali / Muddy/murky
<i>L. lethrinus</i>	24	South-East Lake Malawi	SEM	Shallow / Lake-wide / Muddy
	15	Lake Malombe	MAL	Shallow / Lake Malombe
<i>L. parvidens</i>	2	GenBank	PAR	Shallow / East/Southeast LM
<i>L. marginatus</i>	2	GenBank	MAR	Shallow
<i>L. altus</i>	4	GenBank	ATL	Shallow / Lake-wide / Soft substrate
<i>L. furcifer</i>	2	GenBank	FUR	Shallow
<i>L. longimannus</i>	7	GenBank (2) / Trawl (5)	MAN	Moderately-deep / Lake-wide / Pure sand
<i>L. sp. 'deep-water albus'</i>	8	GenBank (3) / Trawl (5)	DWA	Deep
<i>L. gossei</i>	6	GenBank (3) / Trawl (3)	GOS	Deep / Lake-wide / Pure sand
<i>L. olivei</i>	4	GenBank (2) / Trawl (2)	OLI	Deep
<i>L. longipinnis</i>	5	GenBank (3) / Trawl (2)	PIN	Deep / Lake-wide / Mud/diatomaceous-ooze
<i>L. microdon</i>	3	GenBank	MIC	Deep / Southern / Diatomaceous-ooze
<i>L. mylodon</i>	2	GenBank	MYL	Deep / Lake-wide / Sand
<i>L. polli</i>	3	GenBank	POL	



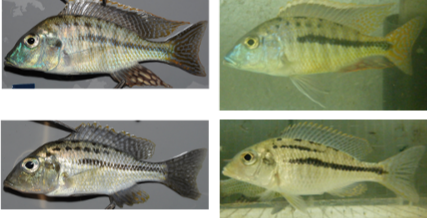
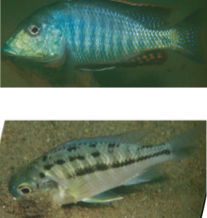
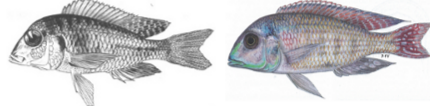

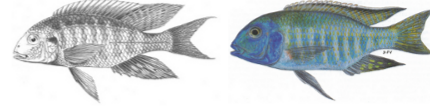
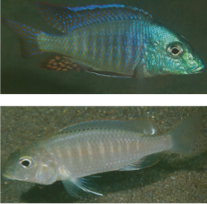
Lectotype / holotype (Eccles & Trewavas 1989)	Colour illustration (Ngatunga & Snoeks 2004)	Lab photos (out of tank / in tank)	Field photos (Konings 2007)
<i>L. sp. 'chilingali'</i>			
<i>L. lethrinus</i> (Gunther 1894)			
<p>160mm SL, deeply sloping wedge shaped snout, characteristic melanin pattern. Widespread in shallow vegetated waters, also enters lower reaches of inflowing rivers</p>			
<i>L. marginatus</i> (Ahl 1926) syn. <i>L. oculatus</i> (Trewavas 1931)		<p>110mm SL, characterised by large eye one third of head length, short snout</p>	
<i>L. furcifer</i> (Trewavas 1931)			
<p>160mm SL, snouth slightly rounded, deeply forked caudal fin. Common off exposed sandy beaches.</p>			

Figure 3. Descriptions (Eccles & Trewavas 1989) and illustrations/photographs of our study taxa (*L. lethrinus* & *L. sp. 'chilingali'*) and other closely related species (*L. marginatus* & *L. furcifer*): Lectotype illustrations (holotype for *L. marginatus*) from Eccles & Trewavas 1989; colour illustrations from (Ngatunga & Snoeks 2004); laboratory photographs from our experimental stocks; field photographs from Konings 2007).

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Appendix III: Data from behavioural experiments

Chapter 2: *Lethrinops* spp.

***Lethrinops* female mate choice experiment:** Percentage of brood genotyped and number of offspring from females of each population assigned to the males of each population in a two-way choice with one male of each population.

<i>L. lethrinus</i> 'mazinzi' vs <i>L. sp.</i> 'chilingali'				
Female population	Generation	Number of offspring assigned to male		% brood genotyped
		Mazinzi	Chilingali	
<i>L. lethrinus</i> 'mazinzi'	Wild caught	10	0	15
	Wild caught	10	0	42
	Wild caught	10	0	40
	Wild caught	10	0	17
	Wild caught	10	0	15
	Wild caught	10	0	33
	1 st Gen lab	9	0	100
<i>Total</i>		69	0	
<i>L. sp.</i> 'chilingali'	Wild caught	0	10	11
	Wild caught	0	10	27
	Wild caught	0	10	9
	Wild caught	0	10	13
	Wild caught	0	10	10
	Wild caught	0	10	11
	1 st Gen lab	0	10	77
	1 st Gen lab	0	10	56
	1 st Gen lab	0	10	30
	1 st Gen lab	10	0	39
	1 st Gen lab	0	10	56
	1 st Gen lab	0	10	40
	1 st Gen lab	0	10	59
	1 st Gen lab	0	10	100
	1 st Gen lab	0	10	50
<i>Total</i>		10	140	

***Lethrinops* male aggression experiment:** Mean number of aggressive behaviours directed towards stimulus males from each population by focal males of each population in pairwise simulated intruder choice tests

<i>L. lethrinus</i> 'mazinzi' vs <i>L. sp.</i> 'chilingali'							
Focal male pop.	Aggression to stimulus						
	Mazinzi			Chilingali			Total
	Displays	Attacks	Total	Displays	Attacks	Total	
<i>L. lethrinus</i> 'mazinzi'	10	46	56	2	22	24	80
	6	6.5	12.5	12	8	20	32.5
	2	32.5	34.5	3.5	24.5	28	62.5
	16	53	69	5	37.5	42.5	111.5
	16.5	42.5	59	0	1	1	60
	15.5	11.5	27	2.5	10.5	13	40
	16.5	20	36.5	7	8.5	15.5	52
	0.5	3.5	4	1.5	5.5	7	11
	10.5	14	24.5	5	5.5	10.5	35
	20.5	21	41.5	2	7.5	9.5	51
	12.5	20	32.5	5.5	2.5	30.5	63
<i>Total</i>	126.5	270.5	397.5	46	155.5	201.5	598.5
<i>L. sp.</i> 'chilingali'	1	11	12	0	26	26	38
	4	26	30	5	19.5	24.5	54.5
	19.5	17.5	37	45	35.5	80.5	117.5
	2	17.5	19.5	14	45	59	78.5
	14	49.5	63.5	8.5	34	42.5	106
	1.5	53	54.5	22	52.5	74.5	129
	4	6	10	21.5	21.5	43	53
	3.5	8	11.5	25.5	22.5	48	59.5
	7.5	22	29.5	51.5	44.5	96	125.5
	6.5	12	18.5	46	29.5	75.5	94
	16	28.5	44.5	26.5	48.5	75	119.5
<i>Total</i>	79.5	251	330.5	265.5	379	644.5	975

Chapter 3: *Astatotilapia calliptera*

***Astatotilapia calliptera* 3-population 3-way female mate choice experiment:** Number of eggs females of different populations laid with males of the different populations in a three-way choice with one males of each population.

<i>A. calliptera</i> Salima / Chizumulu Island / Lake Chilingali				
Female population	Number of eggs laid with males			Total eggs
	Salima	Chizumulu	Chilingali	
Salima	55	0	13	68
	27	0	15	42
	0	0	43	43
	59	0	0	59
	59	0	0	59
	0	92	0	92
	99	0	0	99
	0	0	103	103
	<i>Total</i>	299	92	174
Chizumulu Island	0	10	3	13
	6	21	0	27
	3	18	14	35
	22	12	0	34
	0	50	0	50
	0	19	0	19
	4	35	0	39
	15	24	32	71
	<i>Total</i>	50	189	49
Lake Chilingali	27	0	26	53
	0	0	37	37
	0	0	125	125
	21	0	37	58
	0	30	0	30
	1	0	102	103
	39	0	0	39
	4	96	0	100
	<i>Total</i>	92	126	327

***Astatotilapia calliptera* 2-population 6-way female mate choice experiment:** Number of eggs females of different populations laid with males of the different populations in a six-way choice with three males of each population.

<i>A. calliptera</i> Salima vs Chizumulu Island			
Female population	Eggs caught		
	<i>n</i> with males		total
	Salima	Chizumulu	
Salima	79	4	83
	38	0	38
	40	0	40
	0	69	69
	22	13	35
	1	64	65
	<i>Total</i>	180	150
Chizumulu Island	0	41	41
	32	8	40
	17	14	31
	0	66	66
	34	0	34
	81	0	81
	0	37	37
	1	42	43
	0	71	71
	0	25	25
	0	38	38
	0	30	30
	1	61	62
<i>Total</i>	166	433	599

<i>A. calliptera</i> Salima vs Lake Chilingali				
Female population	Eggs caught			
	<i>n</i> with males		total	
	Salima	Chilingali		
Salima	77	39	116	
	113	0	113	
	11	45	56	
	0	96	96	
	0	70	70	
	0	76	76	
	0	71	71	
	21	54	75	
	<i>Total</i>	222	451	673
	Lake Chilingali	0	76	76
77		0	77	
72		0	72	
0		15	15	
5		40	45	
0		94	94	
0		93	93	
<i>Total</i>	245	324	569	

***Astatotilapia calliptera* male aggression experiment:** Mean number of aggressive behaviours directed towards stimulus males from each population by focal males of each population in pairwise simulated intruder choice tests.

A. calliptera Salima vs Chizumulu Island							
Focal male pop.	Aggression to stimulus						Total
	Salima			Chizumulu			
	Displays	Attacks	Total	Displays	Attacks	Total	
Salima	6	40.5	46.5	0.5	0	0.5	47
	27.5	23	50.5	9.5	13.5	23	73.5
	22	13	35	6.5	2	8.5	43.5
	17.5	22.5	40	16	19	35	75
	26	67.5	93.5	5	5	10	104
	14.5	32	46.5	8.5	22.5	31	77.5
	10.5	8.5	19	15.5	10	25.5	44.5
	35.5	46	81.5	2	0.5	2.5	84
	29.5	26	55.5	7.5	1.5	9	64.5
	23	11.5	34.5	29.5	6.5	36	70.5
	16.5	11.5	28	2.5	4	6.5	34.5
	18.5	11.5	30	10.5	11	21.5	51.5
<i>Total</i>	247	314	560.5	114	95.5	209	769.5
Chizumulu Island	4	7.5	11.5	14	8.5	22.5	34
	7	1.5	8.5	24.5	17.5	42	50.5
	7	4	11	15.5	31	46.5	57.5
	1.5	0	1.5	19.5	12	31.5	33
	2	4.5	6.5	15.5	39.5	55	61.5
	1.5	8.5	10	22.5	35	57.5	67.5
	5	1	6	16.5	8	24.5	30.5
	17.5	18.5	36	1	0.5	1.5	37.5
	5	3.5	8.5	9	2	11	19.5
	3.5	12	15.5	9	12	21	36.5
	19	17	36	10.5	13.5	24	60
	6	8	14	21	62.5	83.5	97.5
<i>Total</i>	79	86	165	178.5	242	420.5	585.5

Chapter 5: *Astatotilapia* spp.

***Astatotilapia* 2-population 4-way female mate choice experiment:** Percentage of brood genotyped and number of offspring from females of each population assigned to the males of each population in a four-way choice with two males of each population.

<i>A. calliptera</i> vs <i>A. sp. 'ilamba'</i>			
Female population	n offspring assigned to male		% brood genotyped
	<i>A. calliptera</i>	<i>A. sp. 'ilamba'</i>	
<i>A. calliptera</i>	10	0	56
	7	3	17
	10	0	18
	10	0	16
	7	0	100
	10	0	15
	7	0	100
	5	0	100
<i>Total</i>	66	3	
<i>A. sp. 'ilamba'</i>	0	10	83
	1	9	59
	0	10	67
	7	2	100
	0	7	50
	0	10	35
	0	10	50
	0	10	26
	0	10	37
	0	10	39
<i>Total</i>	8	88	

Astatotilapia 2-population 2-way female mate choice experiment: Percentage of brood genotyped and number of offspring from females of each population assigned to the males of each population in a two-way choice with one male of each population.

A. sp. 'ilamba' vs A. sp. 'itamba'			
Female population	<i>n</i> offspring assigned to male		% brood genotyped
	A. sp. 'ilamba'	A. sp. 'itamba'	
A. sp. 'ilamba'	0	10	42
	10	0	63
	9	1	31
	9	1	40
	0	10	42
	10	0	33
	9	1	44
	10	0	35
	0	10	63
	10	0	36
	10	0	20
	0	10	42
	10	0	22
	0	10	31
<i>Total</i>	87	53	

Appendix IV: Lake Chilingali – possible loss of endemics

A sampling trip in 2010, during which some of the fish used in this thesis were collected, found the dam maintaining the high water level of Lake Chilingali to be on the brink of collapse, making the future of the lake uncertain and putting its endemic cichlids at risk from habitat loss (see *Practicle Fish Keeping* 2010, issue 6, pp. 10-11). With no action taken it was recently brought to our attention that sometime in 2012 the dam did collapsed, resulting in significant loss of water and perhaps complete drying out of the lake (Genner pers. comm.). We have not yet been able to visit and survey area, but it is possible that the endemic species of *Rhamphochromis* and *Lethrinops* have become extinct in the wild, making Bangor and Hull universities in the UK the last refugia of these species.

