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# The ecological, behavioral, and genetic factors influencing the diversification of Lake Malawi's rock-dwelling cichlids

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**THE ECOLOGICAL, BEHAVIORAL, AND GENETIC FACTORS INFLUENCING THE**

**DIVERSIFICATION OF LAKE MALAWI'S ROCK-DWELLING CICHLIDS**

**BY**

**PATRICK D. DANLEY**

**Bachelor of Science**

**The Pennsylvania State University**

**1995**

**DISSERTATION**

**Submitted to the University of New Hampshire  
in Partial Fulfillment of  
the Requirements for the Degree of**

**DOCTOR OF PHILOSOPHY**

**in**

**ZOOLOGY**

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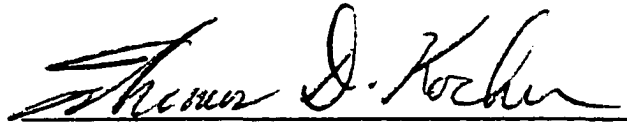
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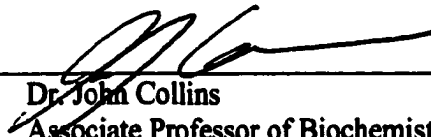
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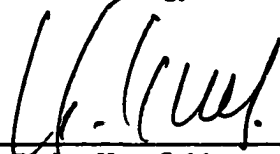
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**To the memory of Joseph Kantekule, a kind friend who gave to me more than I could ever repay.**



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**ABSTRACT**

**THE ECOLOGICAL, BEHAVIORAL, AND GENETIC FACTORS INFLUENCING THE  
DIVERSIFICATION OF LAKE MALAWI'S ROCK-DWELLING CICHLIDS**

by

**PATRICK D. DANLEY**

**UNIVERSITY OF NEW HAMPSHIRE, SEPTEMBER, 2001**

**Rapid evolutionary radiations provide insight into the fundamental processes involved in species formation. Here I examine the diversification of one such group, the rock-dwelling cichlid fishes of Lake Malawi (mbuna), which have radiated from a single ancestor into more than 200 species over the past 700,000 years. Through the examination of several molecular phylogenies, a phylogenetic history of the mbuna is proposed. The diversification of this group has proceeded in three major bursts of cladogenesis each of which has been dominated by a single selective force (selection on**

habitat preferences, trophic morphology, and male nuptial coloration, respectively). A divergence with gene flow model is advocated with reference to this phylogenetic pattern. A population genetic survey of allele frequency distributions at four microsatellite loci is used to quantify levels of gene flow within the mbuna genus *Metriaclima*. Results from this study indicate that migration rates between adjacent populations are low such that slight variation in the selective environment may drive the divergence of populations. Speciation models involving genetic drift, population bottlenecks, founder events, microallopatric divergence, and divergence with gene flow are discussed with respect to these findings. Some of the factors contributing to the existence of highly diverse, densely packed mbuna communities are identified by examining male territorial behavior and habitat utilization within *Metriaclima*. By observing the recolonization of artificially vacated territories, I show that (1) territories are species-specific and (2) this species-specificity is associated with the width of the breeding caves. These results are discussed with respect to character displacement and sexual selection. The speciation of the mbuna has been closely tied to the diversification of male color patterns, yet little is known concerning the genetic basis of such a fundamental character. By (1) examining female mating preferences, (2) describing the cellular basis of color pattern variation, and (3) identifying the genetic basis of this



variation, I show that differential male melanistic patterning, which plays a role in the reproductive isolation of two *Metriaclima* species, is controlled by a limited number of genes. The rapid diversification of the mbuna is discussed with reference to this finding.

## CHAPTER 1

# SPECIATION IN RAPIDLY DIVERGING SYSTEMS: LESSONS FROM LAKE MALAWI

### Abstract

Rapid evolutionary radiations provide insight into the fundamental processes involved in species formation. Here we examine the diversification of one such group, the cichlid fishes of Lake Malawi, which have radiated from a single ancestor into more than 400 species over the past 700,000 years. The phylogenetic history of this group suggests (1) that their divergence has proceeded in three major bursts of cladogenesis and (2) that different selective forces have dominated each cladogenic event. The first episode resulted in the divergence of two major lineages, the sand- and rock-dwellers, each adapted to a major benthic macrohabitat. Among the rock-dwellers, competition for trophic resources then drove a second burst of cladogenesis, which resulted in the differentiation of trophic morphology. The third episode of cladogenesis is associated with differentiation of male nuptial coloration, most likely in response to divergent sexual selection. We discuss models of speciation in relation to this observed pattern. We advocate a model, divergence with gene flow, which reconciles the disparate selective forces responsible for the diversification of this group and suggest that the non-adaptive nature of the tertiary episode has significantly contributed to the extraordinary species richness of this group.

## Introduction

Explosive taxonomic radiations are useful systems in which to study the process of species formation. The extraordinary biological diversity of these systems evolves through multiple cladogenic events. Through comparing such events among closely related taxa, one can begin to identify common factors that influence the speciation process. Identification of these factors is further simplified because these radiations often occur in insular habitats (such as isolated lakes) in which the spectrum of factors affecting diversification is reduced. The young age of most rapid radiations may also simplify the identification of factors involved in the speciation process. Because speciation has occurred relatively recently in these systems, a greater proportion of the phenotypic differences between two lineages are likely to be directly attributable to the forces which cause speciation. For these reasons, an examination of the largest extant vertebrate radiation known, the cichlid fishes of East Africa, should prove informative. In this review we examine the patterns of diversification in a subset of this group, the fishes of Lake Malawi's rock-dwelling assemblage, in an attempt to identify common processes in their radiation. We advocate a model of multiple diversification that may account for the extraordinary biological diversity in this and other explosive radiations.

The East African cichlid radiation is concentrated in the three Great Lakes of Tanganyika, Malawi and Victoria. Lake Tanganyika, the oldest of the three lakes (basin age estimated to be between 9 and 12 million years old) (Cohen et al. 1993) supports at least 197 endemic cichlids in 49 endemic genera (Poll 1986). These genera can be grouped into 12 separate tribes that are thought to have diverged from seven distinct ancestral lineages

(reviewed by Meyer 1993). These tribes are relatively old compared to other East African cichlid lineages; phylogenetic evidence suggests that some of the tribes originated at least five million years ago (Nishida 1991; Sturmbauer and Meyer 1993) making them older than either the Victoria or Malawi lake basins (Fryer and Iles 1972). Lake Tanganyika's cichlids are morphologically and behaviorally more diverse than the cichlids of Lakes Victoria and Malawi (Fryer and Iles 1972), however, the latter two lakes each possess a greater number of cichlid species than Lake Tanganyika.

Lake Victoria was formed 250,000 to 750,000 years ago (Johnson et al. 1996) when the tilting of the Tanzanian shield back-ponded west flowing rivers. It is thus more shallow than Lakes Tanganyika and Malawi which are situated in the cleft of the East African Rift. More than 300 endemic species of cichlids occur in Lake Victoria (Seehausen 1996), all of which were thought to be derived from a single common ancestor (Meyer et al. 1990). However recent molecular data suggest that Lake Victoria was colonized by at least two separate lineages. These two lineages, one representing the Lake Victoria's rock-dwelling cichlids (mbipi) and the other representing all other endemic cichlids from Lake Victoria, each invaded the lake and rapidly diverged within past 12,500 years (Nagl et al. 2000).

The cichlid flock of Lake Malawi is intermediate to those in Lakes Tanganyika and Victoria in almost every respect. Cichlids are thought to have invaded Lake Malawi approximately 700,000 years ago and their morphological diversity is considerably greater than the much younger species flock of Lake Victoria. In addition, the Malawian radiation has produced the greatest number of endemic species of the three species flocks (well over 400 endemic species distributed among 49 endemic genera) (Moran et al. 1994) and appears to be monophyletic in origin (Meyer et al. 1990; Kocher et al. 1993; Moran et al. 1994). For these reasons, the cichlid fishes of Lake Malawi appear ideally suited for the study of rapid radiations.

Early models stressed the importance of vicariant events in generating the species diversity in East African lakes. The multiple invasion model suggested that each lake had

been colonized by multiple lineages which had evolved independently in either space (Mayr 1942) or time (Fryer 1977). Molecular phylogenetic studies provide little support for these models. A study of the Tanganyikan tribes had suggested that their diversification might predate the formation of the lake basin (Nishida 1991) but this notion was later rejected (Meyer 1993) after a reevaluation of the lake basin's age (Cohen et al. 1993). Although it is possible that the 12 Tanganyikan tribes were derived from multiple riverine ancestors, it is clear that most of the subsequent radiation occurred within the lake basin. Likewise, Victorian cichlids appear to have diverged prior to their invasion of the current lake basin. However, the vast majority of the species within this lake (some 300 species) arose *in situ* within the past 13,000 years (Nagl et al. 2000). In contrast, molecular phylogenies suggest the separate, monophyletic origin of the haplochromine cichlids within Lake Malawi (Meyer et al. 1990; Moran et al. 1994). The multiple invasion model is not adequate to explain the extraordinary diversity of East African cichlids.

Vicariance resulting from changes in lake level may have played an important role. East Africa is prone to dramatic changes in climate and precipitation. As a result, lakes in this region experience frequent fluctuations in lake water levels (Owen et al. 1990) and may fragment during dry periods. This is particularly apparent in Lake Tanganyika. Seismic data indicate that approximately 25,000 years ago water levels in Lake Tanganyika were 600m below the current level. During this period, three separate sublakes existed within Lake Tanganyika's current shoreline (Scholz and Rosendahl 1988). This fragmentation is reflected in the phylogeographic patterns of Lake Tanganyika's rock dwelling cichlids (Sturmbauer and Meyer 1992; Verheyen et al. 1996; Rüber et al. 1998; Rüber 1999). Mitochondrial phylogenies indicate that many haplotype lineages are restricted to particular sub-basins. Furthermore, several cross-lake haplotype affinities were identified which correspond to currently inaccessible sub-basin shores. Given the stenotopy of the taxa involved, a previous connection across a paleo-shoreline is the most parsimonious explanation for the observed haplotype affinities.

While the effect of this vicariant event is widespread, it is not universal. Not all taxa in Lake Tanganyika show these phylogeographic patterns (Meyer et al. 1996) and basin morphologies of other lakes do not generate multiple basins during low water stands (Scholz and Rosendahl 1988). The effect of fluctuating lake levels may have been more subtle in these systems (van Oppen et al. 1997; Arnegard et al. 1999; Markert et al. 1999; Danley et al. 2000).

Ecological and behavioral factors appear to have had the largest effect on the diversification of cichlids in all three lakes. It has been difficult, however, to partition and rank the effects of these forces. Below we examine the patterns of diversification in one of these species flocks, the rock-dwelling cichlids of Lake Malawi. Through an examination of the phylogenetic history of this group, we attempt to identify forces that have influenced their diversification and to partition the effect of these forces to particular periods in the radiation. We conclude by suggesting a model that unifies the disparate forces that have acted during each stage of this radiation.

## The Pattern of Diversification

Lake Malawi cichlids have experienced sequential episodes of diversification (Fig. 1.1). Lake Malawi was colonized by a generalized cichlid that first diverged into two major clades (the rock-dwelling *mbuna* and a sand-dwelling clade) plus several oligotypic lineages (Moran et al. 1994; Seehausen et al. 1999). Adaptation to the rock and sand macro-habitats resulted in the divergence of many morphological and behavioral characteristics including body shape, trophic morphology, melanin patterning, reproductive behaviors, and habitat preference (Fryer 1959; Ribbink et al. 1983).

The *mbuna* and non-*mbuna* clades each contain more than 200 species and are thought to be reciprocally monophyletic (Moran et al. 1994). However, determining the relationships of species within these two large clades has been problematic. Morphological characters frequently converge among distantly related lineages within Lake Malawi (Meyer et al. 1990) as well as among the species flocks of different East African lakes (Kocher et al. 1993). Molecular studies have also failed to resolve the phylogenetic relationships within these clades. The paucity of DNA sequence variation (Meyer et al. 1990; Moran et al. 1994), and the retention of ancestral polymorphisms, has stymied attempts to develop fully resolved phylogenies (Moran et al. 1994; Kornfield and Parker 1997).

A recent genome wide survey of several thousand loci has begun to overcome these obstacles. Albertson et al. (1999) reconstructed the inter- and intrageneric phylogenetic relationships of several *mbuna* genera after surveying 2247 Amplified Fragment Length Polymorphism (AFLP) loci. The resulting phylogeny, while limited in scope, confirmed the current  $\beta$  level taxonomy of the *mbuna* and the previous hypotheses concerning the phylogenetic relationships between the *mbuna* and other Lake Malawi cichlids.

The evolutionary relationships among Lake Malawi's cichlid fishes suggest that this group has experienced at least three sequential episodes of diversification (Fig. 1.1). The

primary radiation resulted in the divergence of the rock-dwelling species from the sand-dwelling species. The secondary radiation, responsible for the diversification of mbuna genera, is closely associated with the refinement of the mbuna trophic apparatus. Although the mbuna are generally similar in body shape, melanistic body markings and habitat preferences, there are striking differences in their trophic morphology (Reinthal 1990b). The diversification of species during the tertiary radiations is commonly attributed to the divergence of reproductive characters (Fig. 1.1). Species within genera are nearly identical with respect to most morphological characters and are primarily identified on the basis of male secondary sexual characteristics such as coloration (Stauffer et al. 1997).

This type of phylogenetic history, which is punctuated by multiple diversification events, is a common pattern in rapidly radiating systems (Schluter and McPhail 1993; Sturmbauer 1998). In the sections below we attempt to identify the forces that may explain this evolutionary pattern in Lake Malawi's mbuna. Other rapidly radiating systems are also examined to determine the generality of these processes.



## The Primary Radiation and the Creation of Macrohabitat Clades

The diversification of the mbuna has been episodic and each episode appears to have been dominated by the refinement of phenotypes related to a particular adaptive axis. In the earliest split, adaptation to the two major macrohabitats resulted in two large benthic clades: the sand dwellers and the rock-dwellers. During this period, strong selection on multiple characters resulted in the divergence of many morphological and behavioral characters related to ecological specialization.

An early ecological split appears to be common in cichlid radiations. Lake Victoria's cichlids diverged early in their radiation into rock and sand-dwelling clades (Nagl et al. 2000), as have Lake Tanganyika's cichlids (Sturmbauer 1998). West African cichlids have experienced sympatric divergence of benthic and pelagic forms in multiple crater lakes (Schliewen et al. 1994). This pattern also occurs in a great number of other lacustrine fish groups. Three-spine sticklebacks have diverged into limnetic and benthic forms in multiple North American lakes (Schluter and McPhail 1993). Ecological divergence has also occurred in variety of other fish lineages including lake whitefish, brown trout, arctic char, and rainbow smelts (reviewed by Schluter 1998).

A similar pattern of diversification can be seen in rapidly radiating terrestrial systems. Anoline lizards of the Greater Antilles repeatedly evolved multiple ecomorphs associated with separate habitats (Losos et al. 1998). Darwin's finches diverged into ground finches and tree finches early in their radiation (Lack 1947; Stern and Grant 1996). These studies suggests that ecological divergence in the early stages of a radiation may be a common phenomenon.

## The Secondary Radiation and the Refinement of Mbuna Trophic Apparatus

The secondary radiation leading to the diversification of the mbuna genera represents a refinement of the trophic apparatus. The narrow scope of the morphological and behavioral changes associated with this episode suggest that selection, while strong and divergent, was focused on the trophic morphology and had little impact on other characters.

Two major adaptive innovations common among all cichlid lineages are primarily responsible for the diversification of the mbuna trophic structures; the restructuring of the pharyngeal jaw apparatus (Liem 1974), and the decoupling of certain oral jaw elements (Liem 1980). In their ancestral state, the pharyngeal jaws aid the transportation of food from the buccal cavity to the stomach. Through minor changes in the musculature, skeletal, and nervous systems, the cichlid pharyngeal jaws have adopted a food processing role; a task previously performed by the oral jaws (Liem 1974). The decoupling of upper oral jaw elements allows the independent manipulation of the premaxilla and maxilla thereby increasing the diversity of kinematic pathways associated with jaw movement (Liem 1980).

The combination of these two adaptive innovations increased the diversity of trophic resources available to cichlids and permitted the diversification of trophic structures in the mbuna and other cichlid lineages (Liem 1974). In various mbuna species the pharyngeal jaw apparatus acquired diverse new functions including: shearing prey, stacking scales scraped from other fish, and compacting filamentous algae (Liem 1991). The adoption of food processing functions by the pharyngeal jaw apparatus freed the oral jaws from the dual function of food collection and processing. The oral jaws subsequently diversified and became specialized solely for food collection. These specializations led to a variety of foraging strategies including plucking macroinvertebrates from the algae mat covering the rocks, combing loose algae and diatoms from attached algae, grazing on epiphytic algae, and

plucking scales from the sides of fish. Although the trophic apparatus became more specialized morphologically, trophic versatility persisted through the maintenance of multiple kinematic pathways associated with the manipulation of the oral elements. As a result, cichlids exploit a range of trophic niches usually occupied by several families, if not orders, of fishes (Greenwood 1964).

Trophic versatility, however, is not necessarily expected to promote divergence. On the contrary, morphological and behavioral plasticity might be expected to retard the speciation rate (Meyer 1987; Ribbink 1994). However, many species with extremely versatile jaws subsist on a very limited diet. For instance, *Metriaclima zebra*, which has the greatest number of kinematic pathways associated with jaw movement of all the mbuna (Liem 1980), also has an extremely limited diet (Reinthal 1990a). Furthermore, mbuna trophic resources appear to be narrowly partitioned (Reinthal 1990a; Genner et al. 1999a). The estimated dietary overlap of most mbuna species appears to be extremely limited and, in those cases in which dietary overlap is considerable, species could be distinguished based on other aspects of their feeding ecology (Reinthal 1990a; Genner et al. 1999a). Such strong trophic partitioning is expected to generate the rapid divergence of new species (Robinson and Wilson 1998). The persistence of kinematic pathways associated with trophic versatility, however, may retard the extinction rate during periods of reduced resource availability (McKaye and Marsh 1983).

The resulting picture, generated from morphological, dietary, behavioral, and phylogenetic studies, suggests that the mbuna genera diversified in response to competition for trophic resources, with only minor changes in structures unrelated to trophic morphology. This progression, from ecological diversification to the refinement of trophic structures during a secondary radiation, is well known among other East African cichlid radiations (Seehausen 1996; Sturmbauer 1998). It is also observed in other lacustrine fish groups. For example, arctic charr are known to have diverged into benthic and limnetic forms following their invasion of Icelandic lakes. The limnetic form

then diversified into piscivorous and planktivorous morphs (Snorrason et al. 1989). The differentiation of beak morphologies in Darwin's finches after their adaptation to the major ecological habitats on the Galapagos Islands are probably the best known example of this process in terrestrial vertebrates (Grant 1981).

## The Tertiary Radiation and the Divergence of Reproductive Characters

The tertiary radiations, those resulting in the diversification of extant species, appear to have been strongly influenced by sexual selection (Holzberg 1978; Ribbink et al. 1983; Dominey 1984; McKaye 1991; Hert 1991). The selective pressures operating during this episode resulted in the diversification of male secondary sexual characteristics (primarily color patterns) (Ribbink et al. 1983; McKaye et al. 1984; Deutsch 1997) while the majority of other morphological characters are highly conserved. A similar process appears to have occurred in the sand-dwelling cichlids of Lake Malawi (McKaye et al. 1993; Taylor et al. 1998) and in the diversification Lake Victoria haplochromine species flock (Seehausen and van Alphen 1998).

The reproductive biology of the mbuna suggests that sexual selection may be a particularly potent force. In their lek-like mating system (Barlow 1991), parental investment is highly skewed. Males defend permanent territories (Ribbink et al. 1983), experience a large variance in reproductive success (Hert 1991), and provide the females with no resources other than a place to mate (Holzberg 1978). In contrast, females are free to choose among many males (Parker and Kornfield 1996; Kellogg et al. 1998) but are required to mouthbrood the large yolk rich eggs for several weeks after fertilization. Adult size and reproductive color patterns are sexually dimorphic. Males are larger and are brightly colored whereas females are generally smaller and cryptically colored (Ribbink et al. 1983). The reproductive biology of the sand-dwellers can be characterized in a similar manner (McKaye 1983; McKaye et al. 1990; McKaye et al. 1993; Taylor et al. 1998).

Several studies suggest that male coloration is a target of sexual selection. A comparison of the within to between genera variation in color pattern suggests that male reproductive coloration has diversified more rapidly than other characters such as depth

preference, preferred substrate size and aggression (Deutsch 1997). Deutsch (1997) used this finding to argue that the diversity of male color patterns is the result of sexual selection. Field studies indicate that male color pattern can influence the variance in male reproductive success. Spots on the male's anal fin are similar in shape and color to mbuna eggs and are thought to attract females and increase fertilization rates (Wickler 1962). Hert (1991) noted that male reproductive success varied according to the number of egg spots. Males with more egg spots were generally more successful and males with no egg spots failed to mate.

Laboratory studies of mate recognition indicate that adult color pattern is an important aspect of the mbuna mate recognition system. Males can apparently distinguish con- and hetero-specific females based on visual cues alone. Among these, melanistic color patterns appeared to have the largest effect (Knight and Turner 1999). A similar result was found when examining female mating preferences based on male color patterns (Danley and Kocher, unpublished data).

Male reproductive coloration appears to play a significant role in the reproductive biology of most haplochromine cichlids. Among the rock-dwelling cichlids of Lake Victoria, Seehausen and van Alphen (1998) observed the maintenance of female mating preferences for conspecific males based on visual cues under natural lighting conditions. Their mate recognition broke down, however, when a similar test was performed under monochromatic light that eliminated the differences in male body hue. Under monochromatic light, females of both species preferred the larger and more active species. These results suggest that male body hue is the primary discriminatory factor among a hierarchy of visual cues used by females (Seehausen and van Alphen 1998). Clearly the divergence of male color patterns has significantly contributed to the rapid diversification of the haplochromine cichlids in East Africa's two most species rich lakes.

The dramatic influence of sexual selection is apparent in other rapidly diversifying systems including labrid fishes (Streelman et al. in prep), anoline lizards (Fitch and Henderson 1987, but see Losos and Chu 1998), Hawaiian *Drosophila* (Carson 1997) and

a large number of bird species (Barraclough et al. 1995; Mitra et al. 1996; Prum 1997; Møller and Cuervo 1998; Uy and Borgia 2000). These observations suggest that sexual selection may predominate in the later stages of many radiations.

Remarkably, most members of the mbuna clade appear to have experienced the evolutionary pattern described above. However, this pattern is not without exception. At each cladogenic stage, one (if not several) lineages do not conform to the described pattern. For instance, several oligotypic lineages have been identified which appear to have originated prior to the split between the rock and sand-dwelling clades (Moran et al. 1994; Seehausen et al. 1999). Likewise, a single rock-dwelling genus (*Melanochromis*) appears to have diverged early from the lineage that gave rise to the remaining mbuna (Albertson et al. 1999). Members of this lineage can be distinguished from the other mbuna on the basis of many morphological characteristics including body shape and melanistic body markings (most notably the occurrence of horizontal stripes rather than the typical vertical barring) (Bowers and Stauffer Jr. 1997). This relationship between *Melanochromis* and the remaining mbuna was highly supported in Albertson et al.'s (1999) study, however a more complete sampling of the mbuna genera are needed to test this hypothesis.

An exception to the pattern of diversification during the tertiary radiation can be identified as well. Only two species within the genus *Labeotropheus* have been identified based on a number of morphological and behavioral characteristics. Notably, these species are distinguished based on aspects of body shape and habitat preference (Ribbink et al. 1983) rather than the differences in male color pattern which typically distinguish mbuna congeners. Interestingly, these deviant lineages are remarkably species poor compared to those lineages that follow the typical pattern of diversification.

While a particular divergent selective pressure may predominate during each selective episode, it is not likely that a single force operates to the exclusion of others (Fig. 1.2). Variation in gut contents of closely related species suggests that competition for trophic resources is pervasive throughout the diversification of the mbuna (Reinthal

1990a,b). Variation in microhabitat preference among and within genera suggests that competition for space has also continually influenced their diversification (Hert 1990; Ribbink et al. 1983). Likewise, sexual selection has most likely operated throughout all stages of the radiation; its historical effects are more difficult to identify however. The emerging pattern suggests that while many forces influence the process of diversification at each cladogenic stage, a single divergent selective force predominates during each episode.



## Speciation Models

While most early models of the speciation of Lake Malawi cichlids focused on extrinsic factors, more recent hypotheses are concerned with intrinsic aspects of their biology. The 'key innovation' hypothesis (Liem 1974) argues that certain trophic structures are responsible for the remarkable ability of cichlids to rapidly radiate in novel lacustrine environments. Liem suggests that the cichlid pharyngeal jaw apparatus represents a 'crucial...morphological innovation' (Liem 1974; p. 439) which has granted members of this family a significant competitive advantage and has allowed them to rapidly colonize African lacustrine environments. This key innovation, however, occurs in all labroid fishes, the majority of which (including most other cichlid lineages) do not approach the exceptional species diversity observed in the Great Lake cichlids. This key innovation is clearly not the sole factor influencing the rapid divergence of East African cichlids.

Other hypotheses have focused on the role that the cichlid mating system has played in their rapid radiation (Holzberg 1978). Dominey (1984) was the first to discuss Fisher's process of runaway sexual selection with respect to the diversification of East African cichlids. Several additional authors have extended the discussion with specific respect to Lake Malawi cichlids (Holzberg 1978; McElroy and Kornfield 1990; McElroy et al. 1991; McKaye 1991; Ribbink 1994; Moran and Kornfield 1995; Turner and Burrows 1995; Parker and Kornfield 1996; Deutsch 1997; Knight and Turner 1999). Recently Higashi et al. (1999) proposed a model in which male secondary sexual characteristics and female mating preferences diverge simultaneously. Three outcomes were observed in their simulations; no change in either trait, fixation of a particular male character and the corresponding female preference, and the sympatric fixation of

alternative states of the male and female characters (a result that leads to prezygotic isolation). The authors argue that the clear waters of the East African rift lakes and the reduced levels of natural selection on male characters increase the likelihood of sympatric speciation in these cichlid flocks. Divergent sexual selection, however, is not limited to clear water habitats. Lake Victoria is considerably more turbid than Lakes Malawi and Tanganyika, and yet sexual selection on male color patterns has played an important role in this system (Seehausen et al. 1997, Seehausen and van Alphen 1998). The recent eutrophication and resulting dramatic increase in turbidity of Lake Victoria, however, threatens the diversity of these cichlids which rely heavily on visual cue for their mate recognition system (Seehausen et al. 1997)

Good genes models of sexual selection have also been discussed with reference to Lake Malawi cichlids. Taylor et al. (1998) argue that female mating preferences are influenced by the male's resistance to parasites. In field studies, male mating success was negatively correlated with the number of liver parasites. Given the absence of male parental care, this observation was taken as evidence of a good genes mechanism of female mate choice. Hert (1991) has made similar arguments relating to male foraging efficiency. Male mating success was correlated with the occurrence and frequency of yellow egg spots on the male's anal fin. The production of such carotenoid based color has been linked with foraging efficiency in other fish groups (Endler 1983). Hert (1991) suggests that female mate choice may be dependent on the male's ability to acquire carotenoids, a trait that if heritable may have fitness effects in a female's offspring.

The combined effect of competition and assortative mating in driving the diversification process has recently gained attention. Kondrashov and Kondrashov (1999) examined the sympatric divergence of a population whose members compete for a bimodally distributed resource. Their simulations suggest that populations will diverge when linkage disequilibrium develops between a mating character and characters influencing the ability to compete for a limiting resource. Other models predict that a

similar outcome can occur even if the resource is unimodally distributed (Dieckmann and Doebeli 1999).

### An Integrative Model

Each of the models discussed above fails to adequately address the diversification of Lake Malawi cichlids *in toto*. Most models are applicable to a narrow range of phylogenetic history but do not address the entire process. Models which integrate the various selective forces known to influence the diversification of East African cichlids are needed (Galis and Metz 1998; Sturmbauer 1998). Below we adapt the divergence with gene flow model developed by Rice and Hostert (1993) in which selection and gene flow interact to produce a cyclical process characterized by the reduction of both the strength of divergent selection and rates of gene flow. This model differs from previous dynamic models of cichlid diversification in its reliance on intrinsic (rather than abiotic) triggers of cladogenic events and its recognition of the important role that nonadaptive evolution has played in the diversification of haplochromine cichlids in East Africa.

The diversification of Lake Malawi cichlids, with particular reference to the mbuna, can best be considered in terms of the antagonistic forces of selection and gene flow. Several models have been developed which discuss the divergence of populations with varying rates of ongoing gene flow (Endler 1973; Lande 1982; Rice and Hostert 1993). The model developed by Rice and Hostert (1993) is particularly appealing given the patterns of diversification observed in Lake Malawi. This model predicts that populations will diverge so long as selection is strong (relative to gene flow), divergent, and acting on multiple characters (Fig. 1.3). Prezygotic isolation is expected to develop under these conditions without direct selection on the mate recognition system. Isolation develops as a correlated response (via pleiotropy or genetic hitchhiking) to selection on other characters. The model also predicts that a positive feedback loop might develop (Fig. 1.4). In such a situation, competition for a limiting resource generates divergent selection. Populations respond to

this selection by acquiring adaptations that reduce competition for the contested resource. Consequently, both the strength of divergent selection and the level of interpopulational gene flow decrease, and the strength of stabilizing selection increases. As gene flow is reduced, selective pressures that previously had been too weak to overcome the ongoing levels of gene flow now cause additional divergence. The process is a self-priming engine in which each iteration of the cycle primes subsequent divergence episodes.

Several aspects of this model are relevant to the diversification of Lake Malawi cichlids. The positive feedback nature of the process predicts that multiple episodes of diversification, each related to a separate selection pressure, could occur. This expectation is matched by the proposed phylogenetic history of Lake Malawi's rock-dwelling cichlids as supported by their current taxonomy and recent molecular studies (Meyer et al. 1990; Moran et al. 1994; Albertson et al. 1999). As discussed above, the mbuna diversified in three major episodes; an initial split between sand and rock-dwelling clades, the divergence in trophic morphology, and the diversification of sexually selected characters.

The model also predicts that levels of gene flow will decrease with time. The selection-adaptation process is expected to cause a reduction in gene flow with each cladogenic event. Indirect evidence supports this prediction. The ancestor to Lake Malawi's haplochromine cichlids is thought to have been a generalized riverine cichlid. This precursor to Malawi's extant cichlids was most likely well equipped to disperse great distances through a variety of habitats. Recent estimates of ongoing levels of gene flow indicate, however, that mbuna migration is extremely restricted over limited geographic scales (van Oppen et al. 1997; Arnegard et al. 1999; Markert et al. 1999; Danley et al. 2000). These results clearly indicate that gene flow has decreased from its initially high levels. It is difficult to infer the levels of gene flow that occurred during the secondary radiation; however the lakewide distribution of mbuna genera suggests it may have been significant. In contrast, many species have very narrow distributions, occurring only at a single island or headland. While inferring historical levels of gene flow can be problematic,

the evidence suggests that gene flow has declined since the colonization of the lake.

The model also predicts that the overall divergent selection pressure will decrease with each cladogenic event. Adaptation reduces competition for a particular resource and thereby reduces the overall selective pressure. The residual competition for other limiting resources then drives additional cladogenic events once gene flow is sufficiently lowered (again by adaptation). While the strength of selection at previous cladogenic stages cannot be measured directly, it is possible to infer the relative strength of selective pressures at various stages by the number of the characters involved in the diversification. The diversification of the rock/sand dwelling forms resulted in changes in multiple characters including; body shape, reproductive behavior and morphology, habitat preference, preferred diet, and jaw morphology (Fryer 1959). The divergence of genera resulted primarily in the diversification of trophic structures, while overall morphology, behavior, and color patterns are conserved (Ribbink 1983). Divergence of species within genera is primarily reflected in the diversification of male color patterns; most other morphological characters are conserved (Bowers and Stauffer 1997; Stauffer et al. 1997). The decreasing complexity of the divergent characters at each of the cladogenic episodes suggests that overall selection is declining, in agreement with the predictions of this model.

## The Proliferation of Taxa During the Tertiary Radiation

The diversification that occurred during the tertiary radiation largely accounts for the extraordinary biological richness of the mbuna clade. Ten to thirteen mbuna genera are currently recognized, and many mbuna genera contain over 15 species (likely an underestimate as many species remain undescribed). Species diversity generated during the tertiary radiation exceeds the diversity originating in the two previous episodes.

The potentially nonadaptive nature of sexual selection may be responsible for the extraordinary proliferation of taxa during this radiation. During the initial two cladogenic periods, the Rice and Hostert (1993) model predicts that populations responded to natural selection in a manner that reduces the level of competition for the limiting resource, thereby reducing the strength of divergent selection acting on them. This adaptive response, i.e. a phenotypic change which reduces the impact of divergent selective pressures, is not necessarily expected to develop in response to sexual selection. In fact, the strength of sexual selection may increase as a correlated response to changes in the preferred male trait (Lande 1981). In this regard, evolution during the tertiary period may not have been adaptive; phenotypic changes in male secondary sexual characteristics may not reduce the strength of divergent sexual selection acting on them.

Gene flow, however, is expected to dwindle among populations experiencing sexual selection. Female mating preferences and male secondary sexual characters may diverge among isolated populations. As a result, the likelihood that a migrating male will successfully reproduce decreases. The combination of the continuing selective pressure and the reduction in gene flow means that populations are likely to remain stuck in the 'speciation' domain of the selection vs. gene flow relationship.

Nonadaptive evolution may also permit the evolution of genetic architectures that

accelerate phenotypic divergence. Mutations with large phenotypic effects are expected to increase the rate of phenotypic fixation in a population (Templeton 1982; Coyne 1992; Voss and Shaffer 1997). While little is known concerning the size of allelic effects fixed during adaptive episodes, recent modeling suggests that the size of the largest fixed factor is correlated to the complexity of the phenotypic change (Orr 1998). Complex characters, such as habitat preference (which involves a number of morphological, physiological, and behavioral traits) have a greater dimension in Orr's model. Such characters are expected to evolve through the fixation of alleles with larger effects relative to simpler characters.

The extension of Orr's model into the cichlid system suggests that the size of the largest factor fixed by natural selection during each of these cladogenic events will decrease with time (just as the complexity of the phenotypic changes associated with each episode decreased). However, this model assumes that diverging populations each approach separate fixed optimal phenotypes. If no fixed optimum exists, such as when female preferences are open-ended and natural selection on the preferred male character is weak, the size of allelic effects fixed by sexual selection may exceed those that are fixed under natural selection. Such a genetic architecture may permit the rapid divergence of phenotypic characters and ultimately increase the speciation rate during non-adaptive evolutionary episodes.



## Conclusions

Malawian rock-dwelling cichlids have experienced a minimum of three separate radiations in their phylogenetic history. The first radiation resulted in the differentiation of the sand and rock-dwelling forms and is a classic example of ecological differentiation common in many other rapid radiations. The trophic morphology of the rock-dwelling cichlids differentiated dramatically during the second radiation while most other morphological and behavioral characters were conserved. These observations suggest that strong divergent selection on the ability to acquire trophic resources dominated during the second radiation. Divergence during the third radiation is largely restricted to male nuptial color patterns that have likely diverged in response to sexual selection via female choice. This type of non-adaptive evolution may explain the high rate of species proliferation during the tertiary radiation. All together these radiations have generated over 200 species classified into 12 genera of rock-dwelling cichlids in Lake Malawi.

Future research efforts should focus on a number of areas. First, more detailed phylogenetic analysis is needed to describe the supergeneric, generic, and superspecific groupings of the rock-dwelling cichlids. The phylogenetic model of the mbuna diversification proposed here relies heavily on the currently recognized taxonomic relationships of the mbuna. It is possible, however, that mbuna taxonomy does not accurately reflect their phylogenetic history (see Rüber et al. 1999). More complete taxon sampling is needed to verify the phylogenetic model advocated here and to identify any additional structuring in the radiation. Second, the strength of competition for both trophic and reproductive resources need to be evaluated. Fieldwork that documents the strength of ongoing selection and manipulative laboratory studies, possibly involving hybrids, may be used to quantify the strength of divergent selection on intermediate phenotypes. Third,

female preference functions need to be evaluated within the rock-dwelling cichlids. Such information could be used to evaluate the model proposed here to explain the proliferation of species during the third radiation. Fourth, the genetic basis of convergent characters deserves future attention. Convergence in morphological characters between lakes has been documented (Kocher et al. 1993). Likewise within isolated lake basins, phenotypic convergence in characters such as male coloration (Deutsch 1997; Seehausen et al. 1999) can be considerable. Given the likelihood that gene flow is maintained at low rates between incipient species within Lake Malawi, it is possible that introgressive hybridization may have played a significant role in the diversification of this group (*sensu* Wang et al. 1997). It would be interesting to know whether the convergence of phenotypic characters within Lake Malawi is due to the sharing of ancestral polymorphisms, the result of newly arisen mutations, or introgressive hybridization.

## Acknowledgements

We would like to thank Julie Baldizar, members of the Kocher laboratory, and Lukas Rüber for their insightful comments on previous versions of this paper. Thanks to R. Craig Albertson for providing the line drawings, and Ad Konings for providing the color images, for figure 1.1.

**Figure 1.1 A proposed phylogenetic history of Lake Malawi's rock-dwelling cichlids based on several molecular phylogenies of Lake Malawi cichlids (Meyer et al. 1990; Meyer 1993; Kocher et al. 1993; Moran et al. 1994; Moran and Kornfield 1995; Albertson et al. 1999). Lake Malawi is presumed to have been invaded by a riverine generalist closely allied with Lake Tangyanika's haplochromine tribe approximately 700,000 years ago. This common ancestor subsequently diverged during the primary radiation into the sand-dwelling and rock-dwelling lineages. The rock-dwelling lineage diverged during the secondary radiation into the 10-12 currently recognized mbuna genera. These genera are distinguished primarily on the basis of trophic structure suggesting the importance of trophic competition during this period of the radiation. The spectacular species richness of the mbuna principally arose during the tertiary radiation. During this period, as many as 25 species per genus diverged presumably in response to sexual selection via female choice for male secondary sexual characteristics such as color pattern. Line drawings courtesy of R. C. Albertson, color images courtesy of Konings (1990).**

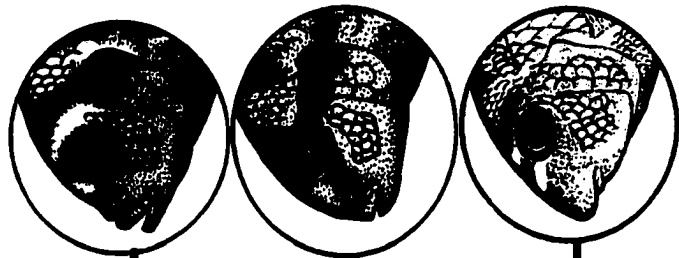
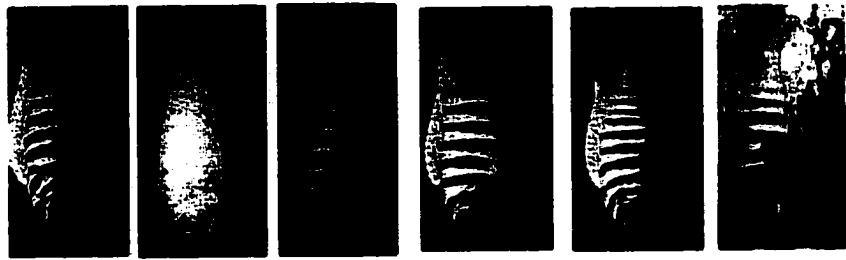


Figure 1.2 The strength and composition of divergent selection operating during each of the three radiations. During the primary radiation, ecological pressures resulted in strong selection on macrohabitat preference (dark gray) and resulted in the divergence of the sand and rock-dwelling clades. Selection on the mbuna trophic apparatus (stippled) dominated during the second radiation. The tertiary radiation appears to have been driven by divergent selection on reproductive characters. Note, however, that while the relative proportion of each selective factor may change during each cladogenic event, none are completely eliminated.

### The Strength and Composition of Divergent Selection

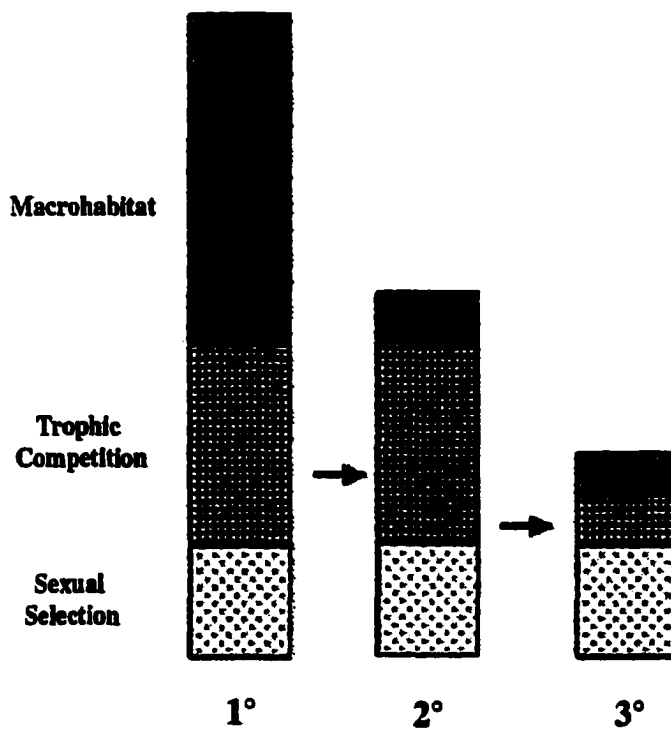
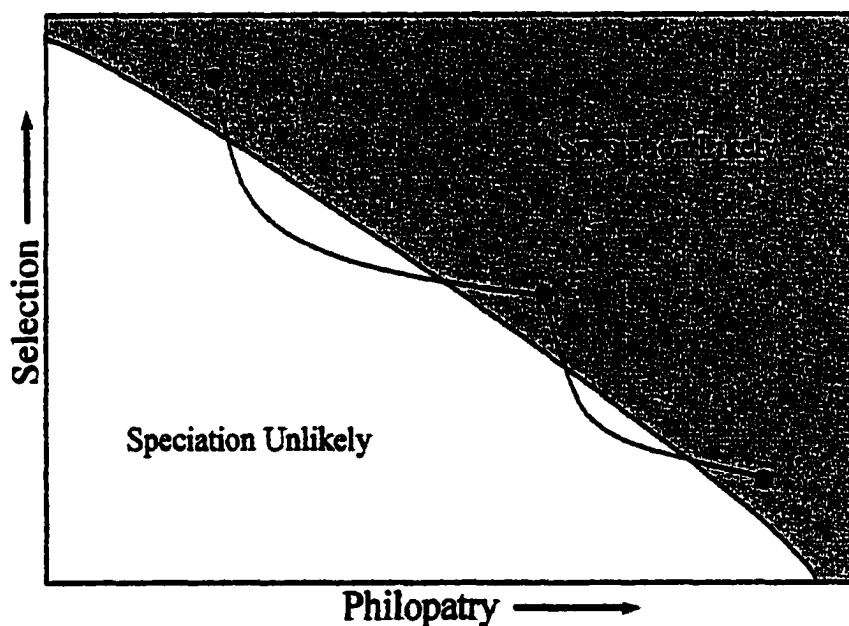
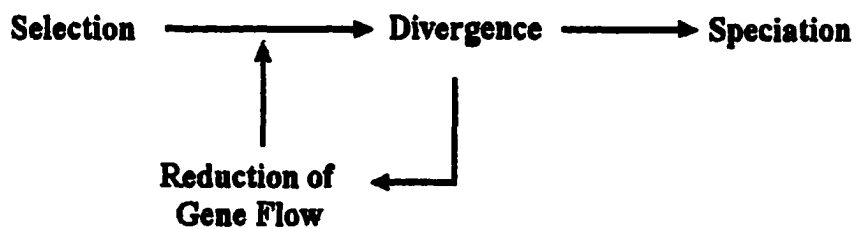


Figure 1.3 Speciation with limited gene flow with reference to Lake Malawi's rock-dwelling cichlids. Populations are expected to diverge so long as divergent selection is strong relative to the levels of ongoing gene flow. Thus if a population's position is plotted on a coordinate grid consisting of selection and philopatry, one can predict the likelihood of a speciation event. Lake Malawi's rock-dwelling cichlids have crossed the curve and entered the speciation domain of the plot at least three times during their evolution. During each radiation, adaptation reduces both the strength of divergent selection and the level of gene flow, thereby allowing subsequent radiations to occur. The size of the arc connecting adjacent cladogenic periods is expected to be roughly proportional to the size of the largest genetic factor fixed during the adaptive episode.



**Figure 1.4** The speciation model advocated here predicts the formation of a positive feedback loop in which each cladogenic event can potentially lead to subsequent episodes. See text for complete details.

## Speciation Engine



*Adapted from Rice and Hostert 1993*



## CHAPTER 2

# DIVERGENCE WITH GENE FLOW IN THE ROCK-DWELLING CICHLIDS OF LAKE MALAWI

### Abstract

Within the past 2 MY, over 450 species of haplochromine cichlids have diverged from a single common ancestor in Lake Malawi. Several factors have been implicated in the diversification of this monophyletic clade, including changes in lake-level and low levels of gene flow across limited geographic scales. The objectives of this study were to determine the effect of recent lake-level fluctuations on patterns of allelic diversity in the genus *Metriaclima*, to describe the patterns of population structure within this genus, and to identify barriers to migration. This was accomplished through an analysis of allele frequencies at four microsatellite loci. Twelve populations spanning four species within *Metriaclima* were surveyed. The effect of lake level fluctuations can be seen in the reduced genetic diversity of the most recently colonized sites; however, genetic diversity is not depressed at the species level. Low levels of population structure exist among populations, yet some gene flow persists across long stretches of inhospitable habitat. No general barrier to migration was identified. The results of this study are interpreted with respect to several speciation models. Divergence via population bottlenecks is unlikely due to the large allelic diversity observed within each species. Genetic drift and microallopatric divergence are also rejected because some gene flow does occur between adjacent

**populations. However, the reduced levels of gene flow between populations does suggest that minor changes in the selective environment could cause the divergence of populations.**

## Introduction

Lake Malawi provides a unique natural laboratory to study the process of species formation (Fryer and Iles 1972). Since the origin of the lake basin 2 MY ago, over 450 species of haplochromine cichlids have radiated from a single common ancestor (Meyer 1993). The diversity within this clade is particularly apparent with respect to trophic morphology, color patterns and reproductive behavior (Fryer 1959a).

The source of this rapid diversification has been debated for decades. Early models focused on vicariant processes involving multiple invasions (Mayr 1942; Fryer 1977) or the isolation of populations into separate lake basins due to fluctuating lake levels (Trewavas 1947; Fryer and Iles 1972; Fryer 1977). Other discussions have focussed on the adaptive radiation of jaw morphologies (Liem and Osse 1975). Sexually dimorphic color patterns and high variance in male reproductive success suggest that sexual selection may also have played a role (Dominey 1984; McElroy and Kornfield 1990; McKaye et al. 1993; Taylor et al. 1998).

Reduced gene flow among populations facilitates divergence and the evolution of new species (Endler 1973; Lande 1982; Rice and Hostert 1993). Limited migration has long been suspected in Malawi cichlids, particularly in the rock-dwelling forms (locally known as 'mbuna'). These species often have restricted geographic distributions; many species are endemic to a single rock outcropping (Ribbink et al. 1983; Lewis et al. 1986; Konings 1990). Species relocated by fish collectors (a practice which is now illegal) tend to have limited distributions adjacent to the site of introduction (Ribbink et al. 1983; Hert 1990).

Molecular studies support the idea that population differentiation occurs over extremely short distances. Analyses of allozyme (McKaye et al. 1984) and mtDNA

haplotype frequencies (Moran and Kornfield 1995) have detected genetic differentiation between northern and southern populations separated by over 200 km. van Oppen et al. (1997) examined populations of four mbuna species using six microsatellite loci and detected significant levels of genetic differentiation among mbuna populations separated as little as 3 km in northern Lake Malawi. Arnegard et al. (1999) and Markert et al. (1999) detected significant levels of population structure in *Labeotropheus fuelleborni* and *Melanochromis auratus*, respectively, over a 43 km transect in Lake Malawi's southeast arm.

Several factors are thought to contribute to population fragmentation in the mbuna. The mbuna are philopatric. They rarely leave the rocky habitats (Fryer and Iles 1972; Holzberg 1978) which are highly fragmented (Fryer 1959b) and the least prevalent substrate in the mosaic of Lake Malawi's shoreline (McKaye and Gray 1984). Furthermore, all Malawian cichlids maternally mouthbrood their young and thus lack a dispersing larval stage. The dynamic geologic history of the lake also contributes to population fragmentation. Geologic evidence suggests that Lake Malawi is prone to frequent changes in water level (Crossley et al. 1984; McKaye and Gray 1984; Scholz and Rosendahl 1988). At least four major desiccation/inundation cycles have occurred within the past 25,000 years. The most recent recession occurred between 1390 and 1860 AD when the lake surface was at least 121m below its current level (Owen et al. 1990). A drop of this magnitude would have drained the entire southern portion of the lake, including both the southeastern and southwestern basins. Such temporal variability would result in the repeated extinction and creation of populations at a particular site.

The objective of this study was to describe the pattern of population structure within the rock-dwelling cichlids of Lake Malawi. Specifically, we wanted to determine the effect of recent lake level fluctuations on patterns of genetic diversity, to measure gene flow at different geographic scales, and to identify migration barriers. These objectives were addressed by examining allele frequencies at microsatellite loci in populations of four *Metriaclima* species from the southeast arm of Lake Malawi.

## Materials and Methods

### Collection Sites

All of the collection sites were located on or adjacent to the Nankumba Peninsula in the southeast basin of Lake Malawi (Fig. 2.1). The habitat at these sites is of extremely recent origin. Most sites are known to have been dry land within the past 500 years (Owen et al. 1990) and those less than 7m deep are known to have been exposed as recently as 1900 AD (Crossley 1982). The collection sites consist of rocky habitats separated by a variety of intervening substrates. The northern sites (Mumbo Island, Ilala Gap, Mvunguti West, Tsano Rock, Harbour Island) are located in areas of large boulders which slope down to the sandy lake bottom (>30 m) (Fig. 2.1). The boulder habitat in this area is generally continuous except for a narrow (350m), sandy beach between Mvunguti West and Tsano Rock, two sandy bays between Tsano Rock and Harbour Island, and a deep-water trench between Mumbo Island and Ilala Gap. The southern sites generally consist of smaller rocks and a shallow rock/sand interface (10–15 m). Substrates between the southern sites generally consist of alternating sandy and rocky coast and include long stretches of shallow sandy bottom (Songwe Hill to Mazinzi Reef and Kanchedza Island to Nkhudzi Hills). Detailed descriptions of the collection sites can be found in Arnegard et al. (1999) and Markert et al. (1999).

Habitat depth and size of the available habitat patch were estimated at each collection site. Habitat depth was measured as the depth of the rock-sand boundary. The size of the available habitat was calculated as the product of the depth of available rocky habitat (corrected for the slope of the habitat) and the shoreline length of interrupted rocky coast (following Arnegard et al. 1999).

## Study Species

*Metriaclima*. *Metriaclima*, like most mbuna genera, diversified rapidly. As a result this genus is exceptionally species rich, even among the mbuna. While interesting from an evolutionary perspective, this rapid and extensive diversification can be problematic. The phylogenetic relationships among the mbuna and within *Metriaclima* are poorly understood (Albertson et al. 1999). In addition, the alpha level taxonomy is currently incomplete. Stauffer et al. (1997) formally recognized 15 species, ten of which were previously undescribed, in their description of this genus. They recognized, however, that at least seven currently recognized species in the genus *Pseudotropheus* and as many as 20 undescribed forms will be included in *Metriaclima* in the future.

The nomenclature used in this study largely follows that established by Stauffer et al. (1997) with one exception. Stauffer et al. (1997) recognized the *Metriaclima* population at Mumbo Island as a distinct species (*Metriaclima melabbranchion*), while the current study includes this group within *Metriaclima zebra*. The *Metriaclima* population at Mumbo Island has generally been considered as a conspecific of *M. zebra* (Ribbink et al. 1983; Konings 1990). Furthermore, the sampled individuals generally lacked the markings (lateral body bars extending into the dorsal fin) which distinguish *M. melabbranchion* from *M. zebra*.

*Metriaclima zebra*. *Metriaclima zebra* is one of the few mbuna species which is widely distributed throughout the rocky areas of the entire lake (Konings 1990). The absence of pigment in its pale blue dorsal fin and the presence of 6-8 black vertical bars on its pale blue flank distinguish it from other members of the genus. Based on its distribution

and its morphological features, Stauffer et al. (1997) consider *M. zebra* to be the most basal member of the genus.

A total of 447 individuals were sampled from seven collection sites (Table 2.1). The sites ranged from Mumbo Island in the north to Mazinzi Reef in the south (Fig. 2.1). The number of individuals sampled per collection site ranged from 28 (Mumbo Island) to 115 (Mazinzi Reef) with a mean sample size of approximately 56 individuals per collection site. The entire study area for *M. zebra* spanned 30.3 km.

*Metriaclima sandaracinos*. This species can be identified by its red/orange dorsal fin as well as 6-7 black vertical bars on its pale blue flank. Its distribution is limited to Kanchedza Island, Chirombo Bay, Nkhudzi Hills, and Mphande Island (Fig. 2.1) (Stauffer et al. 1997).

*Metriaclima sandaracinos* were sampled from three sites which ranged from Kanchedza Island in the north to Mphande Island in the south (Table 2.1). The number of individuals sampled ranged from 42 (Kanchedza Island) to 61 (Nkhudzi Hills). Mean sample size equaled approximately 53 individuals.

*Metriaclima xanostomachus*. *Metriaclima xanostomachus* is readily identified by its bright yellow gular region and dorsal fins. It is distributed throughout the Maleri Islands and at Kanchedza Island (Stauffer et al. 1997), where it is sympatric with *M. sandaracinos* (Fig. 2.1). Twenty-three individuals were collected from Kanchedza Island (Table 2.1).

*Metriaclima benetos*. *Metriaclima benetos* males closely resemble *M. zebra* males but can be distinguished on the basis of male breeding color. Unlike *M. zebra* males,

whose pale blue flanks are interrupted by 6 – 8 black bars, *M. benetos* males are uniformly pale blue. This species is endemic to Mazinzi Reef (Stauffer et al. 1997) where it is sympatric with *M. zebra* (Fig. 2.1). Phylogenetic evidence suggests that *M. benetos* diverged from the ancestral *Metriaclima* prior to the divergence of *M. zebra* and *M. sandaracinos* (Albertson 1999). One hundred twenty individuals were sampled.

Individual fish were collected using monofilament nets while SCUBA diving. For collections made outside of Lake Malawi National Park, fin clips of the right pectoral fin were collected and preserved in >90 % ethanol. The remainder of each fish was preserved as voucher specimens in a 10 % formalin solution. Fish collected from within Lake Malawi National Park were immediately released after clipping an unpaired fin (collection license # 684658).

### Molecular Analysis

Four microsatellite loci were examined: UNH001 (Genbank accession number U17044), UNH002 (U17045), UNH050 (AF036714), UNH231 (G12382). Each locus consisted of perfect dinucleotide (CA) repeats. DNA extraction, locus amplification, and allele identification were performed as described by Markert et al. (1999). A number of these loci have been used to study population structure in other mbuna species (van Oppen et al. 1997; Arnegard et al. 1999; Markert et al. 1999). Previous studies indicate that these loci are not in linkage disequilibrium.



## Statistical Analysis

Each locus was examined for evidence of Hardy-Weinberg equilibrium, the presence of null alleles, and linkage disequilibrium using Genepop 3.1 (Raymond and Rousset 1995). Significance thresholds were established for the tests of Hardy-Weinberg equilibrium and linkage disequilibrium through a Bonferroni correction for twelve comparisons. For example, the  $\alpha = 0.05$  level after correcting for 12 comparisons is  $(0.05/12) 0.0042$ .

Overall estimates of population heterozygosity and its standard error were calculated using DISPAN (Ota 1993). A second estimate of allelic diversity, the effective number of alleles ( $n_e$ ), was calculated as the inverse of the expected homozygosity (Hartl and Clark 1989). Wright's F-statistics were calculated to assess the level of population substructure using FSTAT (Goudet 1995). FSTAT follows the methods of Weir and Cockerham (1984) in calculating F-statistics. Standard deviations and p-values were calculated for the F-statistics by permuting the data for 5000 replicates.

Hedrick (1999) has suggested that, when examining highly variable loci, variation-dependent estimators of population differentiation, such as Wright's F-statistic ( $F_{st}$ ), may identify statistically significant differences in allele frequency distributions that lack biological meaning. To address this issue, we have calculated a second, variation-independent estimator of population subdivision, as suggested by Hedrick (1999). A multilocus estimate of the effective number of migrants ( $N_m$ ) was calculated based on the frequency of private alleles (Slatkin 1985a; Barton and Slatkin 1986) using Genepop 3.1. Exact tests of allelic differentiation between adjacent populations were also performed using Genepop 3.1 to provide a third estimate of population subdivision. The significance levels for the exact tests were corrected for eight comparisons using a Bonferroni correction (see above).

The identification of migration barriers was carried out by regressing an estimate of per generation migration rates ( $M = (1/F_{st} - 1)/4$ ) on geographic distance (Slatkin 1993). Mantel tests performed by Genepop 3.1 were used to assess the significance of this relationship.

## Results

### Locus Characteristics

**Hardy-Weinberg equilibrium.** Each population was tested for random union of gametes at each locus using an exact test (Guo and Thompson 1992). *Metriaclima sandaracinos*, *M. xanostomachus* and *M. benetos* populations did not deviate from random union of gametes at any loci. Three of eight *M. zebra* populations deviated from Hardy-Weinberg expectations at UNH002 (Mvunguti West, Songwe Hill, and Mazinzi Reef); two deviated at UNH001 (Tsano Rock and Mazinzi Reef); and one deviated at UNH231 (Tsano Rock). UNH050 was at Hardy-Weinberg equilibrium in all populations.

Those populations that deviated from Hardy-Weinberg expectations were also examined for evidence of heterozygote deficiency. Only two populations were found to lack heterozygous individuals at the loci not in Hardy-Weinberg equilibrium (Mvunguti West and Songwe Hill at UNH002). A population may be deficient in heterozygotes for several reasons. Inbreeding resulting from limited population sizes may reduce the occurrence of heterozygotes. Population substructure within a collecting location may result in an excess of homozygotes (Wahlund effect); however, efforts were made to mitigate the Wahlund effect by collecting samples within 100 m at each collection site. The observation of excess homozygotes may also suggest that a null allele may be present in the population.

**Null alleles.** Alleles that cannot be amplified due to mutations at the PCR priming site can affect estimates of both heterozygosity and levels of population subdivision. A

previous study detected the presence of a true breeding null allele at UNH002 (van Oppen et al. 1997). Maximum likelihood estimates of the frequency of null alleles were calculated using the EM algorithm of Dempster et al. (1977) in Genepop 3.1. This algorithm bases its null allele frequency estimates on the occurrence of apparent null homozygotes in the data set. Null alleles were assumed to occur when only three of the four loci amplified after multiple attempts. The estimated frequencies suggest that null alleles are rare, with an estimated global average frequency ( $\pm 1$  SD) of 0.039 (0.043). At this frequency they do not significantly alter the observed trends in allelic diversity. For example, the highest estimated null allele frequency was 0.16 at UNH001 at the *M. zebra* collection at Songwe Hill. The estimated frequency of the most common allele at this locus in this population differed from its calculated value by only 0.03 when corrected for the presence of the null allele. Estimated frequencies of the remaining alleles generally differed by less than 0.005.

Linkage disequilibrium. The four loci used in this study were examined for evidence of linkage disequilibrium. Linked loci experience similar evolutionary processes and, as a result, will not provide independent estimates of heterozygosity and measures of population subdivision. Linkage disequilibrium was calculated separately for each population. Only two populations, Mazinzi Reef *M. zebra* and *M. benetos*, show disequilibrium between UNH001 and UNH002 ( $p < 0.002$ ,  $p < 0.001$ , respectively, Fisher's exact test). These results, combined with evidence from an analysis of this pair of loci in other closely related species (Arnegard et al. 1999, Markert et al. 1999), suggests that these loci are not physically linked in the genome. The apparent linkage disequilibrium of these loci in the Mazinzi Reef populations is most likely due to genetic drift because of their limited population size or a recent founding event.

## Allelic Diversity

***Metriaclima zebra* populations.** *Metriaclima zebra*, like all other mbuna species surveyed to date, contains high levels of allelic diversity at all four microsatellite loci. The number of alleles at a locus ranged from 30 at UNH050 to 45 at UNH231, with an average of 39.25 alleles per locus (Table 2.1). The effective number of alleles ( $n_e$ ) within populations ranged from 8.05 (Mazinzi Reef) to 18.12 (Mvunguti West). The corresponding heterozygosities were 0.87 (Mazinzi Reef) to 0.95 (Mvunguti West).

The geographic distribution of genetic diversity reveals some general patterns. Heterozygosities and  $n_e$  are largest at collecting sites surrounded by long stretches of rocky coasts, e.g. between Ilala Gap and Mvunguti West (Fig. 2.2). South of these collecting sites diversity declines. The least diverse population occurs at Mazinzi Reef, which is a moderately sized rock reef nearly 3 km from the nearest shore in Madzidzi Bay. This population has the lowest heterozygosity (0.868) and the smallest effective number of alleles (8.05).

Differences in the observed allelic diversity could result from a number of factors. We investigated the relationship between two measures of allelic diversity (average heterozygosity and  $n_e$ ) and (1) the number of individuals sampled, (2) the age of the collecting site estimated by its depth, and (3) the size of the population as estimated by the size of the available habitat patch. Average heterozygosity did not correlate with sample size or depth ( $p = 0.48, 0.20$ , respectively). Average heterozygosity tends to increase with the size of available habitat but this trend was not statistically significant ( $p = 0.09$ ). The effective number of alleles did not correlate with sample size ( $p = 0.89$ ) or depth ( $p = 0.31$ ), but an increase in the effective number of alleles was associated with an increase in available habitat at a collecting site ( $p = 0.02$ ). Since no intermediate patch sizes were sampled, it is difficult to estimate the true relationship between patch size and allelic diversity.

*Metriaclima sandaracinos* populations. The *M. sandaracinos* samples were only slightly less diverse than the *M. zebra* collection. Thirty-two alleles were observed for each locus except UNH231, which had 33 (Table 2.1). Observed heterozygosities were high, ranging from 0.90 (Kanchedza Island) to 0.945 (Nkhudzi Hills). Values for  $n_e$  ranged from 9.25 (Kanchedza Island) to 16.51 (Nkhudzi Hills).

General geographic patterns are difficult to detect since only three populations of *M. sandaracinos* occur in the study area. However, the site with the largest population, as estimated from the habitat size, is also the most genetically diverse (Nkhudzi Hills) (Table 2.1).

*Metriaclima xanstomachus*. The single *M. xanstomachus* population sampled generally exhibited lower genetic diversity than either *M. zebra* or *M. sandaracinos* as estimated by the average number of alleles per locus (14.5), average heterozygosity (0.91), or the effective number of alleles (9.55). However, only the average number of alleles in *M. xanstomachus* was lower than the range found in either *M. zebra* or *M. sandaracinos*.

*Metriaclima benetos*. Despite being the most extensively sampled population in this study, *M. benetos* exhibited the most limited genetic diversity of all the species examined. Its heterozygosity (0.836) and effective number of alleles (6.12) was below the range found in any of the other species (Table 2.1). The number of alleles present was comparable to that found in the other three species, but the allelic distributions of each locus tended to be dominated by one or two alleles.

## Population Differentiation

There is considerable uncertainty concerning the appropriate estimator of population differentiation for examining allele frequency distributions of highly polymorphic loci. Such loci have an extraordinary power to detect fine differences in allele frequencies between populations, but these differences may not reflect biologically meaningful differences in the populations (Hedrick 1999). In contrast, statistics that compare the sharing of alleles in different populations, such as Slatkin's (1985a) rare alleles method of estimating the effective number of migrants ( $N_m$ ), may more accurately estimate population differentiation when using highly variable markers (Hedrick 1999). Estimates of population differentiation based on allele frequency distributions ( $F_{st}$ , following Weir and Cockerham 1984) and shared alleles ( $N_m$ , following Slatkin 1985a) produce concordant results (Pearson  $p = 0.015$ ) and are presented in Table 2.2.

*Metriaclima zebra* populations. Significant levels of structure were detected among the *M. zebra* populations (overall  $F_{st} = 0.041 \pm 0.008$ ,  $N_m = 3.83$ ). Pairwise comparisons also indicate that gene flow is limited between adjacent populations (Table 2.2). All comparisons of adjacent populations yielded small, but significant,  $F_{st}$  values and low estimates of the effective number of migrants ( $N_m$ ) between populations. Population differentiation is also supported by Fisher's exact test of allele frequency homogeneity. All pairwise comparisons of adjacent populations revealed heterogeneity in allele frequency data, at all four loci, at the  $p < 0.05$  level (Table 2.2).

Estimates of  $F_{st}$  and  $N_m$  suggest that genetic differentiation is lowest among samples collected along nearly continuous rocky coasts (Fig. 2.3). The lowest estimated  $F_{st}$  (four locus  $F_{st} = 0.0105 \pm 0.002$ , estimate  $\pm$  std. dev.) occurred between two populations (Ilala Gap and Mvunguti West) separated by 6.6 km of continuous rocky coast. Similar  $F_{st}$

estimates were calculated for populations separated by 1.5 km of rocky coast and a narrow (350m), shallow beach (Mvunguti West and Tsano Rock,  $F_{st} = 0.0119 \pm 0.002$ ). Although the  $F_{st}$  estimate suggests that this narrow beach may limit migration, the estimated  $Nm$  (4.63) between these populations is the largest of all *M. zebra* comparisons.

Population comparisons across deep water (Mumbo Island to Ilala Gap) yielded a larger four locus  $F_{st}$  value ( $0.0240 \pm 0.008$ ) and a smaller  $Nm$  (3.04). There is a minor increase in population subdivision when the intervening substrate consists of alternating rocky and sandy coasts (Tsano Rock to Harbour Island, four locus  $F_{st} = 0.0250 \pm 0.006$ ,  $Nm = 2.71$ ). Estimates of population substructure dramatically increase when the intervening substrate is dominated by sand. Songwe Hill is separated from Harbour Island by 2.2 km, nearly half of which is sand. The four locus  $F_{st}$  ( $0.0508 \pm 0.007$ ) for this comparison is nearly five times greater than populations separated by a similar distance of rocky coast (Mvunguti West to Tsano Rock). The greatest level of population subdivision occurs between the Songwe Hill population and the Mazinzi Reef population (four locus  $F_{st} = 0.087 \pm 0.01$ ,  $Nm = 1.18$ ). These populations are separated by 5.9 km of uninterrupted sand, highlighting the important role this barrier plays in isolating *M. zebra* populations.

*Metriaclima sandaracinos* populations. *Metriaclima sandaracinos* populations show low, but statistically significant, levels of population substructure (overall four locus  $F_{st} = 0.017 \pm 0.003$ ,  $Nm = 9.46$ ). Estimates of pairwise  $F_{st}$  values suggest that genetic structure exists between all adjacent populations (Table 2.2). The Kanchedza Island and Nkhudzi Hills population comparison yielded a larger four locus  $F_{st}$  ( $0.0271 \pm 0.002$ ) than the Nkhudzi Hills to Mphande Island comparison ( $0.0048 \pm 0.003$ ). This suggests that greater migration occurs between the latter two sites.

This observation is further supported by Fisher's exact tests for homogeneity of allele frequencies. The Kanchedza Island to Nkhudzi Hills comparison indicated significant



heterogeneity at all four loci ( $p < 0.001$ ). The Nkhudzi Hills to Mphande Island comparison indicated that allele frequencies differ at UNH001 ( $p < 0.005$ ) while the allele frequency distributions of the remaining loci are statistically homogeneous.

This observation is consistent with the estimated number of migrants between adjacent *M. sandaracinos* populations. Migration appears to occur more frequently between Nkhudzi Hills and Mphande Island ( $N_m = 11.42$ ) than between Kanchedza Island and Nkhudzi Hills ( $N_m = 4.68$ ).

### Barriers to Migration

The relationship between population substructure and geographic distance was examined in two ways. Following Slatkin (1993), the estimated number of individuals moving between populations each generation ( $M$ ) was plotted against geographic distance on a log/log scale. Mantel tests were performed to test for a relationship between  $F_{st}$  and geographic distance. Geographic distance explained very little of the variation in  $M$  (*M. zebra*  $R^2_{adj} = 0.098$ ), and the Mantel tests failed to detect a significant relationship between geographic distance and the level of population structure in *M. zebra* ( $p = 0.157$ ). The relationship between distance and the level of population structure was stronger in *M. sandaracinos* ( $R^2_{adj} = 0.816$ ), but the Mantel test failed to detect a significant relationship between geographic distance and the level of population structure ( $p = 0.167$ ). The results for *M. sandaracinos* are questionable due to the small number of comparisons.

Estimators of population subdivision suggests that the nature of the intervening substrate plays an important role in isolating populations. To test the significance of this relationship, Mantel tests were performed using a matrix of  $F_{st}$  values from all possible pairwise *M. zebra* population comparisons and a matrix of indicator values describing the

nature of the intervening substrate. The indicator variable ranged from 1 (indicating continuous rock between collection sites) to 7 (indicating continuous sand between collection sites). Three of the authors (JAM, MEA, TDK) separately generated substrate matrices based on their knowledge of the region prior to a discussion of the data. A Mantel test was performed separately for each of the substrate matrices. Each Mantel test revealed a significant relationship between the level of population structure between populations and the nature of the intervening substrate ( $p = 0.04, 0.008, 0.02$ ). Tests were not performed on *M. sandaracinos* due to the limited number of populations sampled and the similarity of substrates between all three populations.

## Discussion

### Patterns of Allelic Diversity

**Metriaclima zebra.** *Metriaclima zebra* collections with the highest diversity occur along the continuous rocky coast of the Nankumba peninsula (Ilala Gap, Mvunguti West, Tsano Rock) (Fig. 2.2). These populations are generally the largest, thought to be the oldest based on the depth of suitable habitat, and occur in areas of high migration. In contrast, populations with the lowest allelic diversity generally occur at sites in the southern end of the transect. These sites are smaller, more recently available, and separated from other sites by inhospitable habitat. It is clear that the analysis of *M. zebra* diversity cannot deconvolute the influences of mutation rate, population size and migration. However, a lack of a correlation between the estimated age of a collection site and the allelic diversity of that site's sample suggests that a population's genetic diversity is not greatly influenced by the accumulation of new mutations in persistent populations relative to historical influences i.e. its source population.

**Metriaclima sandaracinos.** Samples collected from Nkhudzi Hills, presumably the oldest location based on depth, were the most genetically diverse at the four microsatellite loci examined. The two remaining collections differed in their allelic diversity. The southern samples (Mphande Island) are nearly as diverse as the Nkhudzi Hills samples ( $H_t = 0.937$ ,  $n_s = 13.95$ ) whereas the northern samples (Kanchedza Island) are some of the least diverse in the entire study ( $H_t = 0.900$ ,  $n_s = 9.25$ ).

There may be several reasons for the difference in diversity of these two

populations. First, the considerable genetic diversity of the southern (Mphande Island) collection may be the result of a recent colonization event by a large and diverse founding population. Second, more migration may (have) occurred between Mphande Island and Nkhudzi Hills than between Nkhudzi Hills and Kanchedza Island. This hypothesis is supported by both the data ( $N_m = 11.42$  vs.  $4.68$ , respectively), and the geographic arrangement of the sites (Mphande Island is 4 km closer to Nkhudzi Hills than Kanchedza Island). Finally, Kanchedza Island may support smaller numbers of individuals than would be expected by its patch size. As discussed above, *M. sandaracinos* co-occurs with a closely related species, *M. xanstomachus*, at Kanchedza Island. These species occupy similar ecological niches and the resulting competition may limit the population sizes of both species, allowing for the stochastic loss of genetic diversity.

*Metriaclima xanstomachus*. The reduced genetic diversity in this species may be due to a number of factors. First, Kanchedza Island, the only known site for this species in the southeast arm of the lake, is one of the smallest habitat patches in the study area. Second, since the total population is extremely limited in number, only 23 animals were collected. Any estimates derived from such a limited sample of individuals should be considered preliminary. To fully address the genetic diversity of this species, other populations need to be studied.

*Metriaclima benetos*. The genetic diversity of *M. benetos* appears to be limited despite the large number of individuals which were sampled ( $N = 120$ , the largest sample in the study). Furthermore, the single location from which individuals were sampled (Mazinzi Reef) represents the only known area in which this species occurs (Stauffer et al. 1997). The allelic diversity of the entire species should be well represented by this sample. The

limited diversity in this species is most likely due to a genetically reduced founder population and/or a population bottleneck that has occurred during the dynamic history of the southeast arm of the lake. However, the possibility that this species was derived from a genetically depauperate ancestral species cannot be ruled out.

### Scale of Population Structure

The analysis of allele frequency data indicates that gene flow is restricted over limited geographical scales in both *M. zebra* and *M. sandaracinos*. Low, but statistically significant, levels of population structure were detected over the entire study area for both species (*M. zebra*  $F_{st} = 0.068$ ,  $Nm = 3.83$ ; *M. sandaracinos*  $F_{st} = 0.016$ ,  $Nm = 9.46$ ). Pairwise comparisons suggest that gene flow is limited among all adjacent *M. zebra* populations, even when separated by less than 2 km of nearly continuous rocky coast (e.g. the populations of *M. zebra* at Mvunguti West and Tsano Rock). Pairwise comparisons of *M. sandaracinos* populations suggest that gene flow is limited between Kanchedza Island and Nkhudzi Hills, while gene flow is greater between Nkhudzi Hills and Mphande Island.

Significant levels of population structure were detected in *Labeotropheus fuelleborni* and *Melanochromis auratus* over a similar geographic area using the same loci ( $F_{st} = 0.063$ ,  $0.151$ , respectively) (Arnegard et al. 1999; Markert et al. 1999). van Oppen et al. (1997) detected structure over limited geographic scales among populations of four mbuna species, including *Metriaclima zebra* and *Metriaclima callainos*, in the Nkhata Bay area. These results suggest that structured populations are a general feature of the mbuna. The biological importance of this finding is discussed below.

## **Barriers to Migration**

Although limited migration appears to be a general feature of mbuna biology, it is not clear that a single universal barrier to their migration exists. Previous studies have identified several environmental factors that limit migration including deep water, distance between populations (Arnegard et al. 1999; Markert et al. 1999), and the nature of the intervening substrate (van Oppen et al. 1997; Arnegard et al. 1999; Markert et al. 1999).

**Deep water.** Previous examinations of the mbuna species *Labeotropheus fuelleborni* and *Melanochromis auratus* have concluded that migration is primarily limited by their inability to cross deep water (Arnegard et al. 1999; Markert et al. 1999). These species tend to remain closely associated with the substrate and appear to be physiologically incapable of compensating for the change in pressure associated with the rapid change in depth (Hill and Ribbink 1978; Marsh and Ribbink 1981; Ribbink et al. 1983). In the current study, the effect of depth was less pronounced than other physical barriers (i.e. sand).

*Metriaclima zebra* may be more likely to cross areas of deep water because their feeding mode is not closely tied to the substrate. *Metriaclima zebra* males feed much higher (>5m, pers. obs.) in the water column than other mbuna, and gut contents analysis suggest that their diet includes planktonic diatoms (Reinthal 1990). *Metriaclima zebra* may occasionally traverse deep water trenches while feeding high in the water column and so avoid having to compensate for changes in depth.

**Distance.** Arnegard et al. (1999) and Markert et al. (1999) also detected a significant relationship between the level of population structure and the distance separating

populations across the same geographic area. Although distance most likely plays some role in disrupting migration between populations of *Metriaclima* (e.g. Ilala Gap and Mvunguti West *M. zebra* populations), a significant relationship between distance and isolation was not detected in either *M. zebra* or *M. sandaracinos*. Isolation by distance might not be detected if genetic equilibrium has not been established at these recently colonized sites. Other factors may have contributed to the observed lack of association between the level of population structure and distance.

**Substrate.** It appears that the nature of the intervening substrate plays an important role in limiting *M. zebra* migration.  $F_{st}$  values dramatically increase as the amount of sand separating populations increased (Fig. 2.3). The number of migrants crossing long stretches of open sand were estimated to be only 1 migrant per generation (Table 2.2), which approaches the limit which would allow populations to diverge by drift (Wright 1931; Slatkin 1985b). It is unclear why this species appears to disperse across deep water but not across shallow sandy areas.

Sand does not appear to be a general migration barrier within *Metriaclima*, however. The greatest differentiation between adjacent populations of *M. zebra* occurred over 5.9 km of nearly continuous sand (Songwe Hill to Mazinzi Reef, four loci  $F_{st} = 0.087$ ,  $Nm = 1.18$ ). A similar comparison among *M. sandaracinos* samples which are separated by 5.6 km of nearly continuous sand showed no consistent differentiation across loci and had the largest estimated number of migrants in the entire study ( $Nm = 11.42$ ). While the observed similarity of the two *M. sandaracinos* samples may have resulted from number of different processes (e.g. recent colonization of one or both sites by a diverse founding population, or high levels of ongoing migration) the data suggest that a large number of individuals crossed (or currently cross) sandy barriers that nearly extinguish migration in *M. zebra*. The ability of *M. sandaracinos* to cross open sand may be related to their habitat

preference; they were generally found in sediment rich areas near the sand-rock interface.

Although movement across sand by *M. sandaracinos* may be consistent with their habitat preference, it highlights two surprising features. First, it emphasizes the rapid diversification of behavioral characteristics within a group of fish with extremely limited morphological diversity. Rapid divergence of other behavioral characteristics within this genus (e.g. mating behavior, habitat choice; Ribbink et al. 1983; Danley and Kocher in prep.) suggests that selection on behavioral, rather than morphological, characters is the primary force splitting species within this genera. Second, it identifies a paradox between migration rates and species distribution: the species with the highest migration rate (*M. sandaracinos*) is also the more geographically restricted. The observed distribution of *M. sandaracinos* may represent only a limited portion of a historically larger distribution, some populations of which may have been driven to extinction either due to changes in the habitat and/or competition.

### Inferences Concerning Mbuna Diversification

Population bottlenecks / founder effects. The geologic history of Lake Malawi suggests that population bottlenecks and/or founder-flush processes could have played an important role in the diversification of Malawian cichlids (Owen et al. 1990). Geologic studies of the lake basin indicate that Lake Malawi, like other East African great lakes (Johnson et al. 1996), experiences frequent desiccation/inundation cycles which can change the lake level by over 100 m (Owen et al. 1990). Changes in lake-level impact the rocky habitats in particular, and populations that exist in these areas are prone to frequent extinction and recolonization. The low migration rate of the mbuna suggests that recently available habitats would be colonized by a limited number of founders that could then



rapidly repopulate an area.

In contrast, genetic studies of mbuna suggest that species divergence rarely occurs via founder events or population bottlenecks. Although reduced genetic diversity has been observed in recently colonized and/or isolated population (e.g. Mazinzi Reef *M. zebra* and *M. benetos* populations), most species maintain high levels of genetic diversity at microsatellite loci (van Oppen et al. 1997; Arnegard et al. 1999; Markert et al. 1999; Table 2.1). The analysis of mitochondrial genes also fails to detect the widespread effects of founder events/population bottlenecks. Moran and Kornfield (1995) examined mtDNA haplotype diversity in four *Metriaclima* species. Three out of the four species examined showed no reduction in genetic diversity. Furthermore, the retention of ancestral polymorphisms for mitochondrial DNA (Moran and Kornfield 1995) strongly suggests that population bottlenecks/founder flush processes have not played a significant role in the diversification of the mbuna.

The observed genetic diversity of mbuna populations is interesting given the temporal instability of mbuna habitats. Although genetic diversity is expected to decrease because of frequent sampling events associated with multiple extinction/recolonization cycles, allelic diversity is not depressed in most mbuna species.

Genetic diversity within these species may be maintained by several means. Large, genetically diverse populations may have survived low lake-level periods by migrating to sites currently located in deep water. Migrants from several of these deep-water refugia may have colonized the sites sampled in this study (Owen et al. 1990; Arnegard et al. 1999). Mutation may have also played a role in generating the large genetic diversity of our samples. However, mutation is expected to have played a minor role given the recent availability of the collection sites.

Founder effect speciation continues to generate considerable controversy. Theoretical predictions are often contradictory (Barton and Charlesworth 1984; Goodnight 1988; Wagner et al. 1994; Cheverud and Routman 1996; Barton 1998) as are conclusions

from empirical studies (Powell 1978; Ringo et al. 1985; Dodd and Powell 1985; Moya et al. 1995; Bryant and Meffert 1996; Galiana et al. 1996). The results from the genetic analysis of mbuna populations, however, are clear. Founder effect speciation, if it occurs, is rare and likely has not played an important role in the diversification of the mbuna.

**Divergence by drift.** Although population bottlenecks severe enough to generate reproductive isolation have not been detected, several lines of evidence suggest that mbuna populations may be particularly susceptible to the action of genetic drift. The apparent sedentary nature of the mbuna contributes to fine scale geographic isolation that may create many isolated populations (van Oppen et al. 1997). In some cases these populations can be relatively small (e.g. *M. zebra* at Songwe Hill). The high variance in male reproductive success (McElroy and Kornfield 1990) may further reduce the effective population size (Hartl and Clark 1989). Some researchers have argued, however, that the polygynandrous mating system of the mbuna may inflate the effective population size. Multiple matings by both males and females each season may maintain a population's allelic diversity above what would be expected based on a census alone (Parker and Kornfield 1996).

The microsatellite data suggest that stochastic sampling events, particularly associated with the founding of populations and migration between populations, can lead to statistically significant differences in microsatellite allele frequencies (Arnegard et al. 1999; Markert et al. 1999; van Oppen et al. 1997). However, the estimated number of migrants between populations in each of these studies exceeds the one migrant every other generation needed to prevent populations from diverging purely by drift (Wright 1931; Slatkin 1985b).

Drift, however, may play a significant secondary role in the diversification process. Lande's (1981) model of runaway sexual selection, which has often been discussed with reference to the diversification of East African cichlids (Dominey 1984; McElroy and Kornfield 1990; McKaye et al. 1993; Stauffer et al. 1997), predicts that random changes in

either the males' sexually selected trait or the females' mating preference can drastically influence the divergence of populations. More recently, Kondrashov and Kondrashov (1999) have suggested that transient linkage disequilibrium resulting from drift can drive species divergence even in the absence of a reduction in gene flow.

Microallopatric speciation. Previous analyses of the mbuna (including *M. zebra*) have detected statistically significant differences in microsatellite allele frequencies over three kilometers (van Oppen et al. 1997). These authors conclude that mbuna populations are isolated over extremely limited geographic scales and that such populations could diverge via drift or selection. Our studies (Arnegard et al. 1999; Markert et al. 1999; this paper) suggest that population divergence does not occur on microgeographic scales due solely to drift. In all three studies, migration between adjacent sites exceeds one migrant every other generation. Furthermore, the number of migrants moving between the two end points of the transect (43 km) were estimated to be less than one migrant per generation in only one of the species (*Melanochromis auratus*  $Nm = 0.32$ , Markert et al. 1999). These migration estimates are too high for divergence by drift on microgeographic scales. The depressed migration rates of the mbuna, however, may facilitate the diversification of populations which experience low levels of divergence selection.

Divergence with limited gene flow. Sympatric speciation models have been used to explain the diversification of Lake Malawi's cichlids (McKaye et al. 1984; Turner and Burrows 1995; Dieckmann and Doebelli 1999; Kondrashov and Kondrashov 1999). Simulations have shown that sympatric speciation can result from a variety of selective forces including sexual selection (Taylor et al. 1998) and divergent selection on ecological characters (Dieckmann and Doebelli 1999; Kondrashov and Kondrashov 1999).

Furthermore, there is clear evidence that sympatric speciation has occurred in other cichlid systems, most notably the cichlids of Cameroon's crater lakes (Schliewen et al. 1994).

However, models that incorporate the highly fragmented nature of the mbuna, such as Rice and Hostert's (1993) divergence with gene flow model, may be more compatible with the Lake Malawi system. Under this model, reproductive isolation develops via pleiotropy when populations experience strong, divergent selection on multiple characters. So long as selection is strong relative to gene flow, diversification will occur in the presence of considerable migration. Experimental evidence supports this model (Rice 1984; Rice and Salt 1990) and has indicated that prezygotic isolation will develop when habitats are discrete and different, when selection is strong and multifarious, and when bridging populations (populations existing in intermediate habitats and exhibiting intermediate phenotypes) are not expected. This model also suggests that a positive feedback loop can develop when strong selection initiates a divergence process that reduces gene flow between populations. As gene flow is reduced, traits, which could not initially differentiate due to the homogenizing effect of gene flow, will diverge. With each iteration of this process, populations are freed to adapt in response to incrementally lower selection pressures.

Several hypotheses can be generated through the application of this model. One such hypothesis concerns the variation in species richness among mbuna genera. Some genera contain as few as one or two species (e.g. *Genyochromis* and *Labeotropheus*, respectively) while others may have over 30 (e.g. *Metriaclima*). Rice and Hostert's model (1993) predicts that diversification is dependent on two factors: the level of gene flow and the strength of selection (Rice and Hostert 1993, Fig. 2.2). Because estimated migration rates are similar among the mbuna (van oppen 1997; Arnegard et al. 1999; Markert et al. 1999; this paper), it seems unlikely that the variation in species richness has resulted from differential rates of gene flow. However, little is known concerning the strength of selection in natural populations. It is possible that species rich genera, like *Metriaclima*, experience higher levels of selection than species poor genera. *In situ* comparative studies of selection

pressures are needed to test this hypothesis.

Rice and Hostert's (1993) model also recognized sexual selection's ability to drive speciation. Diversification is expected to follow a hierarchical pattern in which sexual selection predominates after divergent natural selection reduces the impact of interspecific competition. Phylogenetic analysis of the mbuna supports this notion. Albertson et al. (1999) investigated the phylogenetic relationships of eight mbuna species. They found an early divergence of genera which have been distinguished on the basis of trophic morphology. The subsequent divergence of species within genera corresponded with the diversification of male secondary sexual characteristics (e.g. male color pattern).

Variation in sexual selection pressures among mbuna species is poorly understood. Future research is needed to quantify the strength of female mating preferences in the field, to identify the male characters under selection, to determine if the targets of female preferences are different among populations, and to assess the mutability of male and female traits under sexual selection. The potential for natural selection to drive diversification in the rock-dwelling cichlids of Lake Malawi has been under-appreciated in recent years. However there remain many aspects of sexual selection that need to be quantified in order to characterize the selective forces acting to differentiate the mbuna.

## Conclusions

Several conclusions can be drawn from this study. First, the impact of recent lake-level fluctuations can clearly be seen in the patterns of allelic diversity among collection locations. The samples collected at the most recently available sites possess reduced allelic diversity compared to samples drawn from deeper, older locations. The reduction in genetic diversity at these locations suggests that stochastic events, including population bottlenecks

and drift, influence the patterns of allelic diversity. However, it is unlikely that these factors alone have contributed significantly to the process of species divergence. Second, reduced gene flow appears to be a general feature of the mbuna. The reduced gene flow of this group has most likely had a strong influence on their diversification by allowing minor changes in the selective environment to drive the divergence of populations. However, it is unclear what role the reduction in gene flow has played in generating species diversity within the mbuna. The data suggest that the more species rich genera experience stronger selective pressures rather than reduced levels of gene flow. Future work is needed to determine if and how selection pressures vary across populations and species.

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Figure 2.1 Geographic distribution of collection sites and the number of individuals sampled (adapted from Markert et al. 1999). (A) The Nankumba peninsula divides the southern aspect of Lake Malawi into two shallow basins. The white area represents that portion of the lake that was dry during a recent desiccation event that lowered the lake level 200m below its current level (from Owen et al. 1990). (B) The geographic distribution of the ten collection locations. The number of individuals sampled for each species at each location is given by  $N_{Mz}$ ,  $N_{Mr}$ ,  $N_{Mb}$ ,  $N_{Mx}$ , for *M. zebra*, *M. sandaracinos*, *M. benetos*, and *M. xanostomachus*, respectively.

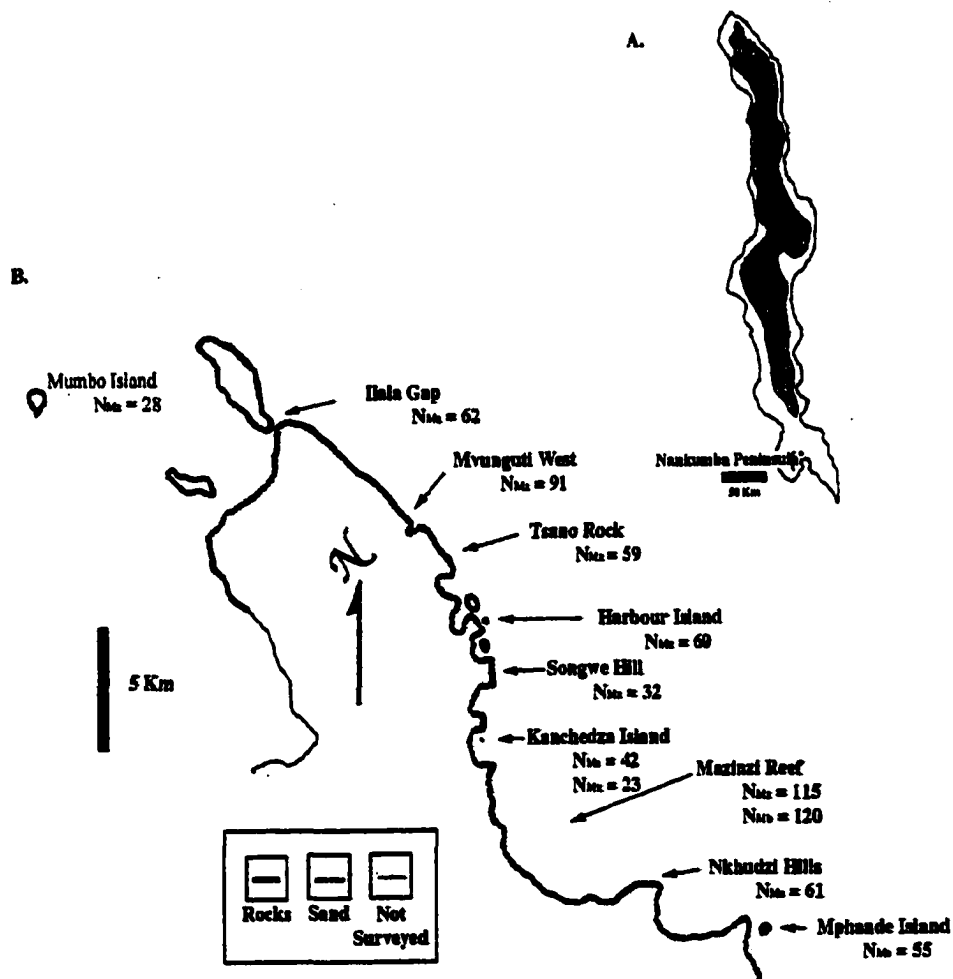




Figure 2.2 The distributions of population heterozygosities for both *M. zebra* (filled square) and *M. sandaracinos* (open circles) are plotted against the distance from the northernmost collection site (Mumbo Island). The error bars indicate one standard error. The shaded area beneath the horizontal axis represents the nature of the substrate along the transect where the striped area indicates deep (>50 m) water, the black area represents rocks, and the gray area indicates shallow sand.

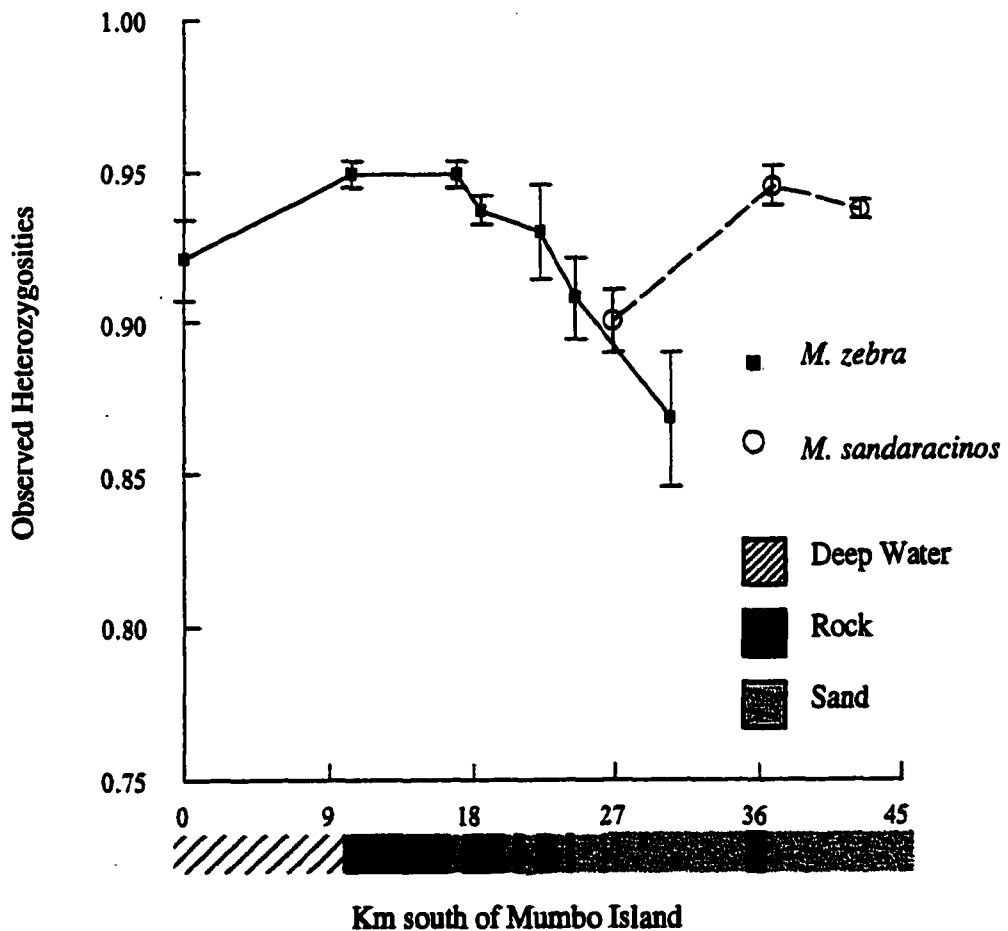
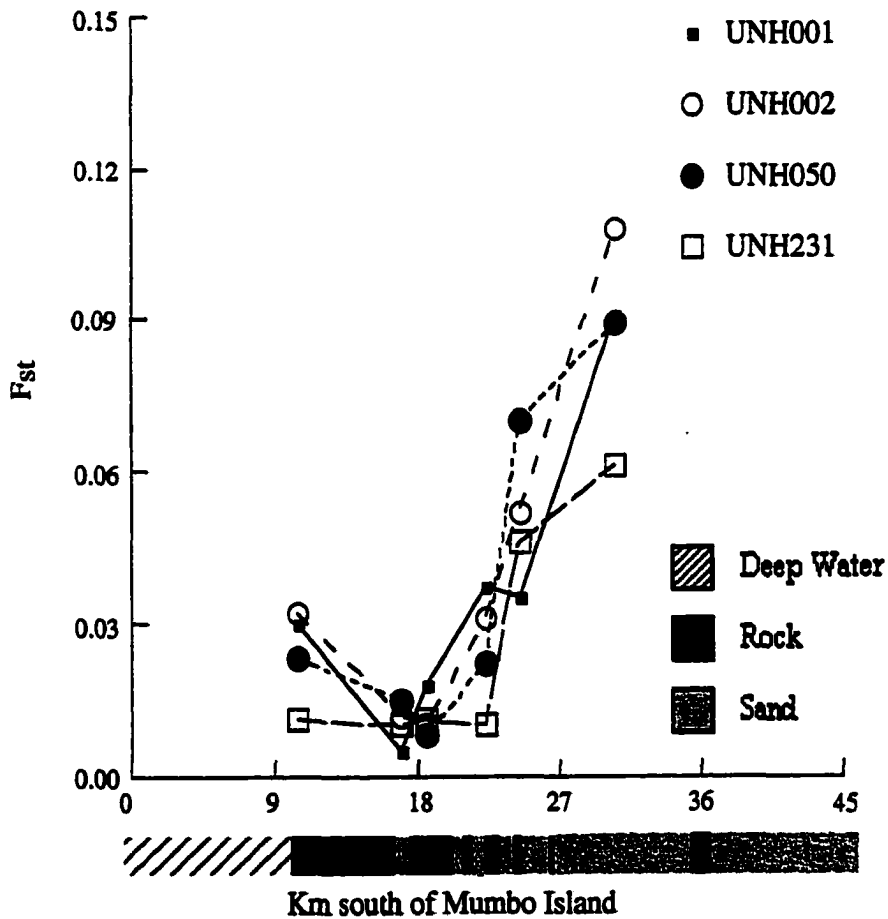


Figure 2.3 A plot of pairwise *M. zebra*  $F_{st}$  values between adjacent sites versus the distance from the northernmost (Mumbo Island) collection site.  $F_{st}$  values for a given comparison are plotted above the more southern of the two populations for each of the loci examined. The closed squares represent UNH001, open circles UNH002, closed circles UNH050, opens squares UNH231. The shaded area beneath the horizontal axis represents the nature of the substrate along the transect where the striped area indicates deep (>50 m) water, the stippled area represents rocks, and the gray area indicates shallow sand.



**Table 2.1 Population characteristics. N = number of individuals sampled, Habitat Size = size of available habitat (m<sup>2</sup>/10<sup>4</sup>),**

**Depth = depth of the rock-sand interface (m), Ho = observed heterozygosity (standard deviation),**

**n<sub>e</sub> = effective number of alleles, UNH001-UNH231 = number of alleles per locus per population,**

**Average = average number of alleles per locus per collection location.**

Population	N	Habitat Size	Depth	Ho	n <sub>e</sub>	UNH001	UNH002	UNH050	UNH231	Average
<b>M. zebra</b>										
Mumbo	28	52.31	46	0.921 (0.0133)	10.94	14	16	19	18	16.75
Ilala Gap	62	212.85	36	0.949 (0.0043)	17.20	25	29	23	31	27.00
Mvunguti	91	212.85	27	0.949 (0.0044)	18.12	27	36	25	34	30.50
Tsano Rock	59	28.30	32	0.937 (0.0048)	14.32	26	28	19	31	26.00
Harbour Island	60	16.67	30	0.930 (0.0156)	14.31	23	28	22	27	25.00
Songwe Hill	32	3.08	4	0.908 (0.0133)	9.81	16	21	15	17	17.25
Mazinzi Reef	115	1.00	15	0.868 (0.0221)	8.05	23	21	18	23	21.25
All populations	447					38	44	30	45	39.25
<b>M. sandaracinos</b>										
Kanchedza Island	42	0.16	3	0.900 (0.0104)	9.25	14	17	17	20	17.00
Nkhudzi Hills	61	15.88	11	0.946 (0.0063)	16.51	25	27	28	26	26.50
Mphande Island	55	0.82	4	0.937 (0.0031)	13.95	23	20	22	22	21.75
All populations	158					32	32	33	32	32.25
<b>M. xanstomachus</b>										
Kanchedza Island	23	0.16	3	0.910 (0.0152)	9.55	13	11	16	18	14.50
<b>M. benetos</b>										
Mazinzi Reef	120		15	0.836 (0.0151)	6.12	21	17	16	21	18.75

**Table 2.2 Results of allele frequency distribution comparisons of adjacent populations. Depth is the maximum depth separating populations. Substrate indicates the nature of the intervening substrate between the two populations. Exact is the probability that the two populations have homogeneous allele frequency distributions after a Bonferroni correction for eight comparisons.  $F_{st}$  is calculated following Weir and Cockerham 1984 and  $N_m$  is the estimated number of migrants estimated using Slatkin 1985a.  $M$  was calculated following Slatkin 1993. A \* indicates the value at all loci, \* indicates  $p < 0.005$**

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	Comparison	Distance (km)	Depth (m)	Substrate	Exact	$F_{st}$ *(std. dev.)	$N_m$	$M$	
	M. zebra	Mumbo Island - Ilala Gap	10.4	>50	Deep sand (50 m)	p<0.05 *	0.0240 (0.005)	3.04	10.16
	M. zebra	Ilala Gap - Mvunguti West	6.6	-36	Large rocks	p<0.005 *	0.0105 (0.002)	3.45	23.56
	M. zebra	Mvunguti West - Tsano Rock	1.5	-36	Large rocks, small beach	p<0.05 *	0.0119 (0.002)	4.63	20.75
	M. zebra	Tsano Rock - Harbour Island	3.7	-30	Rocky coast, deep sand channel	p<0.05 *	0.0250 (0.006)	2.71	9.75
	M. zebra	Harbour Island - Songwe Hill	2.2	-30	Shallow alternating sand and rock	p<0.001 *	0.0508 (0.007)	2.38	4.67
	M. zebra	Songwe Hill - Mazinzi Reef	5.9	-15	Open shallow sand	p<0.001 *	0.0869 (0.010)	1.18	2.63
	M. sandaracinos	Kachedza Island - Nkhudzi Hills	10.0	-11	Open shallow sand	p<0.001 *	0.0271 (0.002)	4.68	11.27
	M. sandaracinos	Nkhudzi Hills - Mphande Island	5.6	-11	Shallow alternating sand and rock	UNH001 p<0.005	0.0048 (0.003)	11.42	51.833

## CHAPTER 3

# SPECIES-SPECIFIC TERRITORIAL BEHAVIOR OF TWO MALAWIAN CICHLIDS

### Abstract

Fish communities of the rocky habitats of Lake Malawi are incredibly speciose and tend to be tightly packed. As a result, males potentially encounter high levels of competition for establishing breeding territories. This project examines the inter- and intraspecific competition for breeding sites in two closely related, sympatric, rock-dwelling cichlids, *Metriaclima zebra* and *Metriaclima benetos*. Territorial males of both species were removed from three quadrats on an isolated rocky reef in the southeast arm of the lake. The pattern of recolonization suggests that territories are species-specific. The width of the breeding cave appears to be associated with the observed species-specificity of the breeding territories. Males of both species were significantly more aggressive when defending a new territory than when defending an established territory. The results of this study suggest that strong competition for breeding sites may have driven the divergence of male cave preferences. Alternatively, male cave preferences may have diverged in response to sexual selection. The effect of these two processes are discussed in terms of the evolution of Lake Malawi's rock-dwelling cichlids.

## Introduction

Resource partitioning has played a major role in the diversification of many biological systems. Early in their radiation, Darwin's finches split into ground and tree clades (Stern and Grant 1996). Among *Anolis* lizards of the Caribbean, as many as five ecomorphotypes have independently evolved on multiple islands (Losos et al. 1998). Both sticklebacks (Taylor and McPhail 2000) and arctic char (Snorrason et al. 1989) have repeatedly formed benthic and limnetic sister species in multiple North American lakes. No where else is this pattern more apparent than in the cichlids of East Africa. Within the three major lakes in East Africa (Lakes Tanganyika, Victoria, and Malawi) rock-dwelling and sand-dwelling specialists have evolved independently (Meyer et al. 1990; Seehausen 1996; Sturmbauer 1998). Within Lake Malawi, this sand-rock split appears to have occurred relatively early in the evolution of this species flock and created two essentially monophyletic clades each containing 200+ species (Meyer 1993).

While large scale habitat partitioning is well known for cichlids in all three lakes, fine scale resource partitioning is also common. Long term studies of Lake Tanganyika's cichlids have identified four broad trophic groups; scale eaters, piscivores, herbivores, and insectivores (Hori 1983). Remarkably, these groups have been further resolved into 12 trophic classes consistently found throughout the lake. Behavioral observations indicate that sympatric members of the same trophic group utilize distinct aspects of the microhabitat to reduce within group competition (Hori 1983). Among Lake Victoria's cichlids, trophic partitioning is plastic and tends to fluctuate in response to the seasonal availability of food items (Bouton et al. 1997). However, clear dietary segregation can be observed and five dietary groups have been identified; loose algae feeders, attached algae feeders, zooplanktivores, insectivores, and molluscivores. In Lake Malawi, fine scale trophic

partitioning is thought to play an important, if not dominant, role in maintaining the extraordinary species richness of the rocky habitats.

Fine scale resource partitioning among cichlids is not limited to their trophic ecology. The partitioning of spawning sites may also contribute to the maintenance of rich, multispecific assemblages in all three lakes. Lake Tanganyika's substrate-breeding species, which guard their developing offspring among the rocks, exhibit preferences for spawning sites based on a number of physical characteristics (Gashagaza 1991). Removal experiments further demonstrated that the reproductive characteristics of species influence the nature of their preferred breeding caves (Gashagaza 1991). Partitioning of breeding sites is thought to be less important to the mouthbrooding cichlids of all three lakes (Genner et al. 1999b; Hori 1983; Turner 1999). Mouthbrooding cichlids may mate in rocky caves, but their offspring develop completely in the oral cavity of one or both of the parents. As a result, they are not as dependent on the substrate for the protection of their young as substrate-spawners. However, habitat partitioning has been observed in mouthbrooding species from Lake Victoria and Lake Malawi (Bouton et al. 1997; Ribbink et al. 1983). Furthermore, removal experiments in Lake Malawi indicate that breeding sites appear to be species-specific for at least one species (*Metriaclima aurora*) (Hert 1990).

Several studies have argued, however, that the partitioning of space, and breeding territories in particular, have played little if any role in the maintenance of complex rock-dwelling communities in Lake Malawi (Genner et al. 1999b; Turner 1999). Parental investment within Lake Malawi's rock-dwelling cichlids, or mbuna, like all maternal mouthbrooding cichlid species, is highly skewed. Females develop large yolk-rich eggs that they incubate for three weeks after fertilization. During this incubation period, females are unable to feed. As a result, females are limited to one to two matings per year. In contrast, males form lek-like aggregations on the rocks and display to passing females from a centralized breeding area in their territory. Males may mate multiple times per year and contribute only gametes to their offspring. Among males, there is a large variance in

reproductive success (Hert 1990). Turner (1999) and Genner (1999b) argue that competition for breeding space is unlikely to develop under this reproductive system since the survival of offspring is not directly related to the territorial behavior of males.

Territory acquisition, however, directly influences male reproductive success. Male mbuna require territories to successfully mate. Furthermore, the rocky habitat is limited in size (McKaye and Gray 1984). Given the central role of territories in male mating success and the finite availability of suitable habitat on which to establish a territory, strong competition for this necessary and limited resource may exist. As a result, species may have evolved territory preferences through which the effect of competition is reduced.

The objective of this study is to examine patterns of species-specific territoriality in two closely related rock-dwelling cichlids, *Metriaclima zebra* and *Metriaclima benetos*, on an isolated rocky reef in the southeast basin of Lake Malawi. I examined the territorial behavior of these species by (1) performing artificial removals of territorial males and observing the pattern of recolonization and (2) quantifying the physical features of male breeding caves.



## Materials and Methods

### Study Site

This study was carried out at Mazinzi Reef, a submerged rocky reef in the southeast arm of Lake Malawi. This reef is approximately 10,000 m<sup>2</sup> in size and located approximately 3.2 km from the nearest shore. Estimated migration rates of *Metriaclima* species indicate that Mazinzi Reef populations are relatively isolated from mainland populations (Danley et al. 2000). There are at least 12 species of rock dwelling cichlids (mbuna) on this reef, four of which belong to *Metriaclima*.

### Study Species

The genus *Metriaclima* is an exceptionally species rich mbuna genus. A total of 24 species are currently recognized (Stauffer et al. 1997). However, this figure is likely to be an underestimate due to a number of undescribed species (Stauffer et al. 1997). Four *Metriaclima* species occur at Mazinzi Reef; *Metriaclima barlowi*, *Metriaclima livingstonii*, *M. zebra*, and *M. benetos*. *Metriaclima barlowi* and *M. livingstonii* can be distinguished from *M. zebra* and *M. benetos* by the microhabitats in which they occur. *Metriaclima barlowi* occurs at the sand-rock interface; *M. livingstonii* occurs over the sandy habitat surrounding Mazinzi Reef where individuals occupy discarded snail (*Lanistes*) shells. Only *M. zebra* and *M. benetos* commonly co-occur on the rocky habitats where their

territories often interdigitate (Fig. 3.1). In addition to their similar habitat preferences, these species are strikingly similar morphologically (Stauffer et al. 1997), however they can be readily distinguished based on male breeding coloration. Male *M. zebra* are bright blue with 6-8 vertical black bars. *Metriaclima benetos* males are bright blue as well but appear to lack all melanistic markings. *Metriaclima zebra* is commonly found throughout the rocky habitats around the lake; it is one of the few rock-dwelling species with a lake-wide distribution. In contrast, *M. benetos* is a narrow endemic that is thought to occur only at Mazinzi Reef (Stauffer et al. 1995).

### Cave Measurements

Males of both species occupy and defend territories from which they court passing females. Males approach females in the water column, display their erect fins, and swim with exaggerated body movements toward a breeding cave in the center of their territory where courtship continues and spawning occurs. The male chases the female from the cave and territory when the mating bout ends.

Several males of both species (*M. zebra*, N = 24; *M. benetos*, N = 38) were observed courting and their breeding caves were identified. The length, width, and depth of the breeding caves of both species were measured. Each dimension was measured at a right angle to the remaining dimensions.

## Removals

Three 4m by 4m quadrats were established on Mazinzi Reef. These quadrats were separated by at least 35m and supported roughly equal numbers of males of both species prior to the removals. The territorial *M. zebra* and *M. benetos* males were identified based on their body markings (parasite scars, egg spots, natural fin clips made by fin predators, and head barring pattern). The breeding cave of each male was located on a hand drawn map of the rock substrate. All territorial males in each quadrat were removed using monofilament nets (6 mm mesh size) 6m long x 2m high while SCUBA diving. Captured fish were sacrificed in a 10% Formalin solution for future morphometric analysis and to prevent the reclamation of a territory by a dislodged male.

## Territorial Behavior

The re-colonization of the quadrats was monitored on days 1, 2, 4, 6, 10, and 19 post-removal. On these days each male defending a territory was identified and his breeding cave was located on the map. The number of aggressive actions (e.g. chasing another fish from its territory) made by each new resident male was recorded during a ten minute observation period on those days when the quadrats were monitored. Empty territories, territories which were occupied prior to the removal but were apparently empty during the recolonization, were observed for at least two minutes to verify that the territory was truly vacant. Territories that either remained vacant at the end of 19 days or were occupied by species other than *M. zebra* or *M. benetos* were not included in the analysis.

Three removals occurred at each of the three quadrats. Each removal was conducted

21 days after the previous removal. All removals occurred between 10:00 and 16:00 during the months of April-July 1996.

In order to compare the observed aggression on a removal site to the aggression of a typical male, males of both species (*M. zebra* N=36; *M. benetos* N=47) were observed between 08:00 and 17:00. Each aggressive act that took place in a ten minute period was recorded. All males that were observed for the behavior controls maintained territories on areas of the reef separate from the removal quadrats. I assumed, therefore, that these males were defending "established" territories. Additional observations of established males were made to determine whether con- or heterospecifics were the primary target of male aggression. Fifteen males of both species were observed for 10 minutes. During this time, the number of aggressive acts directed towards con- and heterospecifics (including males and females of any species found on the reef) was recorded.

### Relative Abundance

Testing whether the probability of occupying a territory was random by species or not depends on the relative abundance of each species. The relative abundance of both species was estimated by counting the numbers of males and females of each species found within a 2m x 25m transect. The transect was established for two minutes before counting began. Females of both species were counted first during one pass over the transect, then the males of both species were counted during a second pass. Each pass was separated by at least two minutes. The relative abundance of both species was estimated in this manner for 12 haphazardly established transects. The relative abundance of males of both species was used to estimate the expected number of males likely to colonized vacated caves. This value was compared to the observed number of recolonizing males to determine if breeding caves were species-specific.

## Results

The territorial fidelity observed in both species indicated that a given site is (Table 3.1). Of 28 territories originally occupied by *M. zebra* males, only 2 were subsequently colonized by *M. benetos* males. Likewise, only 6 of 35 territories occupied by a *M. benetos* male prior to the removal were subsequently occupied by a *M. zebra* male. The pattern of recolonization of both types of caves suggests that species-specific preferences for breeding sites exist in both *M. zebra* and *M. benetos* (Chi-squared = 24.7,  $p < 0.001$ ; Chi-squared = 11.73,  $p < 0.001$ , respectively). Species other than *M. zebra* and *benetos* colonized a small number (1-3) of vacated territories per removal.

The dimensions of the breeding cave are highly correlated with the observed site fidelity (Table 3.2). An analysis of the breeding caves of both species reveals that *M. zebra* males occupy significantly wider caves than *M. benetos* males ( $t = 3.522$ ,  $p < 0.001$ ). Their caves overlapped for the two remaining dimensions (Length  $t = 0.01$ ,  $p = 0.99$ ; Depth  $t = 0.993$ ,  $p = 0.32$ ).

The expression of this species-specific territory occupation was expressed primarily through microhabitat segregation. *Metriaclima zebra* tended to occur over large rock piles with many square shaped cave openings. *Metriaclima benetos* occurred more frequently over exposed bedrock in which parts of the strata had eroded more quickly than others creating the long, narrow caves that *M. benetos* males frequently occupied.

Patterns of male aggression indicated that interspecific competition for territories may be high. Males of both species were more aggressive on newly acquired territories than on established territories (Table 3.3). Among male *M. zebra*, aggressive acts on a newly acquired territory were nearly twice as frequent than on an established territory ( $t = 2.87$ ,  $p < 0.01$ ). *Metriaclima benetos* males also tended to be more aggressive on a newly

acquired territory ( $t = 1.80$   $p = 0.034$ ). *M. benetos* males were 3-4 times more aggressive as *M. zebra* males on both new ( $t = 2.704$ ,  $p < 0.005$ ) and established ( $t = 6.66$ ,  $p < 0.001$ ) territories. In both species, aggression was directed primarily towards heterospecifics (*M. zebra*  $z = 2.63$ ,  $p = 0.008$ ; *M. benetos*  $z = 3.41$ ,  $p = 0.0007$ ; Table 3.4).

## Discussion

The partitioning of breeding sites is a common phenomenon in cichlid fishes. Cichlids from Lake Nicaragua to Lake Tanganyika are known to partition breeding sites based on a number of physical characteristics including: type of substrate, the dimensions of the breeding area, and the slope of breeding surfaces (McKaye 1977; Gashagaza 1991). However this type of resource partitioning is not expected to occur in the cichlid species flock of Lake Malawi (Turner 1999; Gashagaza 1991). All of Lake Malawi's cichlids are maternal mouthbrooders and the survival of their offspring is not dependent on the male territory. This observation has been used to argue against the evolution of species-specific territory partitioning in the mbuna. The breeding caves within the genus *Metriaclima*, however, can be distinguished based on their dimensions and removal experiments suggests that these areas are species-specific (Hert 1990; this study). These findings suggest that factors, in addition to parental care, shape patterns of habitat utilization in East African cichlids.

Interspecific competition for breeding territories may have led to the evolution of character displacement in the mbuna. In the past, the existence of character displacement has been widely disputed. In response, six tests have been outlined in order to determine whether or not the process has contributed to the observed ecological characteristics of sympatric species (Losos 2000). First, the differences between sympatric taxa are greater than expected by chance. Second, the observed difference in character state are related to resource use. Third, resources are limiting and interspecific competition is a function of character similarity. Fourth, the differences are genetically based. Fifth, resource distributions are the same in areas of sympatry and allopatry such that differences in

character states do not reflect differences in resource availability. Sixth, the differences between competing species evolved as a consequence of competition while in sympatry.

The results of this study clearly address tests one through three. The difference in territory use is greater than expected by chance, each species' apparent cave preference are directly related to resource use, and the increase in interspecific competition following the colonization of vacated territories indicates that cave availability is limiting. Furthermore, one would expect that competition for caves would increase as interspecific cave preferences overlap.

Future work is needed to address tests four through six. An ongoing project aims to establish the genetic basis of cave preferences. Lab reared males of both species are being examined for their relative preference of square versus rectangular caves. Additional field work is needed to address test five. The cave occupancy of other populations of *M. zebra* must be examined to establish that occupancy at the Mazinzi Reef population differ from the species mean cave occupancy. Such a study would also need to determine if the potential cave sizes at Mazinzi Reef are equivalent to caves found elsewhere in the lake. Ideally test six would be addressed in a phylogenetic context. By demonstrating that the observed occupancies are not the result of a pre-existing condition through a phylogenetic examination of populations and species within *Metriaclima*, one could establish that the divergent cave preferences evolved in sympatry. An examination of these final three tests would provide strong support for the character displacement hypothesis.

Alternatively, sexual selection, rather than natural selection, may have shaped the observed cave preferences. The likelihood that sexual selection has shaped male phenotypes among Lake Malawi cichlids is well recognized (Hert 1991; McElroy and Kornfield 1990; McKaye 1991). In addition, studies of Lake Malawi's sand-dwelling cichlids have documented the importance of the dimensions of male breeding areas in determining reproductive success. It is possible that male breeding caves in the mbuna, like the bowers of sand-dwelling cichlids (McKaye et al. 1990), represent extended phenotypes



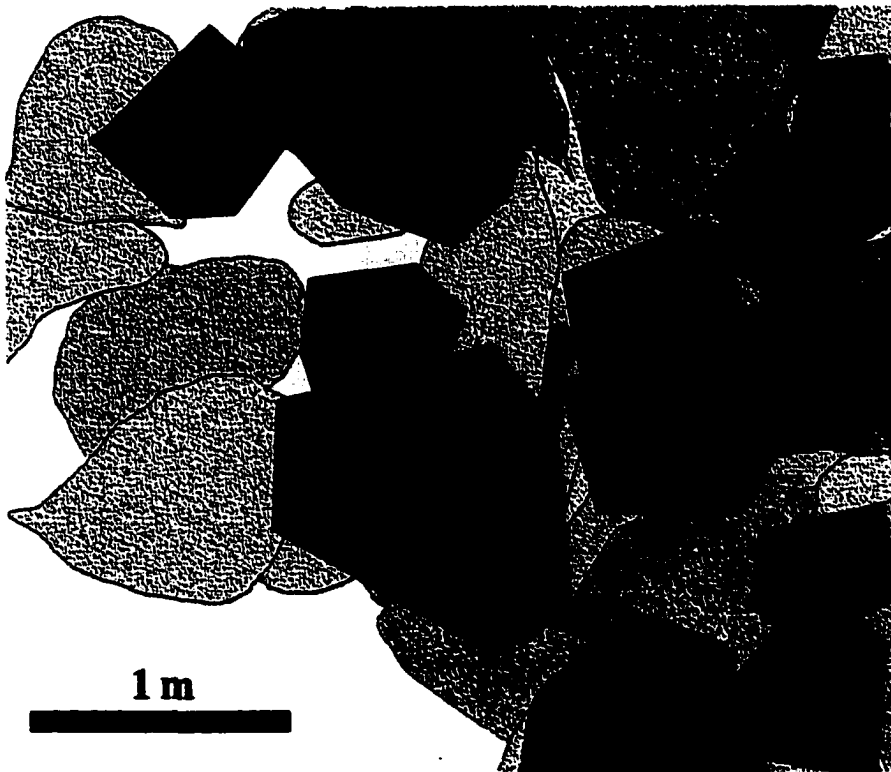
which experience sexual selection. Research is needed to determine if there is a correlation between male breeding cave dimensions and male reproductive success. If such a correlation exists, manipulative laboratory experiments would be warranted.

Natural and sexual selection are not mutually exclusive. Documenting the operation of one mode does not preclude the influence of the other. In fact, they may produce such similar effects that distinguishing the two may be difficult. Their synergistic influence, however, may be a particularly potent diversifying force. If both natural and sexual selection are driving the divergence of male breeding cave preferences, rapid speciation may occur even in the presence of considerable gene flow. Sympatric speciation models in which one trait is involved in both interspecific competition and reproductive isolation are generally recognized as the most likely to occur (Via 2001).

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Figure 3.1 A map of *M. zebra* and *M. benetos* territories. Grey polygons represent rocks, red polygons represent *M. zebra* territories; blue polygons denote *M. benetos* territories. The scale bar (1m) is an approximation.



**Table 3.1 Breeding site preferences. The recolonization of *M. zebra* and *M. benetos* caves were observed following the artificial removal of territorial males. Chi squared tests were used to examine the independence of male breeding cave preferences. Observed recolonizers are the number of males, of each species, occupying a cave previously held by either (a) a *M. zebra* male or (b) a *M. benetos* male. The expected number of colonizers was estimated based on the relative abundance of males of both species (Table 5) and the number of caves originally held by both species.**

<sup>1</sup>  $p < 0.001$

a. *M. zebra* caves <sup>1</sup>

	<i>M. zebra</i>	<i>M. benetos</i>
Observed recolonizers	26	2
Expected recolonizers	12.9	15.1

b. *M. benetos* caves <sup>1</sup>

	<i>M. zebra</i>	<i>M. benetos</i>
Observed recolonizers	6	29
Expected recolonizers	16.1	18.9

**Table 3.2 The mean (variance) of male breeding caves in both species  
Differences in male cave preferences for *M.zebra* (N=24) and *M. benetos* (N=38)  
were determined by two-sample t tests with unequal variances.**

<sup>1</sup> p<0.001

	<i>M. zebra</i>	<i>M. benetos</i>
Width <sup>1</sup>	13.96 (21.88)	9.97 (5.87)
Length	29.5 (255.59)	29.54 (186.66)
Depth	26.06 (240.93)	22.38 (167.64)

**Table 3.3** The mean number of aggressive acts performed during a 10 minute observation on established (*M. zebra* N = 36, *M. benetos* N = 47) and new (*M. zebra* N = 16, *M. benetos* N = 34) territories. Significant difference were detected both between species (t test with unequal variances) and within species (t test with equal variances).

<sup>1</sup> p<0.05

<sup>2</sup> p<0.01

<sup>3</sup> p<0.005

<sup>4</sup> p<0.001

	Established <sup>4</sup>	New <sup>3</sup>
<i>M. zebra</i> <sup>2</sup>	2.1	3.8
<i>M. benetos</i> <sup>1</sup>	8.1	11

**Table 3.4 Median conspecific and heterospecific aggression in *M. zebra* and *M. benetos* . Fifteen males of both species were observed for 10 minutes. All aggressive acts directed towards con- and heterospecifics were scored during this observation period. Wilcoxon signed rank tests were used to determine if aggression was directed more towards one class or the other.**

<sup>1</sup> p < 0.01  
<sup>2</sup> p < 0.001

	Conspecific	Heterospecific
<i>M. zebra</i> <sup>1</sup>	1	4
<i>M. benetos</i> <sup>2</sup>	2	10

**Table 3.5 Mazinzi Reef Transects.** The males and females of *M. zebra* and *M. benetos* which occurred within a 25m x 2m transects were counted. Females of both species were counted during a first pass. Males of both species were counted during a second pass. Transects were established hapazardly but did not overlap.

Transect #	<i>M. zebra</i> female	<i>M. benetos</i> female	<i>M. zebra</i> male	<i>M. benetos</i> male
1	14	17	21	15
2	10	42	21	15
3	5	12	9	22
4	7	48	9	19
5	2	16	10	31
6	4	5	13	16
7	12	16	9	15
8	3	1	7	6
9	0	29	1	6
10	29	29	14	15
11	38	14	28	24
12	45	4	19	4
<b>Total</b>	<b>169</b>	<b>233</b>	<b>161</b>	<b>188</b>



## CHAPTER 4

# THE GENETIC BASIS OF COLOR PATTERN EVOLUTION IN LAKE MALAWI'S ROCK-DWELLING CICHLIDS

### ABSTRACT

Sexual selection on male color patterns is thought to have played a major role in the diversification of Lake Malawi's rock-dwelling cichlids. While this model of diversification has been discussed in the literature for over 15 years, there are few empirical studies documenting the genetic basis of this critical phenotypic character. The objective of this study is to identify the putative genes involved in the diversification of Lake Malawi's rock-dwelling cichlids. Three projects were undertaken in order to identify these genes. First, the importance of male color pattern in establishing reproductive isolation between two closely related species is examined. Through the analysis of female mating preferences, it is determined that visual cues related to male color pattern are sufficient to elicit female choice. Second, the cellular basis of interspecific color pattern variation is determined. The two study species differ primarily in the frequency of melanophores in various body regions. Third, the genetic basis of differences in male color pattern is identified. Using biometric and genomic techniques, the minimum number of genes influencing male color pattern are estimated and their putative genomic locations are identified. While the results of this study are preliminary, they provide valuable insight into the genetic basis of Lake Malawi's cichlid radiation and provide a foundation for future studies of complex phenotypes in this system.

## Introduction

Evolutionary biology is largely a retrospective science. It primarily examines patterns of phenotypic or molecular changes to infer the origins of species. While theoretical models and computer simulations have provided additional insight, we are frequently denied the crucial experiments and observations needed to test the assumptions of these models (Coyne 1992).

The genetic characterization and manipulation of complex phenotypes has been particularly elusive. Theorists have developed the verbal and quantitative genetic models describing the evolution of complex characters (Fisher 1930; Lande 1982; Orr 1998) and predictions generated from these models have been widely accepted in the literature. Indeed one such prediction, the polygenic basis of adaptive change, is widely accepted in neodarwinian evolutionary theories. The number, position, effect, and epistatic behavior of the genes underlying complex phenotypic change, however, is known for only a limited number of genetically well-characterized model systems.

Addressing such fundamental questions concerning the molecular basis of speciation in non-model systems is in its infancy. Such systems, while of interest to students of evolution, often lack the genetic tools necessary to perform molecular studies of phenotypic change. The advent of relatively inexpensive genomic techniques will enable a greater number of researchers to quantify the position and effect of putative loci involved in character evolution. This is one such study. Through Quantitative Trait Locus (QTL) mapping, the location of some of the genes involved in the rapid diversification of East African cichlids are identified.

The diversification of East African cichlid fishes has fascinated evolutionary biologists for over a century. Over 2000 species of cichlid fishes have evolved in East

Africa within the past 10 million years. This unparalleled vertebrate diversification is concentrated in the three East African Great Lakes: Lake Tanganyika, Lake Victoria, and Lake Malawi. Each of the cichlid assemblages in these lakes possess interesting evolutionary features. Lake Tanganyika's cichlids are the oldest and appear to have seeded the surrounding lacustrine cichlid radiations. Lake Victoria cichlids are the youngest; the majority of its 300 species have evolved since its reformation 12,500 years ago. The cichlids of Lake Malawi are particularly attractive. More than 400 cichlid species occur in this lake, the majority of which are endemic. Furthermore, the patterns and processes involved in their diversification have been inferred from recent molecular investigations (Albertson et al. 1999; Danley and Kocher 2001).

The diversification of Lake Malawi's cichlid species flock appears to have occurred in three major bursts of cladogenesis. The initial invading lineage appears to have diverged into two clades each associated with either rock or sand. Within the rock-dwelling clade (or mbuna), strong competition for trophic resources spawned a secondary radiation. This radiation resulted in the diversification of the trophic apparatus and created the majority of the currently recognized genera. Sexual selection drove the tertiary radiation and resulted in the differentiation of male sexual characteristics, particularly male color patterns (Danley and Kocher 2001). In contrast, most ecologically important characters were conserved (Deutsch 1997; Stauffer et al. 1997). Diversification during the third period appears to be the most extensive. For example, the primary and secondary radiations produced 2 and 10 lineages, respectively. The tertiary radiation, which produced at least 20 species in certain lineages, produced ten times as many lineages as the primary radiation and twice as many lineages as the secondary radiation (in certain clades). Results from phylogenetic studies suggest that sexual selection, particularly on color pattern, has played a significant role in increasing the biological diversity of this system.

In addition to the phylogenetic evidence, strong empirical and theoretical evidence supports the hypothesis of diversification through sexual selection on male color patterns.

Both field and laboratory studies have documented the importance of colored ornamentation (Hert 1991), body hue (Seehausen and van Alphen 1998), and melanistic patterning (Knight and Turner 1999) on female mate choice and mate recognition. These and other behavioral observations have produced a nearly ubiquitous discussion of speciation via sexual selection in the cichlid literature. A variety of Fisherian (Deutsch 1997; Knight and Turner 1999; McElroy and Kornfield 1990; McElroy et al. 1991; McKaye 1991; Parker and Kornfield 1996) and "good genes" (Hert 1991; Taylor et al. 1998) arguments have been made. In addition, explicit (Turner and Burrows 1995) and quantitative genetic (Dieckmann and Doebelli 1999; Higashi et al. 1999; Kondrashov and Kondrashov 1999) models of sexual selection have been developed with the diversification of East African cichlids in mind. Given the volume of literature on this topic, however, there exists a dearth of empirical evidence documenting the genetic basis of male traits and female preferences in this or any other system.

A subset of the genes involved in the divergence of Lake Malawi's rock-dwelling cichlids are identified in this study. Three projects were completed in order to identify these genes. First, I demonstrate that male color pattern is important in female mate recognition for two closely related species, *Metriaclima zebra* and *Metriaclima benetos* that differ primarily in male color patterns (Fig. 4.1). Second, the histological basis of the male color patterns is investigated. Third, a genetic analysis of male color pattern was performed. The number of genes influencing the color pattern is estimated using biometric methods that compares the variation in male color patterns across the parental species and multiple hybrid generations. The putative locations of the genes influencing male color patterns are identified in a QTL study. The results of these studies are then interpreted in terms of the divergence of *M. zebra* and *M. benetos* and the rapid diversification of East African cichlids.

## Materials and Methods

### Study Species and Cross Design

*Metriaclima zebra* and *M. benetos* diverged extremely recently. This has made attempts to estimate their exact phylogenetic relationship including the time since their divergence, difficult. Data from geologic studies of the lake suggest that these two species may have diverged within the past 1000 years (Owen et al. 1990). During this time, there has been very little morphological differentiation (Stauffer et al. 1997). However, these species can be distinguished on the basis of the coloration of adult males (Fig. 4.1).

*Metriaclima zebra* males are bright blue with 5-7 black body bars which run dorso-ventrally, a black cheek, and a black submarginal band in the pelvic fin. Male *M. benetos* are the same bright blue ground color as *M. zebra* males but appear to lack any melanistic markings. In the field, no interspecific courtships or matings were observed. In the laboratory, attempts at the natural spawning of several male-female heterospecific pairs yielded only one successful brood. These observations suggest the operation of strong behavioral mechanisms for reproductive isolation.

Hybrids were therefore created through the artificial fertilization of a single clutch of eggs collected from a *M. zebra* female with the sperm collected from a single *M. benetos* male. The F<sub>1</sub> progeny of this cross was randomly intercrossed to produce 16 F<sub>2</sub> families. F<sub>2</sub> males were grown for a minimum of 1 year. Once a male's reproductive coloration developed, tissues were collected to quantify his color pattern and extract DNA (see below).

## Mate Recognition and Female Preferences

Design of experimental set-up. Female mating preferences were examined by simultaneously presenting one male of each species to a single female in a large test aquarium. The males were confined in smaller aquaria located outside of the 300cm x 43cm x 30cm (H) test aquarium at opposite ends. The female aquarium was constructed of a PVC frame and a clear plastic liner. Three refugia constructed of cement bricks were placed inside this large aquarium; two at either end and one in the middle. The males were housed in 37.8 l glass aquaria separated from the test aquarium by a sheet of one-way glass (reflective side facing away from the test aquarium) and a narrow air space. The air space and one-way glass between female and male tanks prevented the males from acoustically and visually interacting with the female test subjects. Males, when faced with a mirrored surface, adopt characteristic mating colors and territorial behaviors similar to those expressed in the field (pers. obs.). Each of the smaller aquaria contained a refuge similar to that in the test aquarium that served as a focus for male territorial displays.

The test aquarium also contained approximately 20 juvenile tilapia hybrids. Tilapia were used as “dither” to assist in acclimating female test subjects. In preliminary test that lacked dither, wild caught female subjects took several days to recover from handling stress before they could be tested. Some females never adequately recovered; they did not move from behind a refuge during several hours of observations. With the inclusion of dither, these same females moved throughout the entire tank immediately upon release.

Assay of female choice. One of three males of each species was introduced into either of the smaller aquaria 21 hours prior to the initiation of the female assays. Males were assigned a tank at random and were approximately matched for size.

The following day a female was introduced to the center of the test aquarium. In order for the one-way glass to be effective, the male tanks had to be significantly brighter than the large female test tank. The smaller male tanks were thus illuminated immediately following the introduction of the female to the test aquarium by 90 watt halogen lamps.

The female was given 30 minutes to acclimate before testing. Two measures of female preferences were scored during a 10 minute observation period: time spent (seconds) in the 1/6 of the tank nearest each male and the number of times the female entered the refuge near each male. Time spent in association is expected to be a coarse measure of preference, whereas frequency of entering a refuge is expected to be a more accurate indicator of a female's willingness to mate (McElroy and Kornfield 1990). Ten females of both species were tested. Female responses to different males were tested using a Wilcoxon Signed Rank test.

#### Quantifying Male Color Pattern

Color patterns can be altered through physiological and/or morphological means. Physiological color changes, changes resulting from the neurological manipulation of chromatophore cells in the dermis, occur very rapidly and often take place during social interactions. For example, the lower *M. zebra* in Fig. 4.2 has adopted a dominant social position as observed by the high level of contrast between its bright ground coloration and its dark black body bars. In contrast, the male in the background, which was intensely colored before this interaction, has adopted a less conspicuous, socially subdominant, coloration. This change in male coloration occurred in a matter of seconds.

Male coloration may also differ due to the differential proliferation of chromatophore cell types. Two types of chromatophores may contribute to the differences

in male coloration observed between these two *Metriaclima* species. Melanophores, relatively large dendritic chromatophores (Fig. 4.3b and c), are responsible for the black areas on the body including the body bars, cheek, and submarginal bands in the pelvic fins. Iridophores (Fig. 4.3d and e) are an order of magnitude smaller and produce the bright blue coloration characteristic of males of both species through the reflection of light off stacks of guanine platelets (seen in the TEM).

In order to characterize the differences in male color pattern between these two species, a number of tissues were sampled and analyzed. Tissues were sampled from: the first complete body bar (one scale from immediately above the lateral line), the space between the first and second body bar (one scale from immediately above the lateral line), and the pelvic fin. All tissues were sampled from anesthetized individuals, immediately treated with 5 mM norepinephrine to contract the melanophores, fixed in 10% phosphate buffered formalin (PBF), and stored in dH<sub>2</sub>O until they were examined the following day.

Chromatophore frequencies were assessed from the digital images of the tissues at 100X. Melanophore frequencies were assessed by counting the number of melanophores which occur in a 2.5 mm<sup>2</sup> area. Individual iridophores, due to their small size and transparency to transmitted light, could not be directly counted. Instead, iridophore frequencies were estimated based on the number of pixels which reflected incident light from a digital image of a 2.5 mm<sup>2</sup> area of tissue.



## Genetic Analysis of Male Color Patterns

**Estimating the effective number of factors.** The number of genes contributing to the expression of complex phenotypes can be calculated using traditional biometric methods. In brief, the mean and variance of the parental,  $F_1$  and  $F_2$  generations are compared to provide a minimum estimate of the number of loci influencing the expression of a trait. The number of effective factors contributing to the difference in male color patterns was calculated using Zeng's correction of the Castle-Wright equation (Zeng 1992).

**Identifying color pattern QTL.** The  $F_2$  mapping family was phenotyped for color pattern and genotyped at 133 microsatellite loci randomly distributed throughout the genome. The joint analysis of the phenotypic and genotypic data was used to identify the QTL influencing male color patterns.

Microsatellite markers were developed following Albertson et al. (in prep). A summary of their protocol is presented below. Genomic DNA was extracted from muscle tissue following standard methods. The genomic DNA was restricted, PCR amplified using ligated priming sites, and enriched using biotinylated  $(CA)_n$  probes and magnetic streptavidin beads. The captured DNA was washed and TA cloned with a commercially available kit. Clones containing sufficiently large inserts (200-500 bp) were minipreped and cycle sequenced. Primer 3 (Rozen and Skaletsky 1996) was used to design primers for clones containing 12 or more perfect CA repeats. Primers were designed to anneal to the template DNA at 60° C and produce a total amplicon size between 100 and 250 bp. The primers were florescently labeled with either 6-Fam, Tet, or Hex (Operon). Additional microsatellite loci isolated from the tilapia, *Oreochromis niloticus*, were as well.

Genomic DNA was extracted from the 95 phenotyped  $F_2$  individuals and the *M. zebra* grandmother. Microsatellite alleles were amplified through 30 cycles of PCR with an annealing temperature of 50-55° C. The PCR products were scored on a 4% polyacrylamide gel using a ABI 377 ABI automated DNA sequencer.

CRIMAP (version 2.4, Green et al. 1990) was used to determine the linkage relationships of the microsatellite loci. Allele sizes were coded as 0 (for missing data), 1, 2, 3, or 4 for all individuals. Since Crimap requires genotypic information for all individuals in the complete pedigree of the  $F_2$ , the genotypes for the grandfather and all  $F_1$  parents were entered as missing data. Non-mendelian inheritance errors, which occur when the observed genotypes of the  $F_2$  families do not equal the expected genotypes based on the inferred  $F_1$  cross, were corrected by replacing the offending genotypes with zeros.

QTL Cartographer was used to identify the positions of putative QTL affecting male color pattern. QTL Cartographer assumes that inbred lines were crossed to create the mapping population. All alleles derived from the grandmother (*M. zebra*) were coded as 1 and all other alleles were coded as 2. This coding strategy may reduce the power to detect QTL if both grandparents of the cross share identical alleles at a particular locus. QTL Cartographer also assumes that the parental lines are fixed for alternative states such that all *M. zebra* alleles tend to increase melanophore frequencies. The pattern of inheritance of melanophore frequencies in the  $F_2$  support this assumption. If the alleles which cause an increase in melanophore number occur in both species, one might expect transgressive segregation in the  $F_2$ . This pattern of inheritance was not observed; all  $F_2$  phenotypes fell within the parental range.

Single marker linear regression analysis, QTL Cartographer module Lrmapqtl, was used to detect QTL linked to the microsatellite markers. Other, more powerful, methods are available to map QTL in more complete data sets (e.g. composite interval mapping). The limited number of linked loci in this study, however, allowed only the less powerful single marker approach. Because of the large number of comparisons made to identify QTL, the

**significance level used to evaluate QTL was derived empirically. The data set was permuted 1500 times to determine the appropriate significance levels.**

## Results

### Mate Recognition and Female Preferences

Male visual cues, most likely differential melanistic patterning, appear to play a role in female choice. Females of both species, when simultaneously presented with con- and heterospecific males, exhibited behaviors indicative of mating preferences (Table 4.1). The willingness to mate with conspecifics was more apparent in *M. zebra* females. *Metriaclima zebra* females entered the cave nearest *M. zebra* males more frequently than caves associated with *M. benetos* males (Table 4.1), although this difference was not statistically significant ( $z = -1.43$ ,  $p = 0.153$ ). Female *M. zebra* also spent more time in the *M. zebra* male end of the tank than the *M. benetos* end of the tank ( $z = -2.80$ ,  $p = 0.005$ ).

The behavior of the *M. benetos* females was less clear. *Metriaclima benetos* females exhibited no significant preference based on the frequency of entering a refuge ( $z = 0.089$ ,  $p = 0.929$ ). Females of this species, however, spent a greater amount of time associating with heterospecific males compared to conspecific males ( $z = -1.99$ ,  $p = 0.046$ ). This measure may be misleading in this species however. *Metriaclima benetos* females behaved differently towards males of each species during the time associating with each male. With heterospecifics, *M. benetos* females performed many aggressive displays including lateral displays and frontal displays. They also attempted to bite the *M. zebra* males through the glass. In contrast, *M. benetos* males elicited a more typical response from *M. benetos* females. The females' behavior appeared less aggressive and consisted primarily of hovering either in the water column above the male or near the substrate.

Both species exhibited species-specific mate recognition. The measures of the females willingness to mate in these tests, however, were confounded with an aggressive

response which was more pronounced in *M. benetos*. As a result, species-specific mating preferences were more apparent in *M. zebra*. Nonetheless, these results indicated that visual cues, particularly male melanistic markings, play a significant role in the reproductive isolation of these species.

### Quantifying Male Color Pattern

Melanophores, the melanin-containing cells responsible for the black areas of the body, are more frequent in all tissue types in the prominently barred *M. zebra* (On Bar  $t = 16.50$ ,  $p = 0.4 \times 10^{-9}$ ; Off Bar  $t = 12.65$ ,  $p = 0.60 \times 10^{-8}$ ; Fin  $t = 20.41$ ,  $p = 0.16 \times 10^{-14}$ ; Table 4.2). However, the frequency of iridophores were not significantly different between these two species on either the barred ( $t = 1.28$ ,  $p = 0.21$ ) or unbarred ( $t = 1.00$ ,  $p = 0.33$ ) regions. Melanophores were more frequent in the barred areas of both species compared to the non-barred areas (*M. zebra*  $t = 6.61$ ,  $p = 0.98 \times 10^{-4}$ ) but this difference was not statistically significant in *M. benetos* ( $t = 2.04$ ,  $p = 0.07$ ; Table 4.2). Iridophores were more frequent in both species on areas not covered by a transverse bar (*M. zebra*  $t = 4.21$ ,  $p = 0.002$ ) but again this difference was not statistically significant in *M. benetos* ( $t = 1.16$ ,  $p = 0.27$ ; Table 4.2).

A key determinant of the power to detect QTL is the number of standard deviations separating the parental phenotypes. This measure incorporates not only the differences between the parental phenotypes but also includes an estimate of how variable the phenotype is in the parental lineages. The variation in the parental lineages reflects in part the genetic and environmental effects as well as the measurement error associated with the phenotype. In this respect, characters that possess a limited amount of variation in the parentals, but are radically different between the parentals, are better suited for QTL

analysis. Based on the characterization of the male color patterns, the frequency of melanophores is clearly the best method of quantifying male color patterns. Furthermore, because two measures of body melanophores (on bar and off bar) are likely to be redundant, only the melanophores on the bar and on the pelvic fin are used to characterize the male  $F_2$  color patterns.

### Genetic Analysis of Male Color Patterns

Estimating the effective number of factors. Melanophore frequencies on the first body bar and the pelvic fin were quantified in 10  $F_1$  and 95  $F_2$ . In the  $F_1$  generation, possessing a greater number of melanophores on both the body bar and pelvic band is partially dominant to the reduced melanophore phenotype (Table 4.2). The variance in the  $F_1$  for both phenotypes is equivalent to the variance observed in the parental generations (Table 4.2, Fig. 4.4b and 4e).

Melanophore frequencies in the  $F_2$  are also intermediate between the parentals for both phenotypes, however, the *M. zebra* phenotype is partially dominant in this generation as well (Table 4.2, Fig. 4.4c and d). The variance in the  $F_2$  generation is much greater than the variance in either parental species or the  $F_1$ . This pattern of inheritance, with the  $F_1$  and  $F_2$  means roughly intermediate to the parentals and an increase in the  $F_2$  variance relative to the previous generations, is consistent with the inheritance of a quantitative character controlled by a small number of primarily additive factors.

The biometric analysis of the effective number of genes suggests that certain aspects of the color pattern, namely the difference in bar melanophore frequency, are under simple genetic control. The upper bound of the estimated effective number of factors for bar melanophores (estimate + 2SD) is approximately 4 genes (Table 4.3). Five loci appear to

influence the number of melanophores on the fin. There is a large sampling variance associated with this measure which indicates that the minimum number of genes influencing fin melanophores may be as high as 11 (estimate + 2SD) (Table 4.3).

Identifying color pattern QTL. The identification of QTL influencing the traits of interest requires the joint analysis of the trait data and the genotypic data. The phenotypic data used to identify QTL influencing male color pattern was described in a previous section. Genotypic data were collected for the F<sub>2</sub> at 133 microsatellite loci (Table 4.4). Forty-nine loci were informative. The informative loci were analyzed using CRIMAP to generate a preliminary linkage map. Only 21 of the 49 markers were linked to another marker in the data set (Table 4.5). No significant linkage was detected among the remaining loci. While such low incidence of linkage makes map construction difficult, it suggests that markers are randomly distributed throughout the genome.

Due to the sparse nature of the map, a single marker analysis was performed to identify putative QTL. For the body bar, three QTL were identified (Tables 4.6 and 4.7). These QTL are linked to markers UNH2014, UNH738, and UNH933. For fin melanophores, three QTL were identified linked to UNH2057, UNH2037, and UNH2012. No QTL were identified which influence both traits. In fact, the two sets of QTL never shared the same linkage group. Each of the QTL appear to have a significant influence on the traits of interest at the  $p = 0.05$  level (not corrected for multiple comparisons).

The majority of the QTL appear to be expressed in a dominant fashion. The degree of dominance was calculated, following Falconer and Mackay (1996), as  $d$  (the difference in the heterozygous class mean and the midpoint between the homozygous class means) over  $a$  (the difference between a homozygous class mean and the homozygous class midpoint). Positive dominance values indicate that the heterozygous class resemble *M. zebra*; negative values indicate that the heterozygotes resemble *M. benetos*. Five of the six color pattern

QTL exhibit dominance in the direction of *M. zebra* (Table 4.7). This is consistent with the melanophore distributions observed in the  $F_1$  and  $F_2$ . The mean phenotype of both hybrid generations exhibited dominance in the direction of *M. zebra* (Fig. 4.4). Dominance values greater than (less than) +1 (-1) indicate the action of overdominance. Overdominance occurred at two of the QTL. Heterozygous individuals at Bar QTL 2 tended to have more melanophores than homozygotes possessing two *M. zebra* alleles. Bar QTL 3 heterozygotes possessed fewer melanophores than homozygotes possessing two *M. zebra* alleles (Table 4.6).

None of the QTL, however, were significant at the empirically determined experiment wide significance level. Based on a permuted data set, an experiment wide  $\alpha = 0.05$  threshold was established. For  $\alpha = 0.05$ , likelihood ratios calculated for each significant QTL must be greater than 11.48 and 11.14 for fin and bar melanophore frequencies, respectively. Only one QTL for each trait approached the permuted significance level.

The failure to identify statistically significant QTL lies in the lack of power associated with this study. Given the degree of dominance exhibited by the QTL and the low marker density of the map, 230 and 354  $F_2$  individuals are needed to detect the bar and fin QTL respectively. With an  $F_2$  mapping population of these sizes, a study would have a 90 percent chance of mapping the possible four and eleven QTL (estimated number of factors plus two standard deviations, see Table 4.3) responsible for the bar and fin melanophore frequencies, respectively (Lynch and Walsh 1998). Seventy-five additional markers would have also greatly improved the power of this study. A total of 125 markers, spanning the estimated 2500 cm map (based on the linkage map of the tilapia (Kocher et al. In prep)), would have produced an average inter-marker distance of 20cm. At this density, a study would require 129 and 198 individuals to identify the possible four bar and eleven fin QTL, respectively, with a 90 percent chance (Lynch and Walsh 1998). An increased  $F_2$  population and map density are needed to firmly establish the putative locations of the QTL identified in this study.



## Discussion

### Mate Recognition and Female Preferences

Male melanistic markings play a role in mating. In both field and laboratory settings *M. zebra* and *M. benetos* possess strong premating barriers which prevent their hybridization. Morphological, acoustical, chemical, and behavioral factors may play a role in preventing heterospecific matings. In the assay of female choice performed in this study, only visual cues were available for mate discrimination. Furthermore, the visual cues were restricted primarily to morphological cues. The one way glass prevented interactions between the males and females (although species-specific courtship patterns are not known to occur within the mbuna (McElroy and Kornfield 1990)). Of the available morphological differences between these species, differences in the cheek, body bar, and pelvic fin coloration are most apparent to human observation. Other, more subtle, morphological differences exist between these two species (Stauffer et al. 1997) and may play a role in mate recognition. Additional manipulative experiments are needed to provide conclusive support for this hypothesis. However, these findings support the hypothesis that the evolution of male color pattern has played a major role in the reproductive isolation of these two species.

### Quantifying Male Color Pattern

The observed differences in male color pattern can be attributed, at least in part, to

interspecific variation in chromatophore frequencies. Male *M. zebra* have a greater number of melanophores than *M. benetos* males on every body region. Depending on which area of the body is sampled, *M. zebra* also have a greater number of iridophores than *M. benetos*. Given the common developmental origin of these chromatophore cell types this finding is not surprising.

Chromatophore precursors originate in the neural crest and disperse throughout the embryo along well defined pathways (Hall 1999; Parichy et al. 2000; Parichy et al. 1999). Chromatophore stem cells then proliferate and differentiate once they have reached their target tissue (Bagnara et al. 1979). The data presented here suggest that *M. zebra* may achieve its greater number of chromatophores through the proliferation of chromatophore precursor cells prior to their differentiation into specific chromatophore types.

Alternatively, the mbuna may lack differentiated chromatophores altogether. Six distinct types of chromatophore have been identified in fish: melanophores, iridophores, erythrophores, xanophores, leucophores, and cyanophores. However, fish, unlike higher vertebrates, may possess mosaic chromatophores which possess multiple types of color producing organelles. Preliminary transmission electron microscopy (TEM) investigations of mbuna chromatophore cell structure indicate that *Metriaclima* species may possess mosaic melano-iridophores (PDD, data not shown). Regardless of the mechanism, the variation in color pattern observed between *M. zebra* and *M. benetos* involves the gain (or loss) of chromatophores in *M. zebra* (or *M. benetos*).

### Genetic Analysis of Male Color Patterns

The biometric analysis of color pattern suggests that this trait has a simple genetic basis in the mbuna. The estimated number of factors equaled two for the bar melanophores

and five for the fin melanophores. If this estimate can be confirmed, it may contribute to an explanation for the rapid speciation of Lake Malawi's cichlids. Traits determined by a limited number of loci evolve rapidly. Strong, persistent selection often favors macromutations which, in turn, fix rapidly in a population (Orr and Coyne 1992). This observation is well known in studies of artificial selection and the development of resistance (Orr and Coyne 1992). Evolution via macromutations are also known to occur in response to strong natural selection (Bradshaw et al. 1998; Bradshaw Jr. et al. 1995; Hunt et al. 1995). Reproductive traits (Wyckoff et al. 2000) and strategies (Lank et al. 1995) are also known to evolve rapidly and may be under oligogenic control. As a result, strong selection and macromutations are often thought to lead to speciation (Orr and Coyne 1992).

Sexually selected traits are generally believed to be under the control of many genes. In particular, the analysis of sexually dimorphic characters that distinguish taxa typically reveal a polygenic basis (Orr and Coyne 1992). This result has been explained as the result of vacillating female preferences and the correlated response of many male characters. A reevaluation of genetic models of adaptation suggest that strong, directional sexual selection on independent male characters may result in the evolution of male characters via macromutations (Danley and Kocher 2001). However, the effective number of factors provides only a rough estimate of the minimum number of genes influencing a trait and does not have the power to resolve hypotheses concerning evolution via macromutations. Unfortunately, the power of the QTL study also prevented the examination of this hypothesis.

Three QTL were identified for both aspects of the male color pattern. None of these QTL were statistically significant at the empirically determined threshold. The limited size of the mapping population, the sparseness of the map, and the dominance of the loci contributed to this lack of power. Nonetheless, future studies involving a greater number of  $F_2$  individuals and markers will likely support the approximate locations of the putative QTL identified here as well as possibly identifying additional QTL.

While this data is preliminary, certain observations can be made. First, the two sets of QTL never co-occur on the same linkage group. This suggests that these two aspects of the male color pattern may not be governed solely by overlapping sets of genes. This observation is further supported by the correlation of bar and fin melanophore frequencies in the  $F_2$  and parentals. Within the parental species, an individual's fin melanophore frequency was highly correlated with its bar melanophore frequency (Pearson correlation value 0.923,  $p = 0.35 \times 10^{-9}$ ). In the  $F_2$ , these two aspects of a male's color pattern, while still significantly associated, were correlated to a much lesser degree (Pearson correlation value 0.334,  $p = 0.002$ ). The reduced correlation of fin and bar melanophore frequencies in the  $F_2$  relative to the parentals, combined with the preliminary genetic analysis, suggests that genes may exist which affect specific elements of the male color pattern.

The existence of region specific color pattern genes is consistent with the developmental origins of melanophore patterning. Pigment precursor cells migrate away from the neural crest in multiple waves. Each of these waves may experience independent patterns of dispersion and differentiation (Le Douarin and Kalcheim 1999). Studies of the invasion of the skin by neural crest cells further revealed that pigment stem cells infiltrate trunk and limb buds at separate rates in vertebrate embryos (Le Douarin and Kalcheim 1999). Additional independence of pigmentation patterning is achieved through the reliance of local cues within the extracellular matrix of the dermal layers in determining the specific fate of chromatophore precursor cells (Bagnara et al. 1979). While melanophores of both the fin and trunk share a common developmental origin, the eventual melanophore patterning in these two body elements may be influenced by separate genetic and developmental processes.

The possible developmental and genetic modularity of the male color pattern may, in part, explain the extraordinary diversity of male color patterns observed in this system. Modular characters are free from the constraints of correlated selection. Correlated selection, selection which has a correlated effect on multiple characters in addition to the

character under selection, is a well recognized force retarding the rate of phenotypic evolution (Arnold 1992). Modularity, however, reduces the strength of selective constraints and may promote the rapid divergence of phenotypic characters (Bromham 2001). Furthermore, modularity increases the diversity of possible phenotypes. For instance, the independent inheritance of body and pectoral fin coloration doubles the possible color combinations from two forms if they are pleiotropically controlled (with melanistic markings versus without) to four forms if they are independent (with bars/dark fin; with bars/without dark fin; without bars/with dark fin; without bars/without dark fins). If the remaining three fins (dorsal, caudal, anal) are also independent of other aspects of the male coloration, 20 forms are possible. Modularity therefore may increase both the rate of phenotypic evolution and the diversity of potential phenotypes.

Second, a preliminary estimate of the gene action of the underlying loci can be made. An examination of the melanophore distributions in the hybrid generations suggests that the *M. zebra* phenotype is dominant. The degree of dominance estimates of the QTL generally support this observation. At five of the six QTL, heterozygotes were similar to individuals that were homozygous for *M. zebra* alleles. This finding does not support, however, the rapid evolution via strong selection hypothesis. Traits controlled by genes with additive effects fix more rapidly than genes with dominant alleles (Falconer and Mackay 1996).

Third, an estimate of the allelic effects can be made. Species undergoing strong, persistent, directional selection, such as the selection thought to be operating on the mbuna (Danley and Kocher 2001), tend to fix alleles which exaggerate the preferred phenotype. However, at half of the QTL, *M. zebra* alleles confer fewer numbers of melanophores. This pattern of allelic effects supports balancing selection rather than strong directional selection. There may be many reasons for this observation. Possessing an over-abundance of melanophores may disrupt mate recognition in *M. zebra* or may otherwise reduce fitness (by increasing the probability of developing melanomas, for instance). Melanophore

number may experience correlated selective pressures from a pleiotropically related trait. It is also possible that possessing genes which increase melanophore frequencies is an ancestral condition and selection has not yet purged these alleles from the *M. benetos* genome.

### Future Directions

This study suffered from an unreliable assay of female choice. The mbuna reproductive repertoire does not contain any fixed action patterns indicative of mate preferences thus no reliable behavioral proxy of a female's willingness to mate could be identified. Variation between these species in their response to similar social situations (e.g. the highly aggressive behavior of *M. benetos* females during the assays) further confounded the assay. Once again it appears as if the intrinsic variation of this system, which ultimately makes it attractive, also foils attempts to successfully characterize it. New behavioral assays are being developed which more accurately assess female mbuna reproductive intentions (Kidd, pers. comm.). This assay quantifies the number of eggs laid within close proximity of a male, a measure which has been successfully utilized in studies of Lake Malawi's sand dwelling cichlids for over 15 years, and should facilitate future studies of sexual selection in the mbuna.

Future studies of male color pattern would benefit from a more inclusive measure of male color pattern. This study assessed color pattern through the number of melanophores in a give area. This method is useful in that it: allows for the rapid quantification of color patterns; is quantitative rather than qualitative; is both exact and precise; is directly related to a biochemical and developmental basis; and facilitates the identification of candidate genes. However, the reliance on meristic quantification of color pattern incompletely characterizes

male color patterns. *Metriaclima benetos* males do not achieve their "barless" state solely by eliminating melanophores from the barred areas of their bodies. The examination of fresh tissues indicate that the melanosomes, pigment containing organelles, are aggregated in the melanophore centers of *M. benetos*. In contrast, the melanosomes of *M. zebra* are dispersed, contributing to the dark appearance of their bars. This observation is further supported by observations made during preliminary studies aimed at examining the effect of neurotransmitters on cichlid chromatophores. Alpha and beta adrenergic compounds have been used to study the neuro-physiologic control of chromatophore structure in a number of fish species (Fujii 1993). In order to determine their effects in cichlids, 5mM norepinephire (an alpha adrenergic) and 5mM isoproterenol (a beta adrenergic) were subdermally injected into both *M. zebra* and *M. benetos*. Norepinephrine, which aggregates melanosomes, enhanced the blue ground color of *M. benetos* and ablated the black body bars of *M. zebra*. Isoproterenol, which disperses melanosomes, produced black spots in the tissue surrounding its site of application in the blue regions of both species. These observations suggest that the physiological control of chromatophores may play a significant role in the differentiation of the barred/barless phenotypes.

Phenotypic measurements of male color pattern which assess and distinguish the influences of morphological and physiological color changes are needed to fully characterize the genetic basis of male color patterns. One method which may prove effective is the joint analysis of spectral reflectance and chromatophore cell count data. Spectral reflectance data provide an estimate of the wavelengths of light absorbed and reflected by the chromatophores. It quantifies the physical properties of the color pattern and is therefore the most inclusive method of quantifying male color patterns.

"Reflectance" QTL represent genes that alter both the number of chromatophores and their physiological state. "Chromatophore frequency" QTL represent those genes contributing only to morphological color changes. The identification of both sets of QTL, reflectance and cell counts, will provide a more complete survey of the QTL involved in the evolution of

male color pattern.

Such an analysis will not be without its difficulties. The collection of meaningful reflectance data is contingent on measuring males at the appropriate physiological condition. This is not an insignificant task. However, *in situ* measurements of fish reflectance data have been developed in other systems and may be suitable to African cichlids. Elaborating the phenotypic measurement, while it will require a greater time investment, may provide a more complete characterization of the molecular basis of color pattern evolution in this group.

The genetic analysis of male color patterns presented here is preliminary and suffers from several faults. As discussed previously, the QTL analysis lacks the power to detect the number of genes suggested by the biometric analysis (given the estimated length of the tilapia linkage map and the number of informative loci in this study, there is only a 56.8 percent chance that a QTL is within 20 cM of a marker). Typing additional markers and increasing the size of the mapping population will address this lack of power. This study is also limited by its analysis. Single marker analysis is the least powerful and least precise method for mapping QTL (Doerge et al. 1997). Furthermore, the statistical package, QTL Cartographer, was designed for the study of inbred line crosses. In adapting the *Metriaclima* data set for this package, potentially valuable allelic information was discarded. An analysis which utilizes all the allelic information would be more appropriate and may provide more accurate results. Nonetheless, this first step towards generating a linkage map for the mbuna and identifying the genes involved in their rapid radiation provides a much needed foundation to pursue additional evolutionary genomic studies in this system.

Genome scans, such as QTL mapping studies, are only one step towards identifying the molecular basis of complex phenotypic change. Such studies provide valuable information concerning the probable position and effect of genes which likely influence the trait of interest. These studies suffer from several limitations however. The identification of QTL relies on the statistical association of genotype and phenotype. The ability to identify



QTL may be compromised by inaccurate phenotyping or genotyping. It also relies heavily on an accurate genetic linkage map; as the map changes so will the estimated position and effect of QTL. QTL mapping projects rarely identify all of the genes influencing the trait of interest. QTL studies can only identify genes which are segregating in the two parental populations used to generate the hybrid mapping population. While this is problematic for any QTL experiment, it is further exacerbated in studies, such as this one, which rely on outbred parental stocks. Only the subset of the genetic variation in the trait of interest present in the parental species will be observed. The remaining variation potentially available in the two species will be overlooked unless many individuals of both species are used to generate the mapping population. Finally, there is a somewhat nebulous relationship between the loci identified in a QTL mapping project and the underlying genes. A QTL may span several megabases of DNA sequence containing hundreds of genes. Identifying a QTL may limit the universe of potentially important genes within the genome but it generally cannot identify the specific molecular basis of a complex trait.

The utilization of additional genomic techniques may provide greater resolution in the identification of male color patterns (Streelman and Kocher 2000). Candidate gene mapping, locating the position of genes with known function that likely influence the trait of interest, can be used to identify genes influencing complex characters. The identification of candidate genes influencing color pattern has been aided in the past twenty years by the growing understanding of the molecular basis of the variation in color pattern. Hundreds of pigmentation pattern mutations have been identified in zebrafish (Johnson et al. 1995; Kelsh et al. 1996; Lister et al. 1999; Odenthal et al. 1996) and work is underway in identifying the molecular basis of these mutations (Huzar et al. 1991; Porter et al. 1991; Lister et al. 1999; Parichy et al. 1999; Parichy et al. 2000; Rana et al. 1999; Kos et al. 2001). A firm grasp of the developmental patterns of chromatophores is emerging for zebrafish (Kelsh et al. 2000) and other cyprinids (McClure 1999). Scientists are also beginning to understand the biochemical pathways contributing to pigment production (Hearing and Jimenez 1987;

Huzar et al. 1991; Prota 1980; Rana et al. 1999) and the physiological and neurological control of color patterns (Fujii 1993). With a growing understanding of the molecular basis of color pattern differentiation, an increasing number of candidate genes can be identified and mapped in cichlids. The overlap of candidate genes and putative QTL will further resolve the molecular basis of divergence in the mbuna.

The patterns of gene expression may also provide additional insight into the molecular basis of differing male color patterns. Tools such as microarrays which detect minor changes in gene expression between two tissue types at thousands of genes are becoming more accessible to non-model systems and will play an important role in future studies of phenotypic evolution. Studies of gene expression, combined with QTL mapping and candidate gene mapping will further reduce the set of potentially influential genes (Streelman and Kocher 2000). The application of gene expression studies to the question of the diversification of male color pattern will be hampered by the lack of understanding of the chromatophore ontogeny in this group. Studies of gene expression require the understanding of the time and location of specific tissues responsible for alternative phenotypes. Developmental studies, such as those common in zebrafish (Kelsh et al. 2000), are needed before gene expression studies can be pursued in the mbuna.

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Figure 4.1 *Metriaclima zebra* and *Metriaclima benetos* males. *M. zebra* males are characterized by 5-7 black vertical bars along the body, a black cheek, and a black submarginal band in the pelvic fin. Male *M. benetos* males appear to lack all melanistic markings.

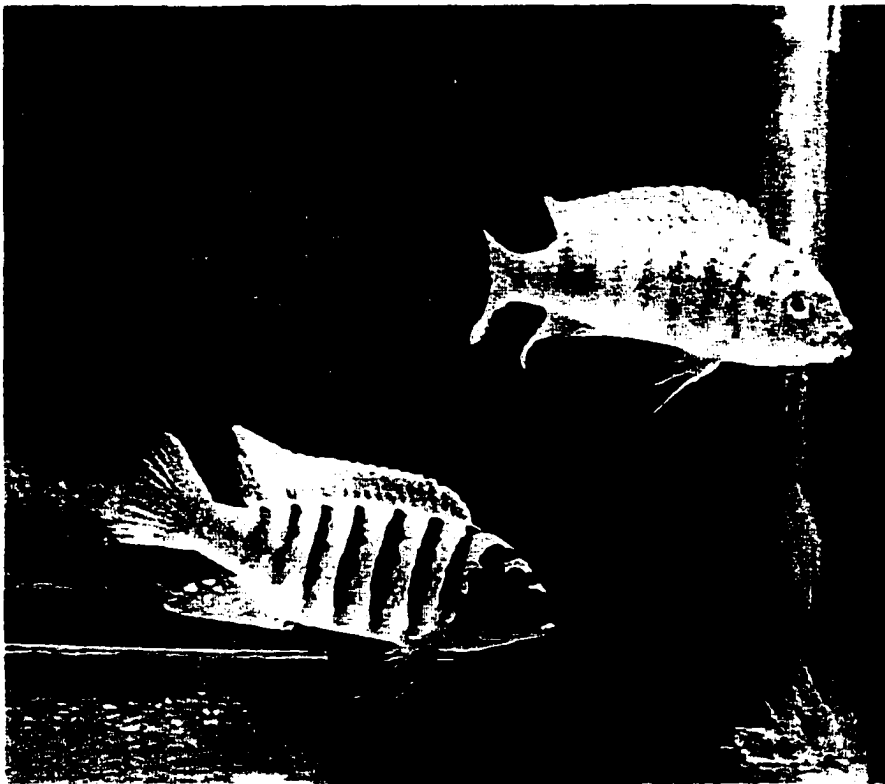
### **M. zebra**



### **M. benetos**



Figure 4.2 The social control of male color pattern. The lower *M. zebra* male is the dominant male in the tank. Note the bright blue ground coloration and dark black bars. The subdominant male, which is less brilliantly colored, displayed the intense colors characteristic of a dominant male until seconds after the dominant male was introduced into the tank.



**Figure 4.3 Chromatophore types influencing *M. zebra* and *M. benetos* color patterns.** A. Two chromatophore types are most common in both *M. zebra* and *M. benetos*. Melanophores contribute to dark areas such as the cheek, body bars, and pelvic fin. Iridophores produce the bright blue ground color common to both species. B. A micrograph of a *M. zebra* scale (on bar) showing the relatively large, dendritic melanophores (100x). C. A close up of a melanophore (400x) emphasizes its dendritic structure. Melanophores darken color patterns through their presence or absence and also by the distribution of melanin with the cell. The melanin in this melanophore, sampled from *M. zebra*, is dispersed throughout the cell. *Metriaclima benetos* melanophores are less apparent on all tissue types because their melanin is aggregated in the center of the melanophore. D. Iridophores generate the characteristic light blue coloration, seen here illuminated by a fiber optic light source at 100x, through thin plate interference. E. Light is reflected of stacks of guanine crystals seen here at 20,000x. Light reflected from deeper crystals in the stack interferes with light reflected from more superficial crystals to produce a broad spectrum of light centered in the blue wavelengths.

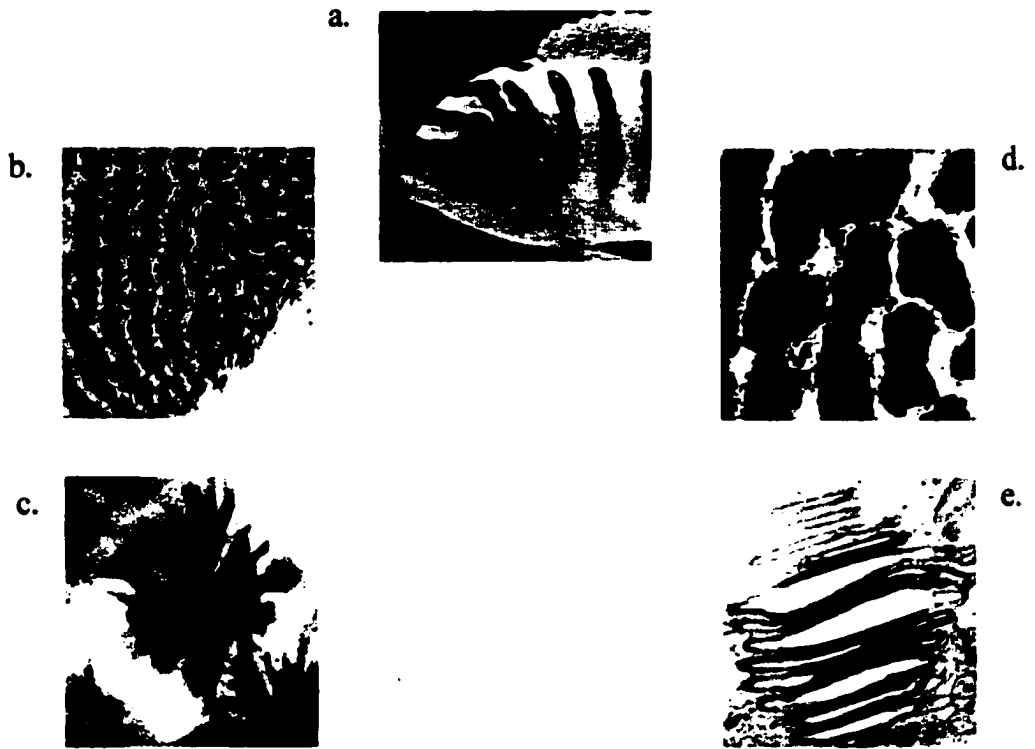
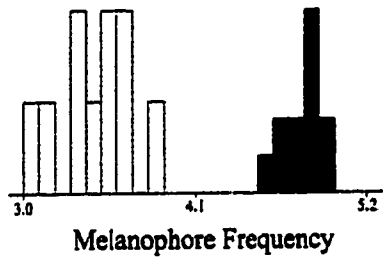


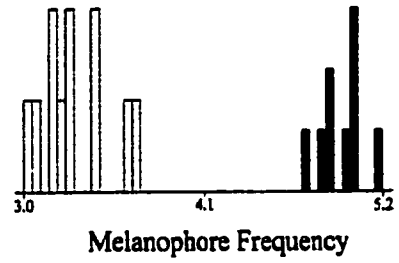
Figure 4.4 Melanophore frequency distributions in the parental,  $F_1$  and  $F_2$  generations for the barred areas and the pelvic fin. A. The distribution of melanophores in the barred areas of both parentals (*M. zebra*  $N = 13$ ; *M. benetos*  $N = 15$ ). B. The distribution of melanophore frequencies in the bars of the  $F_1$  ( $N = 10$ ). C. The distribution of melanophore frequencies in the bars of the  $F_2$  ( $N = 95$ ). D. The distribution of melanophores in the fins of both parentals (*M. zebra*  $N = 13$  ; *M. benetos*  $N = 15$ ). E. The distribution of melanophore frequencies in the fins of the  $F_1$  ( $N = 10$ ). F. The distribution of melanophore frequencies in the fins of the  $F_2$  ( $N = 95$  ). Note the intermediacy of the  $F_1$  and  $F_2$  distributions and the increase in the  $F_2$  variance.



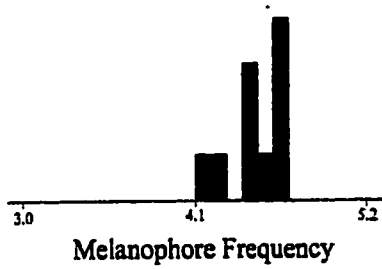
A.



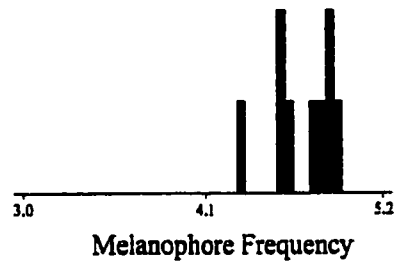
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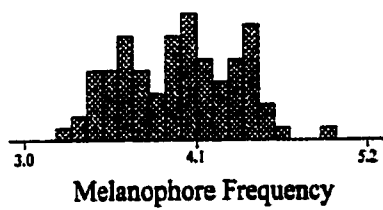
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E.



C.



F.

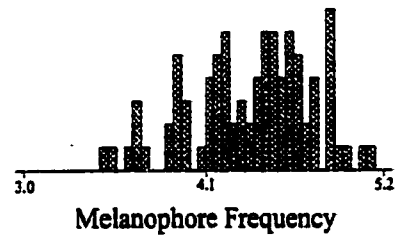


Table 4.1 Female mate choice in *M. zebra* and *M. benetos* . Females of both species were simultaneously presented with males of both species and their preferences were scored based on the time spent associating with each male and the number of times they entered a refuge nearest each male.

# p = 0.005

\* p = 0.046

Female	Male	Refuge		Time		
		<i>M. zebra</i>	<i>M. benetos</i>	<i>M. zebra</i>	<i>M. benetos</i>	
	<i>M. zebra</i>	6.5	0.5	570	45	#
	<i>M. benetos</i>	4	5	506	199	*

**Table 4.2 Chromatophore frequencies.** The mean (and variance) of melanophore and iridophore frequencies were quantified in five tissue types in four populations. Melanophore counts were natural log transformed prior to using t tests to identify significant differences. Iridophore frequencies were estimated based on the number of pixels which reflected incident light from a digital image of a 2.5 mm<sup>2</sup> area of tissue. Pixel counts x 10<sup>-3</sup> are presented. \* indicate that parental phenotypes were significantly different at p<0.001

<b>Population</b>	<b>N</b>	<b>Melanophore On Bar*</b>	<b>Melanophore Off Bar*</b>	<b>Melanophore On Fin*</b>	<b>Iridophore On Bar</b>	<b>Iridophore Off Bar</b>
<i>M. zebra</i>	13	4.81 (0.015)	4.42 (0.020)	5.04 (0.033)	29.72 (508315.13)	60.00 (1200788.45)
<i>M. benetos</i>	15	3.45 (0.055)	3.21 (0.071)	3.25 (0.061)	40.79 (280351.88)	47.78 (429096.81)
F <sub>1</sub>	10	4.48 (0.034)	NA	4.77 (0.054)	NA	NA
F <sub>2</sub>	95	4.00 (0.131)	NA	4.39 (0.147)	NA	NA

**Table 4.3** The biometric estimates of the effective number of genes influencing the number of melanophores on the boy bars and fins based on the Castle-Wright equations.

	<b>Number of Effective Factors</b>	<b>Variance</b>
Bar	2.13	1.4
Fin	5.0	10.0

**Table 4.4** The microsatellite used in the QTL analysis. Mbuna microsatellites were developed from *Metriaclima zebra* genomic DNA. Tilapia microsatellites were developed from *Oreochromis niloticus* genomic DNA.

	<b>Mbuna</b>	<b>Tilapia</b>	<b>Total</b>
<b>Number of Microsatellite Loci Tested</b>	51	82	133
<b>Number of Microsatellite Loci which were Polymorphic</b>	37	12	49
<b>Number of Microsatellite Loci with No Amplification</b>	12	48	60
<b>Number of Microsatellite Loci which were Fixed</b>	2	22	24

**Table 4.5 The genetic map of linked loci. Microsatellites with an \* were developed from tilapia genomic DNA.**

<b>Linkage Group 1</b>	
UNH2014	0 cm
UNH2018	14.3 cm
<b>Linkage Group 2</b>	
UNH2021	0 cm
UNH2016	4.4 cm
UNH2022	26 cm
<b>Linkage Group 3</b>	
UNH2040	0 cm
UNH2023	12 cm
<b>Linkage Group 4</b>	
UNH2044	0 cm
UNH2025	4.3 cm
<b>Linkage Group 5</b>	
UNH2049	0 cm
UNH2054	15.1 cm
UNH2051	28.3 cm
<b>Linkage Group 6</b>	
UNH2055	0 cm
UNH2035	21.2 cm
<b>Linkage Group 7</b>	
UNH2057	0 cm
UNH2059	2.4 cm
UNH2037	9.4 cm
<b>Linkage Group 8</b>	
UNH2058	0 cm
UNH2045	5.6 cm
<b>Linkage Group 9</b>	
UNH738*	0 cm
UNH154*	6.9 cm

**Table 4.6** The location and significance of the six QTL which influence male color patterns. \* The significance level presented here is not corrected for multiple comparisons. Empirically determined significance thresholds equaled Likelihood Ratio = 11.48 and 11.14 for fin and bar QTL, respectively, at the alpha = 0.05 level.

	Marker	Linkage Group	Likelihood Ratio	Sign. Level*	
<b>Fin</b>	QTL 1	UNH933	NA	8.37	0.004
	QTL 2	UNH2014	1	5.46	0.021
	QTL 3	UNH738	9	4.03	0.048
<b>Bar</b>	QTL 1	UNH2057	7	7.66	0.006
	QTL 2	UNH2012	NA	4.38	0.039
	QTL 3	UNH2037	7	2.46	0.021

**Table 4.7 QTL effects.** The mean number of melanophore frequencies for three classes of  $F_2$  are presented. Mean *M. zebra*/*M. benetos* represent the mean melanophore frequencies of those  $F_2$  individuals which are homozygous for either the *M. zebra* or *M. benetos* alleles. The mean melanophore frequency of  $F_2$  is also given as is an estimate of the dominance. Dominance values which are positive indicate that *M. zebra* alleles are dominant to *M. benetos* alleles. Dominance values greater than (less than) 1 (-1) indicate the action of overdominance.

Fin QTL					
	Marker	Mean <i>M. zebra</i>	Mean Heterozygote	Mean <i>M. benetos</i>	Dominance
Fin QTL 1	UNH933	4.47	4.39	4.27	0.209
Fin QTL 2	UNH2014	4.52	4.38	4.33	-0.519
Fin QTL 3	UNH738	4.32	4.33	4.50	0.894

Bar QTL					
	Marker	Mean <i>M. zebra</i>	Mean Heterozygote	Mean <i>M. benetos</i>	Dominance
Bar QTL 1	UNH2057	3.95	3.99	4.44	0.855
Bar QTL 2	UNH2012	4.03	4.05	3.73	1.170
Bar QTL 3	UNH2037	4.05	3.89	4.26	2.628



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