Species recognition and reproductive isolation in Malawi cichlid fishes, *Metriaclima estherae* and *M. callainos* (Teleostei: Cichlidae)

A thesis submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

at

University of the Western Cape

By

Nonhlanhla Precotia Nyalungu

October 2019

Declaration

The following thesis has not been submitted to a university other than University of the Western Cape, Bellville, South Africa. The work presented here is that of the author.

Acknowledgements

Firstly, I would like to thank my two supervisors Dr Vanessa Couldridge and Dr Adriaan Engelbrecht for their assistance throughout my thesis.

I want to thank the following people who assisted me with data analysis: Jabulile Malindi, Sisanda Mayekiso and Moqebelo Morallana. I also want to thank Rekha Sathyan, James Ayuk, Ayanda Zide, Olusola Saibu and Dr Monica Mwale for all their advice and input.

My mother Rally Nyalungu for encouraging and believing in me.

Lastly, I would like to thank the National Research Foundation (NRF) for their financial assistance.

List of Figures

List of Tables

Abstract

Cichlids are one of the most diverse groups of fishes in the world. The highest concentration of cichlids is found in Lake Malawi, where over 1500 species are recorded to occur, which are dominated by the haplochromine tribe. Several authors proposed that sexual selection has been a major contributor in the high rate of speciation of haplochromine cichlids. However, sexual selection alone may not be sufficient to fully explain high speciation rates among cichlids. Many closely related cichlids in Lake Malawi differ only in coloration, yet occur in sympatry. It is still not fully understood how they remain reproductively isolated. Previous studies suggest that visual cues are primarily used for reproductive isolation and species recognition. In the current study, visual, chemical and acoustic cues were investigated to observe how they may potentially influence species recognition and reproductive isolation between two closely related species, *Metriaclima estherae* and *M. callainos*, as well as between alternate colour morphs of *M. estherae*.

Female preference was measured by giving the female a choice between the males of red and blue *M. estherae* and *M. callainos*. Male preference was tested by giving the males a choice between *M. estherae* and *M. callainos* females. Acoustic cues of the males were recorded with a Dolphin Ear Pro hydrophone. Peak frequency and duration sounds were analysed with Raven Pro 1.4 Software. The genetic differences between the two species were investigated by amplifying mitochondrial control region, FISH L15926 and H00650.

The results demonstrate that *M. callainos* females use primarily visual cues for mate recognition whereas *M. callainos* males use both visual and chemical cues. Both *M. estherae* females and males showed no significant preference for their own species based on either visual or chemical cues and *M. estherae* females did not show any preference for either of the two different colour morphs of their conspecific males.

The results of the acoustic analysis demonstrated that there are inter- and intraspecific acoustic differences between *M. estherae* and *M. callainos*. This suggests that acoustic cues can potentially be used for species recognition as well as intraspecific mate choice.

The results of the molecular study confirm that there is genetic variation between *M. estherae* and *M. callainos* and also suggest that hybridisation occurs between the study taxa. Furthermore, there are morphological differences between the two species, between males and females, and also between the two male colour morphs of *M. estherae*.

Chapter 1. General Introduction

1.1 Ecology of African cichlid fishes

Cichlid fishes are spiny-rayed freshwater fish that have high colour diversity (Stiassny and Meyer, 1999). Unlike most fishes, they have a single nostril on each side of the head (Fryer and Iles, 1972). Cichlids are found in Africa, Madagascar, Southern India, Sri Lanka and in South and Central America (Stiassny and Meyer, 1999). Most of these regions were part of the southern continent of Gondwana, which fragmented ~180 million years ago (Stiassny and Meyer, 1999). The Jordan Valley in the Middle East has four species of cichlids, Iran has only one species, Southern India and Sri Lanka have three species, with some in brackish water, Madagascar has 17 species, some also in brackish water, Cuba and Hispaniola have four species, some also in brackish water, North America and Central America have 111 species, and South America has 291 species (Kullander, 1998). The largest number of cichlid species are found in East African Lakes; Lake Tanganyika, Lake Malawi and Lake Victoria, where more than 2000 species are currently endemic (Genner *et al*., 2004) (Figure 1.1). Lake Tanganyika is between 5 - 20 million years old and has ~160 - 185 endemic cichlid species (Genner *et al*., 2004). Lake Malawi is between 2 - 5 million years old (Turner, 1999) and has ~1500 endemic species (Genner *et al*., 2004). Lake Victoria, which is believed to have partially or completely dried up 14500 years ago, has ~ 500 endemic species (Genner *et al*., 2004).

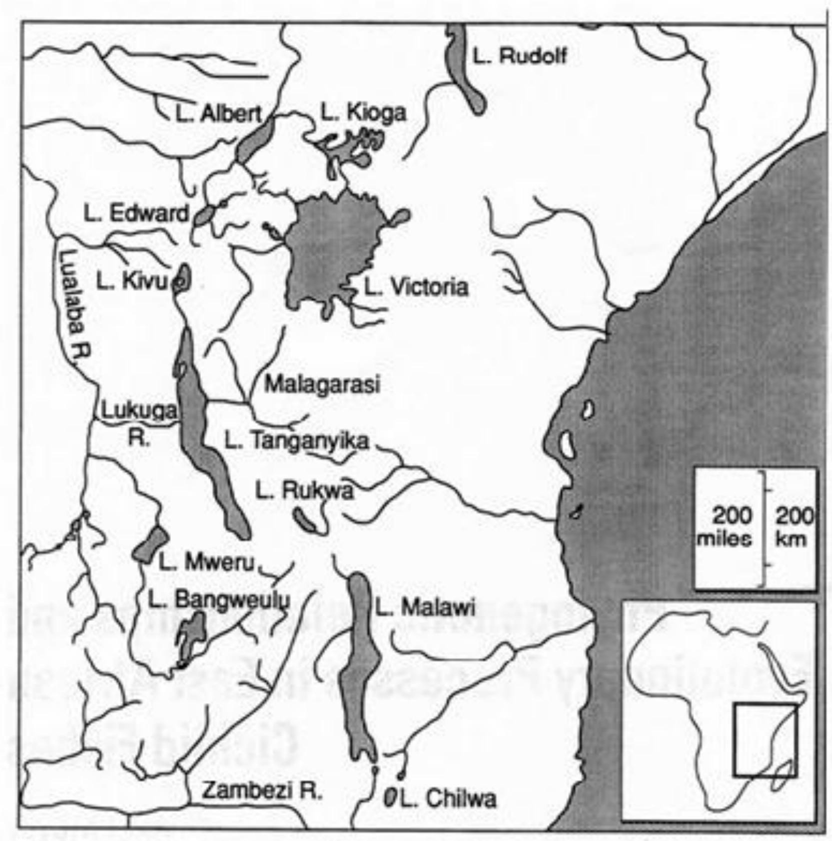

Figure 1.1: Map showing the three Great lakes of East Africa (Lake Tanganyika, Malawi and Victoria) (Meyer, 1993).

Cichlids generally occur in shallow waters and are absent at depths of > 100 - 200 m in Lake Tanganyika and Malawi (Kornfield and Smith, 2000). They are found in one of three major ecological guilds; rocky habitats, sandy habitats and pelagic (Moran *et al*., 1994). In all three lakes, additional habitat specialization occurs, where some species inhabit swamps, abandoned shells and sheltered rocks (Sato and Gashagaza, 1997). Cichlids possess a great diversity of feeding habits, including phytoplankton, deposit, algal, leaf choppers, mollusc, zooplankton, piscivorous, insect and benthic feeders (Fryer and Iles, 1972).

1.2 Breeding behaviour of cichlid fishes

Several species of African cichlids are territorial and males defend their territories against conspecific males (Fryer and Iles, 1972). Males of some cichlid species form territories in rocky substrates where spawning takes place (Fryer and Iles, 1972). Others spawn on sandy substrates, where males build a nest and defend it (Yanagisawa *et al*., 1997; Tweddle *et al*., 1998). Some cichlid species of Lake Tanganyika are substrate guarders, where the young help in taking care of the nest (Sato *et al*., 1984; Gashagaza, 1991). Breeding behaviour of cichlids includes bi-parental care and single mouth-brooding care by either a male or female (Fryer and Iles, 1972). Mouth-brooding care by females is the most common reproductive character for most African cichlids (Fryer and Iles, 1972). During spawning, males perform displays to attract females. The female will then follow a male to its territory, where she deposits one or several eggs, and when the eggs are fertilized, they are immediately deposited in her mouth (Kornfield and Smith, 2000). Some eggs of the rock-dwelling cichlids, such as those found in Lake Malawi, could be fertilized by multiple males (Fryer and Iles, 1972). Microsatallite-based paternity studies indicated that up to six males can fertilize a clutch from a single female's mouth (Kellog *et al*., 1995; Parker and Kornfield, 1996).

1.3 Study species

1.3.1 Taxonomy of the study taxa

All cichlid fish fall under the family Cichlidae (Fryer and Iles, 1972). Taxonomically, four subfamilies of cichlids are recognised: Etroplinae (India and Madagascar), Ptychochrominae (Madagascar), Cichlinae (Neotropical region) and Pseudocrenilabrinae (Africa) (Sparks and Smith, 2004; Smith *et al*., 2008). Molecular data suggests that the species flock of East

African lakes forms a single lineage, haplotilapiines (Schliewen and Stiassny, 2003; Schwarzer *et al*., 2009; Dunz and Schliewen, 2013).

The tilapiine (Trewavas, 1983) and the haplochromine (Poll, 1986) tribe are two major tribes found in Lake Malawi (Turner, 1996). The haplochromine tribe is the most abundant and has ~850 species, with only 400 being formally described (Konings, 1990). The haplochromine tribe can be further divided into three major lineages; the rock-dwelling cichlids, also known as mbuna cichlids, the pelagic cichlids and the sand dwelling cichlids (Stauffer *et al*., 1997). The mbuna tribe has 13 genera: *Abactochromis, Cyathochromis*, *Cynotilapia, Genyochromis, Gephyrochromis, Iodotropheus, Labeotropheus, Labidochromis, Petrotilapia*, *Melanochromis*, *Metriaclima*, *Tropheops*, and *Pseudotropheus* (Trewavas, 1935; Oliver and Loiselle, 1972; Stauffer *et al*., 1997; Oliver and Arnegard, 2010). The term mbuna has no formal taxonomic validation; it is derived from the Chitumbuka language, which is spoken in northern Malawi (Genner *et al*., 2005). Konings (2001) listed 295 mbuna species in a field guide. The mbuna cichlids are recognised by having small scales on their nape and chest, although this characteristic is also found in other haplochromine cichlids (Trewavas, 1935). They are also recognised by a reduction of the left ovary, an abrupt change from large flank scales to small chest scales and possession of true ocelli (Fryer, 1959; Oliver, 1984). Most mbuna species are sexually dimorphic, their males are larger, and have larger pelvic and dorsal fins and exhibit brighter colours (Genner and Turner, 2005). Both sexes of the mbuna are polygamous (Genner and Turner, 2005).

1.3.2. Reclassification of the genus of the study species

The study taxa, *Metriaclima estherae* (Konings, 1995) and *M. callainos* (Stauffer and Hert, 1992) belong to the mbuna haplochromine tribe of Lake Malawi. Previously, both these species were listed in the genus *Pseudotropheus* (Regan, 1922). The genus *Pseudotropheus* initially had 30 species and the genus was subdivided into six species complexes, based on morphology and behaviour (*P. elongatus*, *P. tropheops*, *P. williamsi*, *P. zebra*, *Pseudotropheus* "aggressive" and *Pseudotropheus* "miscellaneous") (Ribbink *et al*., 1983). A new subgenus of *Pseudotropheus* (*Maylandia*) was proposed by Meyer and Foerster (1984) for the members of the *P. zebra* complex, and Trewavas (1984) proposed the subgenus *Pseudotropheus* (*Tropheops*) for members of the *P. tropheops* complex. However, Stauffer *et al*. (1997) declared the subgenus *Maylandia* to be a *nomen nudum* and established the new genus *Metriaclima* for most of the species of *Maylandia*. Later, Conde and Gery (1999) claimed that *Maylandia* was a senior synonym of *Metriaclima*, but Geerts (2002) disputed their argument. However, recent publications have adapted to the subgenus *Metriaclima* for these species (Konings, 2016; Stauffer *et al*., 2016). Therefore, the genus *Metriaclima* will also be adapted for the current study.

1.3.3 Description of the study species

Both *M. estherae* and *M. callainos* are endemic to Lake Malawi (Kasembe, 2017). *Metriaclima estherae* comes in several colour morphs (Table 1.1, Figure 1.2). The males and females of *M. callainos* are blue, but some females are also whitish (Genner *et al*., 2005, Kasembe, 2017). Both species resemble each other in morphology and in coloration of the blue morph (Stauffer and Hert, 1992). They can be distinguished from each other by the fact that the jaws of *M. estherae* do not completely close the gape, their teeth are always visible, and they have thicker lips. The body shape of *M. callainos* is also more laterally compressed than *M. estherae* (Konings, 2001). Males of *M. estherae* can be further recognised by having yellow oscillated spots on the dorsal fin (Konings, 2001). Both species are found in rocky, sediment free habitat with shallow water (Konings, 2001). They both feed by scraping off loose algae from the rocks (Kasembe, 2017). *Metriaclima callainos* occurs naturally in Nkatha Bay and was introduced at Likoma, Namalenje and Thumbi West Island (Kasembe, 2017) (Figure 1.3). They can attain a maximum length of 8.01 cm (Kasembe, 2017). *Metriaclima estherae* is found in Minos Reef, Chilucha Reef, Metangula, Nkhungu and Masinje and has a maximum standard length of 9.77 cm (Kasembe, 2017) (Figure 1.3). Both species are listed as vulnerable on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species. *Metriaclima estherae* used in the current study were collected from Minos Reef while *M. callainos* were collected from Chidunga Rocks (Figure 1.3).

Table 1.1: Different colour morphs of *M. estherae*.

Morphs	**Description (Colour)**	**Sex**
Orange (O)	Orange	Males and females
Orange blotches (OB)	Dark blue blotches on a pinkish, yellowish or orange body	Males and females
Blue body (BB)	Light blue with dark blue bars on body	Males

Figure 1.2: The study taxa, blue *M. estherae* (A), red *M. estherae* male (B), *M. estherae* female (C), *M. callainos* male (D) and *M. callainos* female (E).

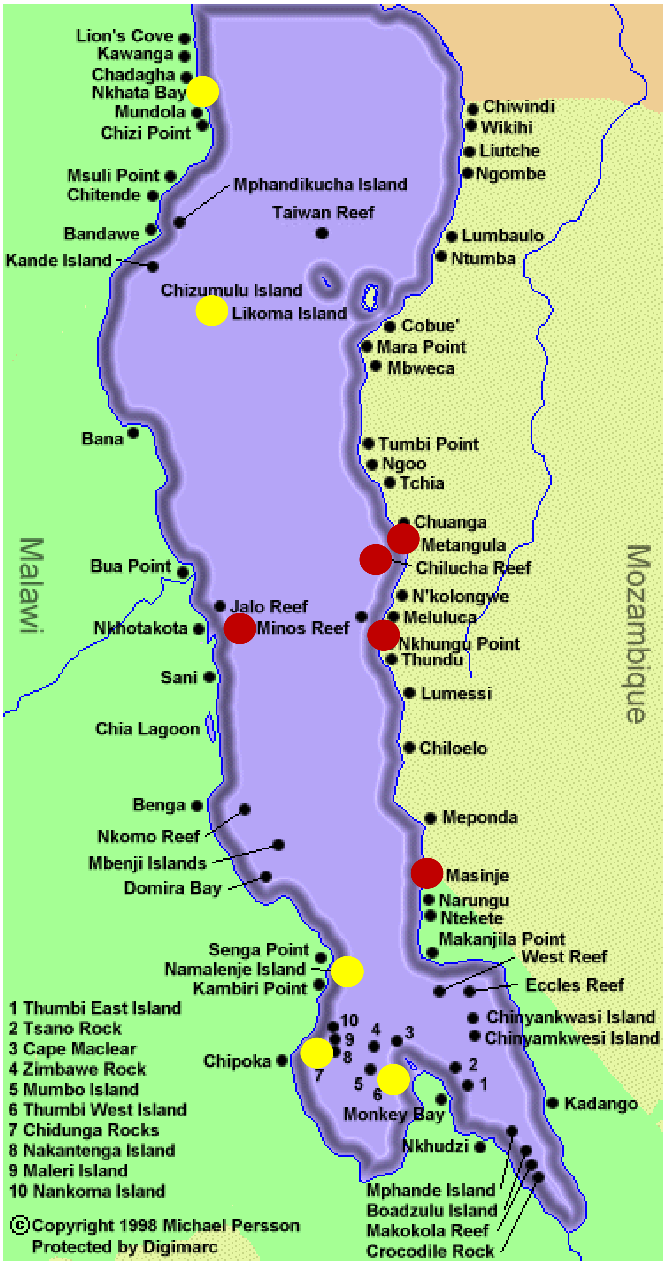

Figure 1.3: Map of Lake Malawi showing the location where the fish were collected, Minos Reef and Chidunga Rocks, as well as other documented localities (©Michael Persson 1997-2006). Yellow = *M. callainos* sites, red = *M. estherae* sites.

1.4 Lake Malawi

Lake Malawi is the southern most of the great African lakes (Lewis *et al.*, 1986). It is a rift lake which lies in a 3000 km splint of the Earth's crust known as the Western Rift Valley (Lewis *et al.*, 1986). The age of the lake is estimated to be between 2 - 5 million years old (Johnson and Ng'ang'a, 1990; Delvaux, 1995). Lake Malawi is 570 km long, and has a surface area of 30,800 km^2 (Delvaux, 1995). This makes Lake Malawi the ninth largest lake in the world (Konings, 1990) and third largest in Africa (Delvaux, 1995). Lake Malawi is relatively narrow, at 60 km wide, and deep, with a maximum depth of 704 m (Fryer and Iles, 1972). The southern parts of the lake are shallow with a depth of less than 100 m. In the lake, sandy shores alternate with rocky shores (Konings, 1990). The lake consists mostly of Pre-Cambrian metamorphic rocks and also Pre-Cambrian and lower Palaezoic granitic syenitic plutons, though less common (Owen *et al.*, 1999). Volcanic rocks are found at the northern end of the lake, in the Rungwe volcanic field (Owen *et al.*, 1990).

Temperatures of the lake follow a seasonal pattern with temperatures of between 15 ºC and 23 ºC being recorded in the winter months and temperatures of between 25 ºC and 40 ºC recorded in the summer months (Konings, 1990). However, the difference between surface waters and deeper waters is very small (Konings, 1990). Wave lengths of between 3 - 4 m are common in the lake during windy weather and the lake level may rise up to 2 m during rainy seasons (Konings, 1990). Lake Malawi generally supports clear waters with visibility of up to 20 m in some parts of the lake. Water pH is constant throughout the year and varies between pH 7.8 and pH 8.5. Life in the lake is restricted to the upper 200 m because vertical circulation does not occur and dissolved oxygen is absent beyond this layer (Konings, 1990). Lake Malawi is sensitive to climatic changes due to the small dimension of the hydrological catchment area and the subtropical climate, with long dry seasons often resulting in water level fluctuations (Delvaux, 1995). Lake Malawi was affected by a dry climate in the late Pliocene/early Pleistocene, ~ 0.57 - 1.6 MYA, causing the lake level to drop by 250 - 500m compared to its present level (Delvaux, 1995; Cohen *et al*., 2007). There is evidence to suggest that this could have even occurred as recently as 25 000 to 135 000 years ago, causing most of the southern side of Lake Malawi to be completely dry (Scholz and Rosendahl, 1988).

1.5 Lake Malawi cichlid fishes

Molecular data suggests that the mbuna flock of Lake Malawi may have evolved ~700 000 years ago from a single common ancestor (Meyer *et al*., 1990). Currently, Lake Malawi is estimated to have ~1500 species of cichlid fish (Turner *et al*., 2001), with only five species belonging to the tilapiine family (Turner, 1996) and the rest to the haplochromine group, with only two species not endemic to the lake (Turner, 1996). Several factors are thought to be behind the rich diversity of the cichlid fishes. One important factor is their anatomy; cichlids are the only fishes in the world that possesses two sets of jaws, one in the mouth used to suck, scrape or bite food and the other in the throat to crush, macerate, slice or pierce morsels before ingestion (Stiassny and Meyer, 1999). These sets of jaws are able to adapt according to different types of food, enabling each species to occupy specific niches and allowing them to co-exist without competition (Stiassny and Meyer, 1999). The second factor has to do with their mouth brooding characteristics, where females keep the fertilized eggs in their mouth (Fryer and Iles 1972, Stiassny and Meyer, 1999). This provides the offspring protection from predators, thus providing a better chance of survival (Stiassny and Meyer, 1999). Water level fluctuations in the lake are also thought to provide opportunity for recolonization of new islands by random individuals that may diverge to form new taxa (Greenwood, 1965; Kaufman, 1997). Other factors include water characteristics and lake age (Ladiges, 1968), habitat complexity (Temple, 1969), predation (Greenwood (1965), hybridization (Capron de Caprona and Fritzsch, 1984) and trophic polymorphism (Sage and Selander, 1975). It is also thought that sexual selection has also contributed to the divergence of these fishes (Dominey, 1984).

1.6 Sexual selection and speciation in African cichlid fishes

Sympatric species of Lake Malawi haplochromine cichlids differ considerably in male colour (Konings, 2007), whereas female colour is often similar (Lande, 1981). The body colour of males is thought to play a role in social communication, mate choice and foraging, and is subjected to strong sexual section by females (Maan and Sefc, 2013). Additionally, haplochromine cichlids have polygynous mating systems with exclusive female parental care; this results in strong mate selection by females (Maan and Sefc, 2013). It is now widely accepted that sexual selection by females acting on male colour is one of the main driving forces responsible for speciation in African cichlids (Dominey, 1984; Seehausen *et al*., 1997; Salzburger, 2009; Sobel, 2010; Maan and Seehausen, 2011; Wagner *et al*., 2012; Maan and Sefc, 2013; Lackey *et al*., 2018). It is thought that sexual selection leads to speciation through the evolution of male signals and that female mating preference for those signals may result in assortative mating and thus reproductive isolation (Lande 1981; Seehausen *et al*., 1997; Coyne and Orr, 2004; Salzburger, 2009; Wagner *et al*., 2012). Sexual selection is thought to cause divergence among populations and the rate of divergence may be greater compared to allopatric speciation without sexual selection (Masta and Maddison, 2002).

Sexual selection results in a rapid divergence of sexually dimorphic traits associated with the acquirement of a mate (Darwin, 1871; Fisher, 1930) and many of these traits are thought to be involved in mate recognition and assortative mating between closely related species (Panhuis *et al*., 2001; Coyne and Orr, 2004). Studies demonstrate that sexual selection can also promote speciation within a single population of African cichlids (Panhuis, 2001; Coyne and Orr, 2004; Ritchie, 2007).

1.7 Conservation and management of African cichlids

Freshwater systems in Africa have a high level of fish diversity and are important to both livelihood and economy of the continent (Dugeon *et al*., 2011). Recently, there has been an increase in development and agriculture close to water bodies. The aim of these developments is to support and improve the livelihood of the communities and they are projected to double by 2050 due to the increase in the number of people (Dugeon *et al*., 2011). However, it is thought that these developments are not well represented in the development planning process and they do not always comply with species conservation within inland waters (Dugeon *et al*., 2011). Additionally, freshwater fishes are recognised as an important source of food for Africans and represent 45 % of harvested food sources on the continent (Dudgeon *et al*., 2011). Overfishing has been reported as the major reason for the decline of the larger cichlid fish in the continent, especially in Lake Victoria (Goudswaard and Ligtvoet, 1988) and Lake Malawi (Tweddle *et al*., 1995). Major threats to African freshwaters include: habitat degradation, agriculture infrastructure development, unsustainable water extraction, water pollution from domestic and agricultural sources, and introduction of alien species, sedimentation and mining (Dudgeon *et al*., 2011).

1.8 Major threats of Lake Malawi cichlids flocks

Lake Malawi supports livelihood by providing transport, a reservoir of freshwater and a source of food for the people (Lewis *et al*., 1986). Approximately 40 000 tonnes of fish are harvested from the lake annually and over 16 000 people make their livelihood from fishing in the lake (Lewis *et al*., 1986). A survey by Snoeks *et al*. (2011) concluded that Lake Malawi has the highest number of threatened fish species with 105 endangered species (contributing 28 % of the total species assessed species estimated to be in the lake). The high level of restricted niches and low numbers of offspring production are thought to make them vulnerable to extinction (Snoeks *et al*., 2011). Additionally, the mbuna species grow slowly, are extremely vulnerable to degradation and recover slowly from population declines (Ribbink, 2001; Thieme *et al*., 2005). Lake Malawi is further impacted by water level fluctuations (Owen *et al*., 1990). This may result in extinction of some species, especially those that are adapted to particular niches (Owen *et al*., 1990). Lake Malawi National Park was established in 1980, to conserve the freshwater fish, particularly the mbuna fish (Hough, 1989). The park covers 9,400 ha and is divided in four conservation zones: special zone, wilderness zone, natural zone and general zone (Glenfell, 1993). The islands and lacustrine areas are designated wilderness zones where no fishing is permitted, while terrestrial areas are designated natural zones. The Park was declared a UNESCO World Heritage site in 1984 (Hough, 1989). A management fishing zone was established 2 km off the mainland shoreline where fishing is prohibited. The major conservation concerns in the park are the possible impact of introduced fish species on natives and illegal fishing by local people around the park area (Croft, 1981).

In conclusion, the establishment of parks such as the Lake Malawi National Park will assist in conserving cichlids. Furthermore, proper development and management of parks and protected areas that specifically address the issues of conservation for lakes and wetlands are required for the conservation of African cichlids (Darwall *et al*., 2011).

1.9 Rationale

Several previous studies suggest that sexual selection is a primary driver of speciation in African cichlids (Carson, 2003; Jordan *et al*., 2003; Knight and Turner, 2004; Maan *et al*., 2004; Salzburger *et al*., 2006; Pauers *et al*., 2010; Egger *et al*., 2010; Sobel *et al*., 2010; Maan and Seehausen, 2011). However, sexual selection can also lead to differences in male traits without resulting in speciation (Price, 1998). This was demonstrated in occasions where signal transmission evolved in order to maximise detectability in different environments (Price, 1998). An additional previous study demonstrated that differences in traits such as male colour are not always related to mate choice, even where sexual selection has been implicated (Llopart *et al.*, 2002). Seehausen and van Alphen (1999) further suggested that sexual selection alone is not sufficient to explain the rapid speciation of African cichlid fishes. To demonstrate that sexual selection is driving speciation, studies must provide evidence that the traits responsible for reproductive isolation between closely related species evolved under sexual selection within species (Lande, 1981; Boeke, 2002).

Despite being the focus of much attention, there is still insufficient information regarding the cues used to maintain reproductive isolation in closely related species of cichlids (Knight and Turner, 1999). Verzilden *et al*. (2010) suggest that it is important to take into account all modes of communication in order to understand how mating decisions can influence sexual selection and reproductive isolation. Multiple cues such as visual (Genner and Turner, 2005; Seehausen *et al*., 2008), acoustic (Rollo and Higgs 2008, Verzijden *et al*. 2010), and chemical

(Plenderleith *et al*., 2005; Blais *et al*., 2009) are thought to play a role in reproductive isolation. Furthermore, it is thought that mate choice experiments based on a single cue may not be sufficient to demonstrate reproductive isolation among closely related species (Blais *et al*., 2009). To date, most studies on mate selection have focused on visual (colour) cues as the main driver of sexual selection and reproductive isolation among closely related species of haplochromine cichlids (Seehausen and Alphen, 1998; Knight and Turner, 1999; Couldridge and Alexander, 2002; Knight and Turner, 2004; Maan *et al*., 2004). Acoustic (Amorim 2004, 2008; Verzilden *et al*., 2010) and chemical (Pleinderleith *et al*., 2005; Blais *et al*., 2009) cues have received far less attention. There has also been very little focus on preferences for alternative colour morphs within the same species, despite this being a proposed catalyst for subsequent divergence. The current thesis aims to investigate species recognition and reproductive isolation between *Metriaclima estherae* and *M. callainos* and also to investigate their genetic and morphological differences.

1.10 Research Objectives

Haplochromine cichlids provide a good model to study evolutionary processes due to their high ecological, behavioural and phenotypical diversity and because of their young age (Seehausen, 2006; Salzburger, 2009; Sturmbauer *et al*., 2011). Because our study taxa possess only minor morphological and behavioural variation (Staufer and Hert, 1992; Konings, 1999), this makes them perfect candidates to study reproductive isolation among closely related species. The aim of this study was to investigate cues that may promote reproductive isolation and species recognition between two closely related species, and between alternative colour morphs of the same species. Experimental mate choice trials based on visual, sound and chemical cues were employed to assess species recognition in *M. estherae* and *M. callainos*. Previous studies have successfully used molecular techniques to resolve the genetic relationship and population structure of the mbuna cichlids (Bowers *et al*., 1994; Reinthal and Meyers, 1997; Shaw *et al*., 2000; Market *et al*., 2001). Some previous studies used morphology to identify mbuna species (Fryer, 1959; Oliver, 1984). Due to similarities between the study taxa, molecular techniques and morphological measurements were employed to eliminate any doubt regarding their taxonomic status.

1.11 Structure of the thesis

Each chapter of this thesis will be independent and will have its own introduction, materials and methods, results and discussion. If the same materials and methods are used, the initial methods will be referred to. The thesis will be structured as follows:

Chapter 1: General Introduction. The literature review and background to the study is discussed in detail. The conservation of African cichlids is also discussed in this chapter.

Chapter 2: Female mate choice and species recognition between two closely related cichlid fish of Lake Malawi, *Metriaclima estherae* and *M. callainos*. Multiple cues are thought to facilitate mate recognition and reproductive isolation (Partan and Marler, 1999; Rowe, 1999). In this chapter, a combination of visual and chemical cues was used to investigate mate selection and species recognition between *Metriaclima estherae* and *M. callainos*.

Chapter 3: The role of males in maintaining reproductive isolation and species recognition in *Metriaclima estherae* and *M. callainos*. Previous studies on mate choice selection focused on female choice acting on male colours as the main driver of speciation (Seehausen and van Alphen, 1998; Couldridge and Alexander, 2002; Maan *et al*., 2004). However, not much is known about the role of males in mate selection and species recognition. Females of many species are brightly coloured too, having either similar or contrasting colours to their conspecific males. Male mate choice and species recognition is investigated in this chapter.

Chapter 4: Inter- and Intra-specific acoustic differences between *Metriaclima estherae* and *M. callainos.* Previous studies found that sounds that are produced by cichlids may be used in species recognition (Amorim, 2004; 2006). The characteristics of acoustic cues produced by *Metriaclima estherae* and *M. callainos* were investigated.

Chapter 5: Genetic and morphological differences between *Metriaclima estherae* and *M. callainos*. The genetic and morphological differences between *Metriaclima estherae* and *M. callainos* are investigated in this chapter.

Chapter 6: General Discussion. General discussion and suggestions about future studies are presented in this chapter.

Chapter 2. Female mate choice and species recognition between two closely related cichlid fish of Lake Malawi *Metriaclima estherae* and *M. callainos*

Abstract

Cichlids are one of the most diverse and colourful groups of freshwater fishes in the world. Female choice acting on male colour is thought to be a major contributing factor to their rich diversity. Colouration among the males of these fishes differs considerably whereas females often have similar coloration. The aim of this study was to examine cues that may drive speciation in African cichlids by using two closely related species, one of which exists as two different male colour morphs. Female preference was tested by giving females a choice between conspecific and heterospecific males, by employing visual cues, a combination of chemical and visual cues, and chemical cues only. The results demonstrate that females of *M. callainos* were able to recognise their conspecific males by using visual or chemical cues. However, females of *M. estherae* were unable to recognise conspecific males. These results suggest that colour alone may be sufficient to promote speciation in some cichlid fish, but not others, and that even closely related species may differ in their discriminatory abilities.

2.1 Introduction

The mbuna (rock-dwelling) cichlids are small colourful group of fishes that occupy the rocky shores of Lake Malawi (Genner *et al*., 2004). There are estimated to be over 400 endemic mbuna species in Lake Malawi, but with only 295 currently described species (Konings, 2001; Konings, 2007). This tribe consists of many sympatric closely related species that share similar morphology and ecology but differ significantly in colouration (Seehausen *et al*., 1997; Seehausen and van Alphen, 1998; van Oppen *et al*., 1998).

While males are very brightly coloured, the body colour of mbuna females is usually less conspicuous compared to the males (Lande, 1981; Maan and Sefc, 2013). It is thought that male colour is an important trait that drives interspecific and intraspecific sexual selection and speciation in these fishes (Jordan *et al*., 2003; Knight and Turner, 2004; Maan *et al*., 2004; Salzburger *et al*., 2006; Stelkens and Seehausen 2009; Egger *et al*., 2010; Pauers *et al*., 2010; Pauers and McKinnon, 2012; Maan and Sefc, 2013; Selz *et al*., 2013; Tyers and Turner, 2013).

However, it was demonstrated that in some cases female preference for male colour is insufficient to account for speciation processes (Ryan and Rand, 1993). To demonstrate that sexual selection is driving speciation, some authors suggest that studies must provide evidence that the sexually selected traits are also used as cues in mate recognition (Boake *et al*., 1997; Boake, 2002). Some studies further suggest that multiple cues involving all forms of sensory modalities must be considered, in order to understand how mating decisions can influence sexual selection and reproductive isolation (Verzilden *et al*., 2010; Hohenlohe and Arnord, 2010; Hebets, 2011). There is evidence to suggest that cichlids are able to use a variety of traits such as colour, territory quality, male size, pattern symmetry, courtship behaviour, length and condition of fins, courtship sounds and chemical cues for species recognition (Genner and Turner, 2005; Genner *et al.* 2008; Young *et al.* 2009).

Not much is known about the cues used for mate recognition and reproductive isolation among closely related cichlid species and the importance of mate selection in the evolution of new species (Knight and Turner, 1999; Maan *et al.*, 2004). It is important to understand the evolutionary processes that are responsible for the rapid divergence of these fishes (Knight and Turner, 1999). Few studies have focused on chemical cues as driver of sexual selection and reproductive isolation in African cichlids (Plenderleith *et al.*, 2005; Blais *et al.*, 2009). The aim of this study was to investigate cues that may promote reproductive isolation and species recognition among closely related cichlid species. We hypothesize that female cichlids use both visual and chemical cues for species recognition and reproductive isolation. Two closely related species, *Metriaclima callainos* (Stauffer and Hert, 1992) and *M. estherae* (Konings, 1995) were used to test this hypothesis. Female mate choice was tested by giving females a choice between heterospecific males and a choice between different colour morphs of conspecific males.

2.2. Materials and methods

2.2.1. Study species and housing

Metriaclima callainos and *M. estherae* are endemic to Lake Malawi. *Metriaclima estherae* females are orange-beige and orange with no vertical bars. The males of *M. estherae* come in orange-red and blue morphs. Female and male *M. callainos* are blue with no vertical bars. The fish were sourced from a South African importer. Only first (F1) and second (F2) generation fish from Lake Malawi, Chidunga Rocks and Minos Reef were used for the experiments (Figure 1.3). Different fish species were kept separately in 200 litre tanks. A layer of gravel was placed to cover the bottom of the tank and rocks provided for shelter and territories. Water quality was maintained by performing a 20 % weekly water change and by adding 10 g of Lake Malawi cichlid pH buffer. Water temperature was maintained at 26 ºC with 300 Watts (W) Life-tech Aquarium aqua heaters and pH maintained between 7.8 and 8.5. The tanks were filtered with Aqua Clear filters. The laboratory ceiling was fitted with a 40-W white fluorescent lighting bulb on a 12:12 hour light: day cycle. Fish were fed daily on algae flakes (protein 46.0 %, crude %, oil and fats 12.05 % and fibre 3.0 %).

2.3. Measuring female preference between males of red and blue *M. estherae* and blue *M. callainos*

2.3.1. Experimental protocol

The methods of the current study were modified from Couldridge and Alexander (2002) and Amorim *et al*. (2008). Only adult fish showing signs of sexual maturity were selected for all experiments. The current study did not involve actual mating. A previous study demonstrated the amount of time females spend with males is a reliable method for investigating mate choice (Couldridge and Alexander, 2001). Female preference was tested by using (a) visual cues only, (b) visual and chemical cues and (c) chemical cues only. The experimental tanks (90×30×30 cm) were divided equally into three compartments. For the visual cues experiments, the experimental tank was divided by a clear sealed Perspex box. For the visual and chemical cues experiment the tank was divided with a clear perforated Perspex box and for the chemical cues experiments, the tank was divided by a blue perforated Perspex box. The blue Perspex box was used to obscure the colour of the fish, thus allowing the presence of chemical cues only. Female preference was measured by giving the female a choice between males of red *M. estherae* and blue *M. callainos*, males of blue *M. estherae* and *M. callainos* and males of red and blue *M. estherae*. A female was placed in the middle compartment. Males of different species were placed in the side compartments and were provided with terra cotta pot to act as territory. The middle compartment was further divided into three equal zones, with the amount of time the female spent in each section recorded as a measure of preference. After the female was introduced in the tank, she was given 15 minutes to acclimatize. Each session was recorded with a Sony camcorder for 30 minutes. Ten replicates were conducted for each experiment with different individuals each time. All fishes were sized matched before each trial, by measuring Total length (TL) to the nearest 0.01 millimetre (mm) with a Vernier digital calliper, and weighed (g) with a microbalance scale before each trial. The amount of time the female spent with each male was recorded from the videos.

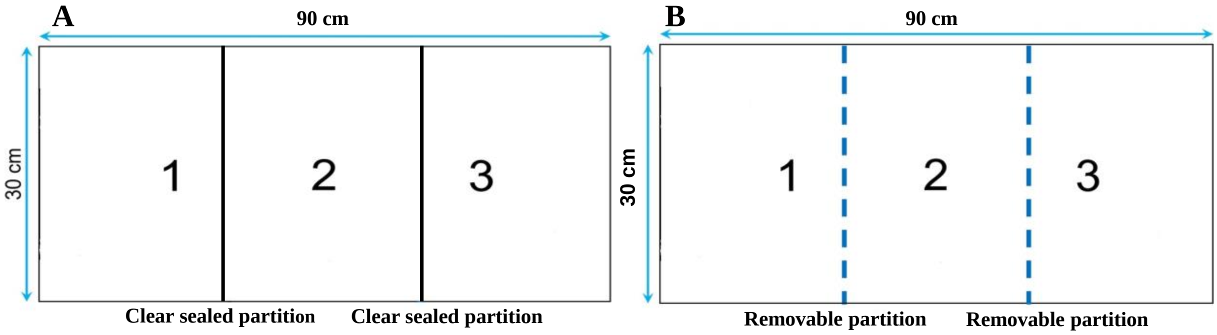

Figure 2.1: Experimental tank divided with a clear sealed partition (A) and an experimental tank divided with a perforated removable partition (B). Diagram modified from Gibelli *et al.*, 2018.

2.4. Statistical analysis

Data was first tested for normality using Shapiro-Wilk's W test and for homogeneity of variances using Levene's test. Repeated measures ANOVA (Analysis of Variance) was used to assess differences in the average time the females spent in each zone, followed with a *post-hoc* test (Fishers LSD) to test for significant differences. All statistics were carried out using Statistica (StaSoft Inc, 2005).

2.5. Results

2.5.1. Red *M. estherae* versus blue *M. callainos*

Average TL (mm) and weight (g) of the fish used for these experiments are presented in Appendix 1 (Table 8.1 - 8.6). There was a significant difference in the amount of time female *M. callainos* spent with the males in the presence of visual cues only ($F_{2, 27}$ = 23.50 p < 0.001, Figure 2.2 a), visual and chremical cues ($F_{2, 27}$ =14.03, p < 0.001, Figure 2.2 b) and chemical cues only ($F_{2, 27}$ = 35.65, p < 0.001, Figure 2.2 c). *Post-hoc* tests show that *M. callainos* females spent more time with conspecific males.

There was a significant difference in the amount of time *M. estherae* females spent between the two males when only visual cues were present ($F_{2, 27}$ = 6.26, p < 0.001). However, a *post-hoc* test revealed that the females spent less time in the centre compartment and an equal amount of time near the two males (Figure 2.2 d). In the presence of both visual and chemical cues, *M. estherae* females likewise spent an equal amount of time with both males ($F_{2, 27}$ = 8.13, p = 0.002; *Post-hoc*, Fishers LSD, Figure 2.2 e). In the presence of chemical cues only, *M. estherae* females also spent equal time with both males ($F_{2, 27}$ = 22.69, p < 0.001 (*Post-hoc*, Fishers LSD, Figure 2.2 f).

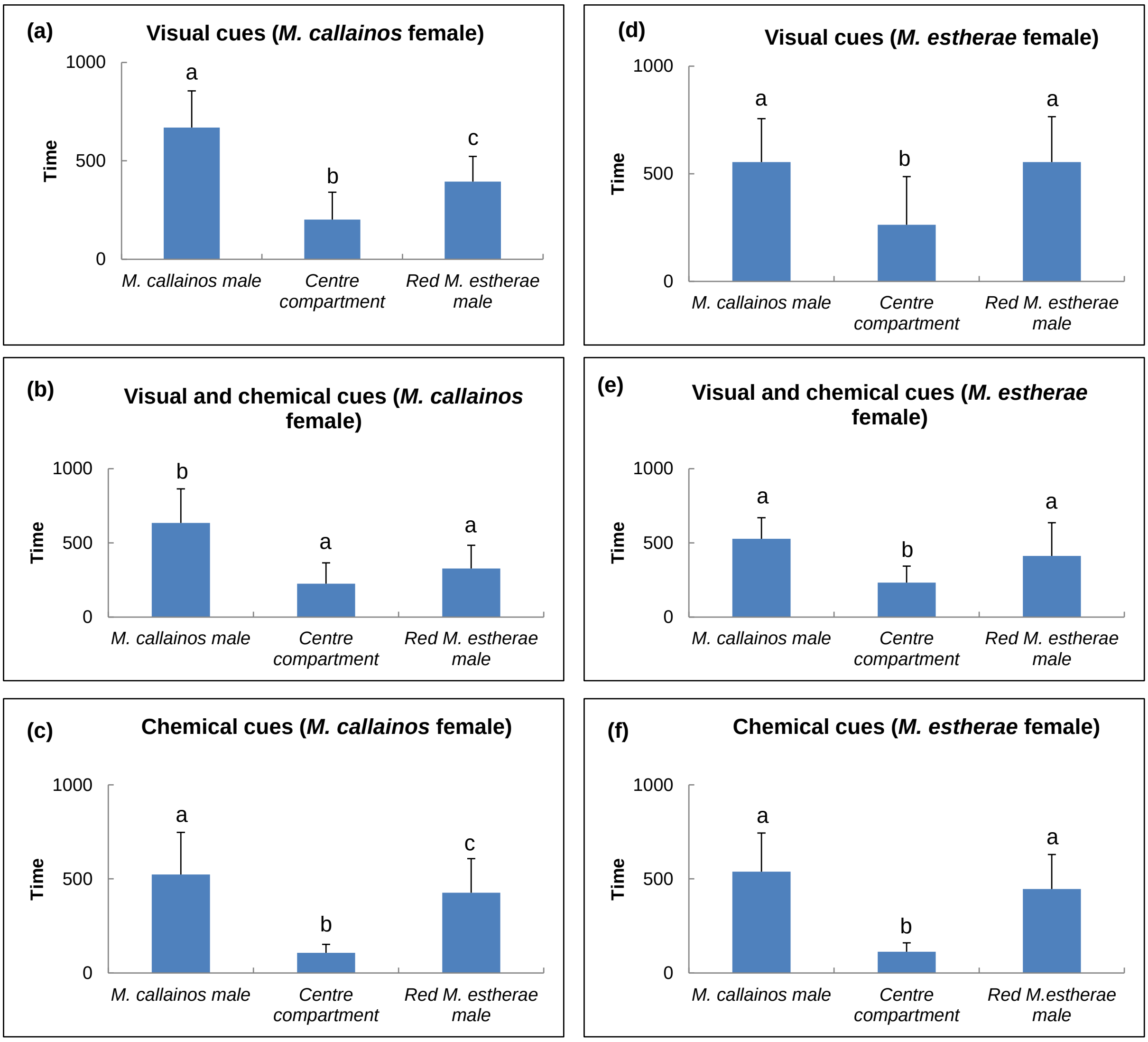

Figure 2.2: Average time females spent with *M. callainos* and red *M. estherae* males. Error bars indicate standard deviation; different letters indicate a significant difference ($p < 0.05$).

2.5.2. Blue *M. estherae* versus *M. callainos*

In the presence of only visual cues, *M. callainos* females spent more time with *M. callainos* males ($F_{2, 27} = 6.89$, $p = 0.004$; *Post-hoc*, Fishers LSD, Figure 2.3 a). In the presence of both visual and chemical cues, there was no significant difference in the amount of time *M. callainos* females spent with both males ($F_{2, 27} = 2.11$, $p = 0.14$; Figure 2.3 b). *Metriaclima callainos* females spent more time with *M. callainos* males in the presence of chemical cues only ($F_{2, 27} = 62.89$, $p < 0.001$; *Post hoc* test, Fishers LSD, Figure, 2.3 c).

When only visual cues were present, blue *M. estherae* females spent equal time with both males, but less time in the centre compartment ($F_{2, 27} = 25.53$, $p < 0.001$; *Post-hoc* test, Fishers LSD, Figure 2.3 d). In the presence of both visual and chemical cues, there was no significant difference in the amount of time *M. estherae* females spent with the different males ($F_{2, 27} = 0.89$, $p = 0.42$; Figure 2.3 e). There was a significant difference in the amount of time *M. estherae* females spent near the males in the presence of chemical cues only ($F_{2, 27} = 48.76$, $p < 0.001$; Figure 2.3 f). *Metriaclima estherae* females spent more time with *M. callainos* males (*Post-hoc*, Fishers LSD).

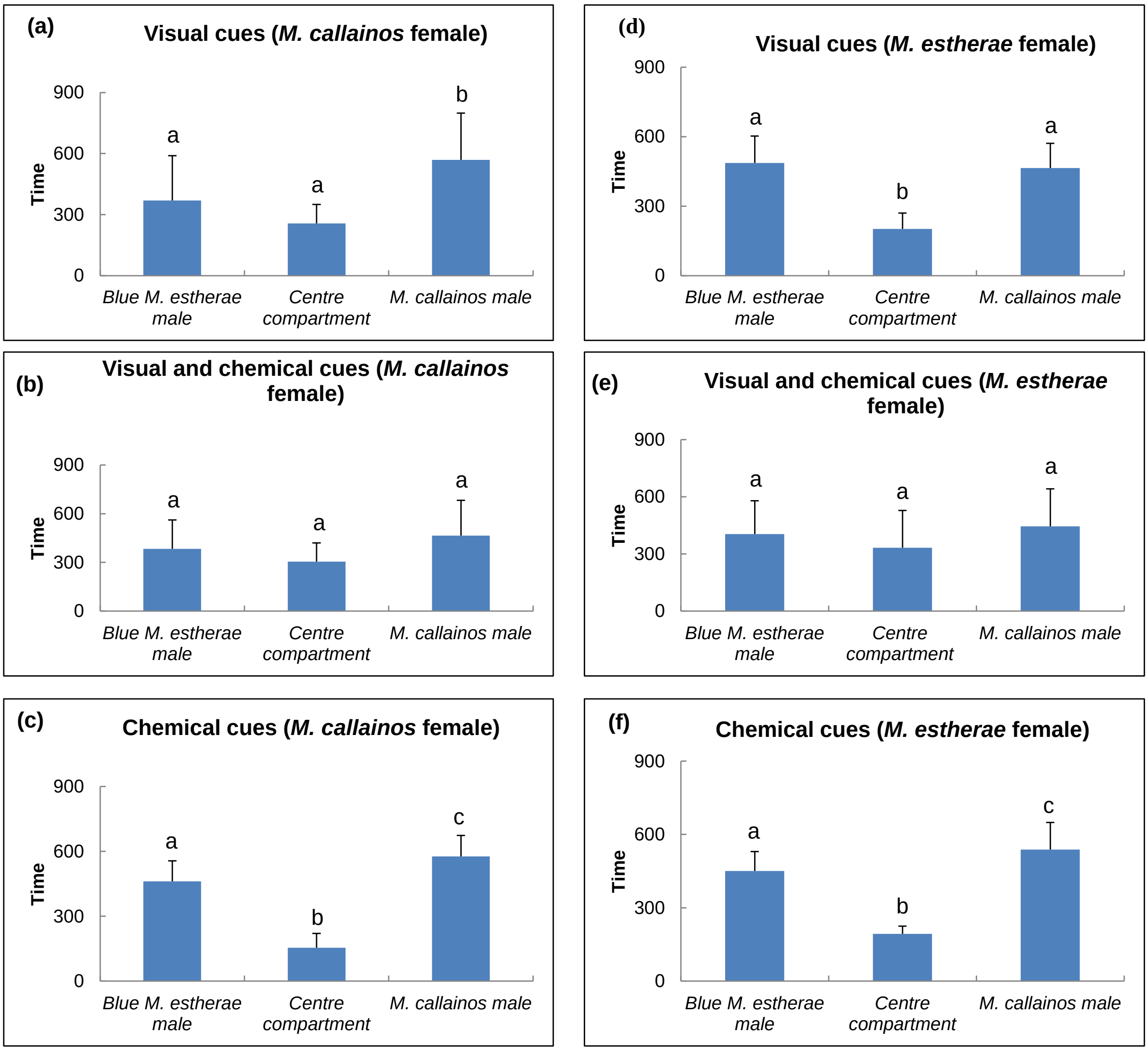

Figure 2.3: Average time females spent with blue *M. estherae* and *M. callainos* males. Error bars indicate standard deviation; different letters indicate a significant difference ($p < 0.05$).

2.5.3. Red *M. estherae* versus blue *M. estherae*

When only visual cues were present, there was no significant difference in the amount of time the females of *M. callainos* spent in the three compartments ($F_{2, 27} = 1.51$, $p = 0.24$, Figure 2.4 a). However, in the presence of visual and chemical cues, *M. callainos* females spent less time in the centre compartment and an equal amount of time near the two males ($F_{2, 27} = 8.19$, $p = 0.002$; *Post-hoc* test, Fishers LSD, Figure 2.4 b). In the presence of chemical cues only, *M. callainos* females spent more time with blue *M. estherae* males ($F_{2, 27} = 29.49$, $p < 0.001$; *Post-hoc* test, Fishers LSD, Figure 2.4 c).

There was no significant difference in the amount of time that *M. estherae* females spent with both males when only visual cues were present ($F_{2, 27} = 1.07$, $p = 0.36$, Figure 2.4 d). In the presence of visual and chemical cues, there was a significant difference in the amount of time female *M. estherae* spent in the central zone, but not between the two males ($F_{2, 27} = 8.33$, $p = 0.002$; Figure 2.4 e). In the presence of chemical cues only, *M. estherae* females spent more time with red *M. estherae* males ($F_{2, 27} = 32.19$, $p < 0.001$; *Post-hoc* test, Fishers LSD, Figure 2.4 f).

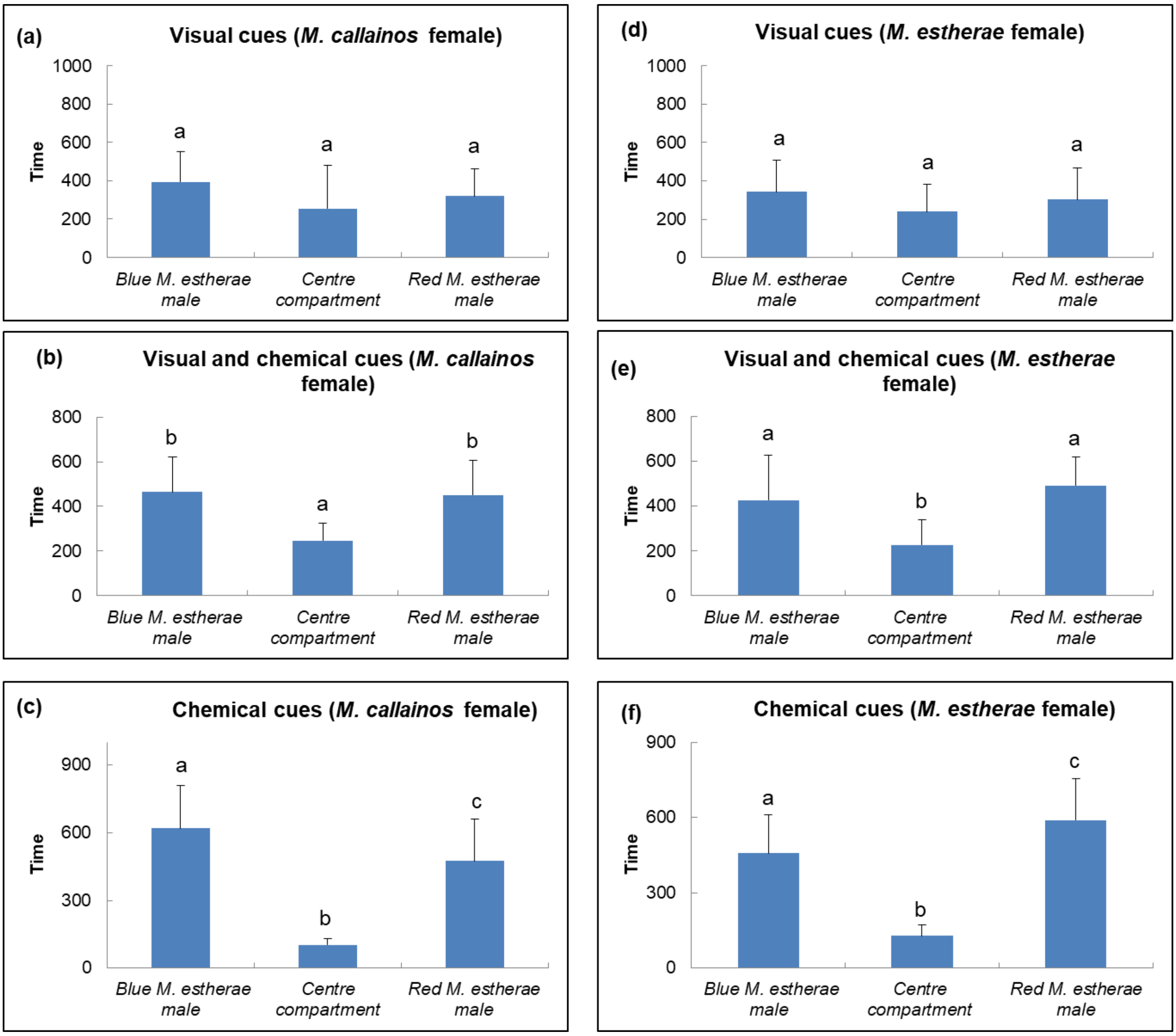

Figure 2.4: Average time females spent with blue and red *M. estherae* males. Error bars indicate standard deviation; different letters indicate a significant difference ($p < 0.05$).

2.6. Discussion

Several studies have demonstrated that sexual selection depends on multiple sensory cues (Partan and Marler, 2005; Candolin, 2003; Hebets and Papaj, 2005; Chenoweth and Blows 2006; Hohenlohe and Arnold, 2010; Verzijden *et al*., 2010; Hebets, 2011). Divergence in sexual selection cues is thought to have resulted in the rapid adaptive radiation of African cichlids (Maan *et al*., 2004). Visual (Knight and Turner 1999; Couldridge and Alexander, 2002; Maan and Sefc, 2013; Selz *et al*., 2013; Tyers and Turner, 2013), acoustic (Verzijden *et al*., 2010) and chemical (Plenderleith *et al*., 2005; Blais *et al*., 2009) cues are important in sexual selection and reproductive isolation in African cichlids. However, some researchers argue that it is not necessary for all cues and their preference for them to diverge in order for reproductive isolation to occur (Selz *et al*., 2014). In some cases, reproductive isolation may operate on divergence of a single cue, such as colour, as demonstrated in *Heliconius* butterflies (Jiggins *et al*., 2001), poison-dart frogs (Reynolds and Fitzpatrick, 2007), treehopper insects (Rodriguez *et al*., 2006) and in North American crossbill birds (Snowberg and Benkman, 2007). In this study, the role of visual and chemical cues was investigated to provide an insight into mate recognition systems between closely related cichlid species.

Proximity tests are commonly used and acceptable methods to measure mate choice in cichlids studies (Seehausen and van Alphen, 1998; Knight and Turner, 1999; Turner *et al*., 2001; Couldridge and Alexander, 2002; Werner and Lotem, 2003; Jordan *et al*., 2003; Knight and Turner, 2004; Maan *et al*, 2004; Plenderleith *et al*., 2005; Egger *et al*., 2008; Blais *et al*., 2009; Maan *et al*., 2010; Selz *et al*., 2014). In general, these studies use the time spent in association with a subject as an indication of mate choice. These studies typically use three classes of experiments to quantify mate choice in cichlids: the free access (Maan *et al*., 2004), female only access (Turner *et al*., 2001; Knight and Turner, 2004; Blais *et al*., 2009) and restrained access (Seehausen and van Alphen, 1998; Knight and Turner 1999; Couldridge and Alexander, 2002; Selz *et al*., 2014). In the current study, a restricted access design was adopted as it allows a manipulation of cues available to females. The method of using proximity to measure mate choice was further proven to be accurate by Couldridge and Alexander (2001). In addition, proximity methods have been previously used for many other fish species (Forsgren, 1992; McLennan and Ryan, 1999; Kodric-Brown and Strecker, 2001; McLennan, 2004; Kodric-Brown and West, 2014; Gozlan *et al*., 2014). We are thus confident that the results obtained here are a reliable measure of mating preferences.

The results of the current study demonstrate that *M. estherae* females did not recognise conspecific males over a closely related species. In none of the treatments did they prefer their own species. The inability of *M. estherae* females to discriminate conspecific males may be due to the fact that males of *M. estherae* have both blue and red morphs and their blue morph is very similar in appearance to males of *M. callainos* (Stauffer and Hert, 1992). The two species are easily confused. Knight and Turner (2004) also demonstrated that some species of African cichlids lack the ability to mate assortatively due to strong similarities of male breeding colours. Females of *M. estherae* also showed no strong preference for either of the two conspecific colour morphs. Indeed, some previous research also found that some cichlid species did not recognise conspecifics (Knight and Turner, 1999; Couldridge and Alexander, 2002). However, the former studies only tested visual cues for species recognition. Knight and Turner (1999) recommended that future studies should investigate both visual and chemical cues. Both visual and chemical cues were considered in the current study. However, the results did not provide clarity about the cues used for species recognition in *M. estherae*.

On the other hand, the females of *M. callainos* were easily able to recognise and choose conspecific males, Figure 2.1 (a - c). When *M. callainos* females were given a choice between blue *M. estherae* and *M. callainos* males, they were still able to recognise conspecific males, even in the presence of visual cues only, Figure 2.3 (a - c). This is in agreement with previous studies, which have also demonstrated that *M. callainos* females (Couldridge and Alexander, 2002) and males (Knight and Turner, 1999) are able to recognise and choose assortatively by employing visual cues. However, when *M. callainos* females were given a choice between red and blue *M. estherae* males, they showed no preference for blue males over red males, Figure 2.4 (a - b). A previous study showed that in the absence of conspecific males, females choose heterospecific males with colour patterns similar to their conspecific males (Couldridge and Alexander, 2002). Therefore, it was expected that the *M. callainos* females spend more time with blue *M. estherae* males.

Previous studies have demonstrated that chemical cues also play a role in mate recognition (Blows and Allan, 1998; Ortiz-Barrientos *et al*., 2004; Plenderleith *et al*., 2005; Blais *et al*., 2009) and in assessing mate quality and compatibility of mates (Penn, 2002). In the current study, chemical cues did play some role in mate preference, as *M. callainos* still preferred conspecifics in the absence of colour cues. A study by Jordan *et al*. (2003) suggests that visual cues other than colour may play an important role in species recognition in cichlids. The latter study demonstrated that even when colour was obscured; the behaviour, shape and pattern of males are not concealed, suggesting that females may still be able to recognise males based on their shape and courtship behaviour. *Metriaclima estherae* and *M. callainos* belong to the *P. zebra* species complex, which consists of ecologically, morphological and behaviourally similar species (Stauffer and Hert, 1992; Stauffer *et al*., 1997). Furthermore, a study found no significant difference in the courtship behaviour of *M. zebra* cichlids, which are closely related to the study species (Mac Elroy and Kornfield, 1990). The previous study further suggested that there might be little or no behavioural differences between the study taxa. However, a study that examined species recognition between the two closely related and morphologically similar species *Pundamilia nyererei* and *Pundamilia pundamilia* found that females solely use colour for species recognition (Salze *et al*., 2014) and were only able to recognize conspecific males when colour was visible (Seehausen and van Alphen, 1998). This suggests that visual cues are indeed important for species recognition and reproductive isolation. With the exception of *M. estherae*, the findings of this study are in agreement with previous studies suggesting that visual cues maintain reproductive isolation in closely related cichlid species of Lake Malawi (Knight and Turner 1999; Couldridge and Alexander, 2002) and of Lake Victoria cichlids (Seehausen, 1997; Seehausen and van Alphen, 1998; Seehausen *et al*., 1999).

2.7. Conclusions

The results of the current study suggest that both visual and chemical cues are utilised in species recognition in *M. callainos*. Previous studies demonstrate that chemical cues also aid species recognition in cichlid species (Plenderleith *et al*., 2005; Blais *et al*., 2009). The results of the current study suggest that chemical cues are probably not as important as visual cues in species recognition. *Metriaclima estherae* females did not prefer conspecific males. The lack of assortative mating has been demonstrated in other cichlids species (Knight and

Turner, 2004), other fish species such as arctic charr (*Salvelinus alpines*) (Jonsson and Jonsson, 2001) and guppies (*Poecilia reticulata*) (Magurran, 2001) as well as in the butterflies *Heliconius erato* and *Heliconius himera* (McMillan *et al*., 1997). Further investigations are required to clarify the mechanism of mate selection and species recognition for *M. estherae*.

Chapter 3. The role of males in maintaining reproductive isolation and species recognition in *Metriaclima estherae* and *M. callainos*

Abstract

African cichlid fishes are one of the most species rich fishes in the world. The males of African cichlids are conspicuously coloured while females are usually cryptically coloured. This has led to the theory that female mate choice acting on male colour is largely responsible for the diversity of African cichlids. However, some species also have brightly coloured females. There is limited knowledge regarding the role of male preferences for female colour patterns in species recognition. The aims of the current study were to test whether males of African cichlids are able to recognise conspecific females. Mate recognition was investigated by employing visual cues, chemical cues and a combination of visual and chemical cues. *Metriaclima estherae* and *M. callainos* males were given a choice between females of both species. The results show that *M. callainos* males were able to recognise conspecific females when both visual and chemical cues were used. However, *M. estherae* males were unable to recognise conspecific females. The results of the study suggest that species differ in their discriminatory abilities and that multiple cues are probably required for species recognition in African cichlids.

3.1 Introduction

The three great Lakes of East Africa (Lakes Tanganyika, Victoria and Malawi) have an extremely high diversity of cichlid species, with ~ 2000 combined endemic species (Genner *et al*., 2004). In Lake Malawi, there are estimated to be as many as 1500 endemic cichlid species (Turner *et al*., 2001) and they are thought to have evolved ~ 700 000 years ago (Meyer, 1993; Stiassny and Meyer, 1999; Turner, 1999). There are several possible contributing factors to their high speciation rates. Liem (1973) suggested that the high species diversity of these fishes is due to allopatric speciation with the aid of specialised feeding apparatus. However, many previous studies suggest that sexual selection is a major factor driving speciation in African cichlids, even in sympatry (Kotcher, 2004; Seehausen *et al*., 2008).

Haplochromine cichlids are polygamous with exclusive female parental care (Werner and Lotem, 2002). After spawning, the mouth brooding female refrains from eating while males replenish their gametes for the next spawning season (Seehausen and van Alphen, 1998). This breeding cycle has resulted in a strong selection for male colours by females (Fisher, 1930; Lande, 1981; Dominey, 1984). It is thought that females of haplochromine cichlids choose males that display the most intensive colouration during courtship displays (Nelissen, 1985), leading to speciation through male competition as sexual selection (Tinghitella *et al*., 2018). Females of haplochromine cichlids generally have less conspicious coloration compared to males, thus the males are expected to be less choosy (Werner and Lotem, 2002). However, evidence suggests that males of some fish species may also be selective if the female quality is highly variable (Owens and Thompson, 1994), or as a result of sperm depletion, time and energy expenditure and loss of mating opportunities (Johnstone *et al*., 1996; Johnstone, 1997).

There is less information about the role of male haplochromine cichlids in the process of mate selection and speciation. Much attention has been given to the role of female visual cues on mate choice and reproductive isolation in haplochromine cichlids (Seehausen and van Alphen, 1998; van Oppen *et al*., 1998; Knight and Turner, 1999; Couldridge and Alexander, 2002; Stelkens and Seehausen, 2009; Pauers and McKinnon, 2012; Maan and Sefc, 2013; Selz *et al*., 2013; Tyers and Turner, 2013). The aim of this study was to investigate whether males are able to recognise their conspecific females and thus maintain reproductive boundries as shown in female haplochromine cichlids. Previous studies that tested the role of males in species recognition, only focused on visual cues as the main driver of reproductive isolation (Knight and Turner, 1999; Werner and Lotem, 2003). However, focusing on a single cue is insufficient to explain species recognition and reproductive isolation in African cichlids (Blias *et al*., 2009). In the current study a combination of visual and olfactory cues were employed to investigate mate recognition in the males of *M. estherae* and *M. callainos*. This study aims to further test the role of male cichlid fish in maintaining reproductive isolation.

3.2 Materials and methods

The experiments were modified from Werner and Lotem (2003). The experimental setup followed the same procedure as Chapter 2, Section 2.2.1. However, the males were placed in the centre compartment of the test tanks and given a choice between females of *M. estherae* and *M. callainos*.

3.2.1. Statistical analysis

Data was converted by square root transformation to meet the assumptions of ANOVA (Analysis of Variance). Shapiro-Wilk's W test was used to test for normality of the data and Levene's test was used to test for homogeneity of variances. Repeated measures ANOVA was used to assess the average time the males spent in each zone followed with a *post-hoc* test (Fishers LSD) to test for any significant differences. All statistics were carried out using Statistica (StaSoft Inc, 2005).

3.3. Results

3.3.1. *Metriaclima callainos* males

Average TL (mm) and weight (g) of the fish used for these experiments are presented in Appendix 2 (Table 9.1 - 9.6). *Metriaclima callainos* males spent an equal amount of time near the two females compartments when visual cues alone were employed ($F_{2, 27}$ =11.7, $p < 0.001$; *Post-hoc* test, Fishers LSD, Figure 3.1 a).

Post-hoc tests show that *M. callainos* males spent more time with *M. callainos* females compared to *M. estherae* females when both visual and chemical cues were employed ($F_{2, 27}$ =15.9, $p < 0.001$, Figure 3.1 b).

In the presence of chemical cues only, *M. callainos* males spent an equal amount of time between *M. estherae* and *M. callainos* females, but more time in the centre comparment ($F_{2, 27}$ = 63.4, $p < 0.001$; *Post-hoc* test, Fishers LSD, Figure 3.1 c).

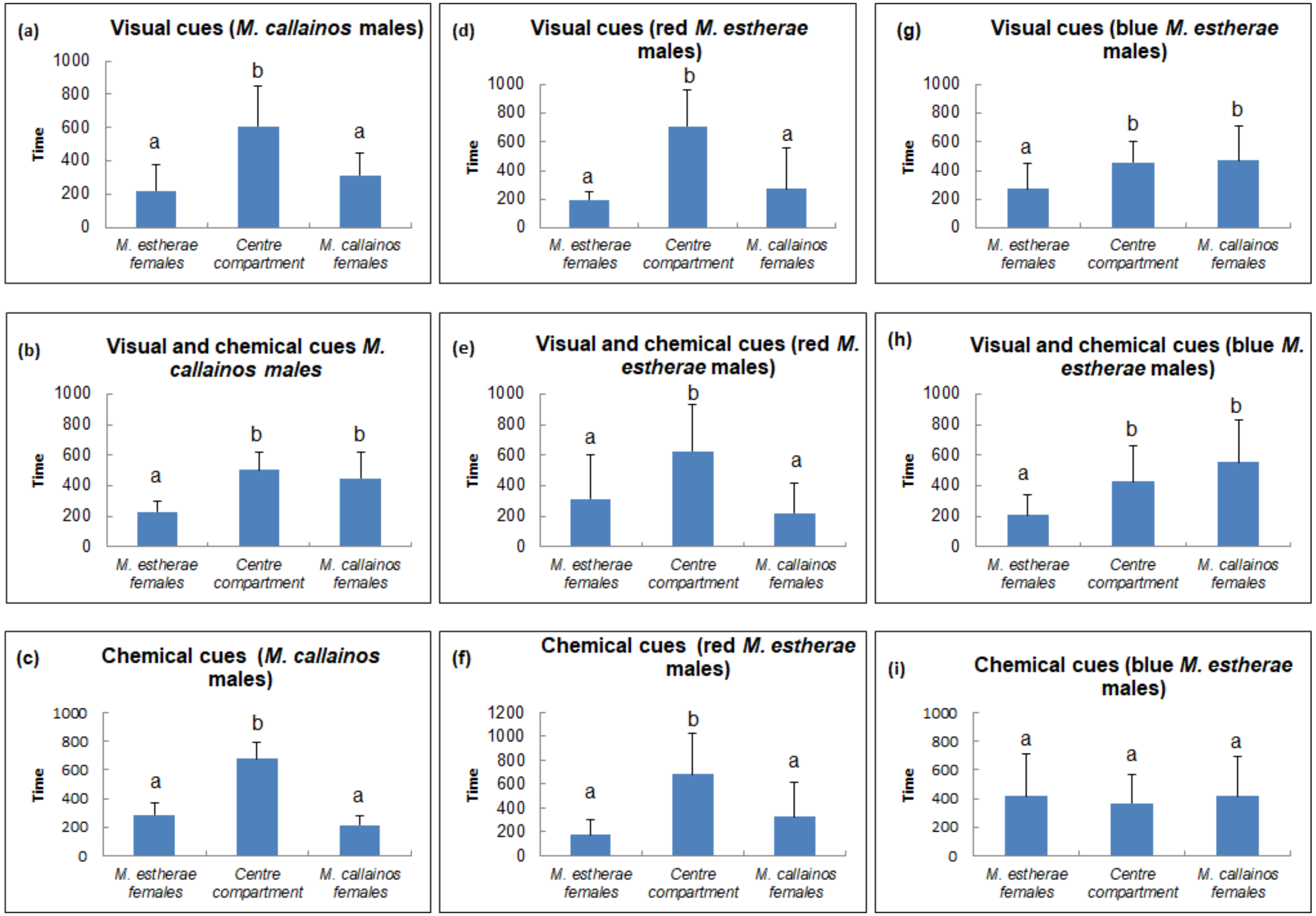

Figure 3.1: Average amount of time males spent with *M. estherae* and *M. callainos* females. Error bars indicate standard deviation; different letters indicate significant difference ($p < 0.05$).

3.3.2. Red *M. estherae* males

There was a significant difference in the amount of time the males of red *M. estherae* spent in each compartment in the presence of visual cues only. A *post-hoc* test (Fishers LSD) showed that the red *M. estherae* males spent an equal amount of time with both females and more time in the middle compartment ($F_{2, 27}$ =13.1, $p < 0.001$, Figure 3.1 d).

Likewise, in the presence of visual and chemical cues, red *M. estherae* males spent an equal amount of time with both females, but more time in the centre compartment ($F_{2, 27}$ =6.5, $p < 0.001$; *Post-hoc* test, Fishers LSD, Figure 3.1 e).

Red *M. estherae* males also spent equal amounts of time with both females when only chemical cues were employed ($F_{2, 27}$ =8.9, $p = 0.001$; *Post-hoc*, Fishers LSD, Figure 3.1 f).

3.3.3. Blue *M. estherae* males

There was a significant difference in the amount of time the males of blue *M. estherae* spent between the two females when only visul cues were employed ($F_{2, 27}$ =5.1, $p < 0.001$). However, a *Post-hoc* test (Fishers LSD) showed that males of blue *M. estherae* spent more time with *M. callainos* females (Figure 3.1 g).

In the presence of visual and chemical cues, blue *M. estherae* males also spent more time with *M. callainos* females ($F_{2, 27}$ = 6.8, $p = 0.004$; *Post-hoc*, Fishers LSD, Figure 3.1 h).

In the presence of chemical cues only, there was no significant difference in the amount of time blue *M. estherae* males spent in each compartment ($F_{2, 27}$ = 0.1, $p = 0.937$, Figure 3.1 i).

3.4 Discussion

Numerous studies on mate selection and recognition systems in African cichlids focused their studies on female mate choice as the main driver of speciation (Seehausen and Alphen, 1998; Couldridge and Alexander, 2002; Knight and Turner, 2004; Maan *et al*., 2004). The males of haplochromine cichlids invest less in parental care which led to the theory that speciation is a result of female choice. There is comparatively little information about whether the males of African cichlids also play a role in mate selection and thus speciation. Previous studies investigating mate choice have used both female proximity to males (Seehausen and van Alphen, 1998; Turner *et al*., 2001; Couldridge and Alexander, 2002; Jordan *et al*., 2003; Knight and Turner, 2004; Maan *et al*., 2010; Selz *et al*., 2014) and male proximity to females (Nuttall and Keenleyside 1993; Beeching and Hopp 1999; Werner and Lotem 2003), as an indicator of female and male choice respectively. The results obtained here are thus believed to be an accurate proxy for mating preferences.

The results of this study show that when visual cues alone and chemical cues alone were employed, respectively, *M. callainos* males were not able to distinguish between *M. estherae* and *M. callainos* females, despite females of these species being dissimilar in colour pattern. Only when both visual and chemical cues were present, were *M. callainos* males able to recognise conpecific females (Figure 3.1 b). This is partly in agreement with a previous study (Knight and Turner, 1999) where the results showed that *M. callainos* males were able recognise conspecific females. However, the latter study reported that *M. callainos* males were able to recognise conspecific females by using visual cues alone, but were unsuccessful in recognising conspecific females when nuptual colour was similar. In the current study, *M.*

callainos males were only able to recognise conspecific females when both visual and chemical cues were present.

The results of the current study showed that the red *M. estherae* males were not able to recognise conspecific females in all experiments, Figure 3.1 (d - f). Blue *M. estherae* males spent more time with *M. callainos* females when visual cues alone were used and when both visual and chemical cues were used, Figure 3.1 (g - h). In the presence of chemical cues only, the blue *M. estherae* males spent an equal amount of time with both females (Figure 3.1 i). Similar results were observed in a previous study (Chapter 2 of the current thesis), where the results show that the females of *M. estherae* were unable to recognise conpecific males. In the latter study, it was suggested that the reason *M. callainos* females were not able to recognise conspecific males was because the blue males of *M. estherae* are similar to *M. callainos*. However, since *M. estherae* females only have orange colour morphs and *M. callainos* females are blue, it would be expected that the males of *M. estherae* would be able to differentiate between the two females. The reason why both sexes of *M. estherae* are not able to recognise conspecific mates is not clear. On the other hand, previous studies found that *M. callainos* females are able to recognise their conspecific males (Couldridge and Alexander, 2002; Chapter 2 of the current thesis). The results suggests that *M. callainos* has a greater visual discriminatory ability or reliance than does *M. estherae*.

There are conflicting reports regarding the cues that the males of haplochromine cichlids use for mate recognition. Knight and Turner (1999) suggest that the some males from the *Pseudotropheus* species complex use visual cues for species recognition, whereas Meyer *et al*. (1990) suggest that chemical cues are important for males in mate recognition. Pauers *et al*. (2010) demonstrated that both males and females of the mbuna cichlid, *Labeotropheus fulleboni* use visual cues for reproductive isolation. The results of this study suggest that both visual and chemical cues are required for male mate choice. This contradiction of the results may suggests that the males of haplochromine cichlids could have a reduced role in sexual selection. However, to confirm this, more studies of male mate selection should be conducted.

Research shows that male breeding colour of haplochromine cichids instensifies during courthsip and in territorial dominance (Nielsien, 1985) and that adult females, even of brighly coloured species, tend to maintain similar body colour as found in juveniles and young males (Genner and Turner, 2005). Genner and Turner (2005) further suggested that female colour has the same role as in juveniles and is not a sexual signal. This further suggests that male colour patterns are the main driver of reproductive isolation and speciation in African cichlids. This is consistent with the theory that male breeding colour is impotant for mate selection and species recogntion (Couldridge and Alexander, 2001; Knight and Turner, 2004; Maan *et al*., 2004; Stelkens and Seehausen, 2009; Pauers and McKinnon, 2012; Maan and Sefc, 2013; Selz *et al*., 2013; Tyers and Turner, 2013).

It should be noted that the role of acoustic signals was not taken into account during the the current study. Recent studies found that females of some haplochromine cichlids are also able to produce sounds (Simões *et al*., 2008). It is possible that accoustic stimuli from females are required by males for species recognition. Furthermore, it is not known whether actual breeding would have occurred between these two species since this study did not involve actual breeding. However, a previous study has shown that distantly related haplochromine cichlids can hybridise and produce healthy fertile offspring (Capron de Caprona and Fritzch, 1984).

A previous study (Knight and Turner, 1999) suggested that males of haplochromine cichlids probably have a reduced role in the speciation process. However, other studies show that females of other haplochromine cichlids of Lake Malawi (Couldridge and Alexander, 2002; Knight and Turner, 2003) and Lake Victoria (Seehausen and van Alphen, 1998) mate assortatively and can identify conspecific males by using only visual cues (colour). A study (Couldridge and Alexander, 2002) further demontrated that in the absence of conspecific males, females prefer males that have colour patterns similar to their conspecific males.

3.5 Conclussions

Much attention has been given to the role of female haplochrome cichlids in maintaining reproductive isolation (Seehausen and van Alphen, 1998; Couldridge and Alexander, 2002; Knight and Turner, 2003). However, males may also evaluate female signals to avoid hybridization. The results of the current study demonstrate that males can identify conspecific females, although more than one cue is required. *Metriaclima callainos* males were better able to recognise conspecific females by using a combination of visual and chemical cues. *Metriaclima estherae* males, on the other hand, do not prefer conspecifics, even in the presence of a combination of cues.

Chapter 4. Inter- and intra-specific acoustic differences between *Metriaclima estherae* and *M. callainos*

Abstract

Although female choice acting on male colour is thought to be a main driver of diversity of Lake Malawi cichlids, recent studies have found that cues such as chemical and acoustics may also be important in species recognition and reproductive isolation. However, it is still not fully understood the role these cues play in mate choice and species recognition. In this study, acoustic signals of *Metriaclima callainos* and *M. estherae* were investigated. Peak frequency and duration of the sounds made by the two species were analysed and compared. There were significant differences in the acoustic signals of these two species. There was also a significant variation in the sound characteristics between individuals within a species. This suggests that acoustic cues can potentially be used for species recognition and as a mate choice cue in some cichlid fishes.

4.1 Introduction

In nature, females must be able to recognise and must prefer to mate with conspecific rather than heterospecific males (Capron de Caprona and Ryan, 1990; Ryan and Rand, 1993). Mate recognition systems involve information from multiple sensory modalities (Escobar-Camacho and Carleton, 2015). Cues such as visual (Jordan *et al*., 2003; Knight and Turner, 2004; Maan *et al*., 2004; Pauers *et al*., 2010; Salzburger *et al*., 2006; Egger *et al*., 2010; Pauers and

McKinnon, 2012; Maan and Sefc, 2013; Selz *et al*., 2013; Tyers and Turner, 2013), acoustic (Amorim *et al*., 2004) and chemical (Plenderleith *et al*., 2005; Blais *et al*., 2009) are important in mate recognition. Previous studies show that fish are able to produce sounds that are often species (Amorim *et al*., 2004; 2008) or population specific (Lamml and Kramer, 2007; Philips and Johnson, 2008). Acoustics cues contain information about mate quality, which may influence mating decisions (de Jong *et al*., 2007; Simões *et al*., 2008; Verzijden *et al*., 2010). Some studies found that some fish are able to discriminate between conspecific and heterospecific sounds (Lobel, 2001; Lugli *et al*., 2004, Rollo and Higgs, 2008; Estramil *et al*., 2013). Another study also demonstrated that some fish could recognise each other based solely on sound (Myrberg and Riggio, 1985).

In haplochromine cichlids, the divergence of signals associated with mate selection is thought to influence reproductive isolation and influence the process of sympatric speciation (Maan *et al*., 2004; Amorim *et al*., 2004; Simões *et al*., 2008; Verzijden *et al*., 2010; Estramil *et al*., 2013). There is not much information about how closely related sympatric cichlid species remain reproductively isolated (Knight and Turner, 1999; Maan *et al*., 2004). The process that drives speciation in haplochromine cichlid is still not fully understood (Knight and Turner, 1999). Research demonstrates that in the early stages of courtship, the males of some species of African cichlids produce low frequency, short pulse sounds (Amorim *et al*., 2004; Pisanski *et al*., 2014). These acoustic signals are often, but not always, accompanied by quiver displays (Amorim *et al*., 2004; Amorim and Almada 2005; Simões *et al*., 2008), which involves fast shaking movements while the fins are raised close to another individual (Verzijden *et al*., 2010). The sound that is produced during the quiver displays is thought to be similar to rapidly repeated pulses (Lobel, 1998; Amorim *et al*., 2004).

Previous studies found significant differences in sound production by cichlid species of the genus *Pseudotropheus*, now formally recognized as *Metriaclima*, in Lake Malawi (Amorim *et al*., 2004; Danley *et al*., 2012). Another study demonstrated that female cichlids prefer to court with males that produce sound (Verzijden *et al*., 2010; Spinks *et al*., 2016). The importance of acoustic signals in species recognition among closely related species is still not fully understood. In this study, courtship acoustic signals of *Metriaclima callainos* and *M. estherae* were investigated. The aim of this study was to investigate if sound may potentially influence reproductive isolation between closely related species, by assessing whether sounds differ between these two species.

4.2 Materials and methods

4.2.1 Housing

The fish were housed and maintained as described in Chapter 2, Section 2.1.1.

4.2.2 Experimental protocol

The experimental protocol for this chapter was modified from Amorim *et al.* (2008). Experiments were conducted in an experimental tank divided into three compartments (90×30×30 cm) with a clear perforated Perspex box. A layer of gravel was placed on the bottom of the test tank. One male was placed in the side compartment and acclimatised for 24 hours before each recording session. Males were provided with a terracotta pot to act as a territory. A Dolphin Ear Pro hydrophone was connected to a Sony camcorder. The sound was recorded by suspending the hydrophone in the male’s compartment, approximately 5 cm above the terracotta pot. Sounds were therefore, recorded upon introducing a group of five to eight conspecific females for 45 minutes. All electrical equipment in the laboratory was temporarily switched off to reduce background noise. A total of eight *M. callainos*, eight red *M. estherae* and six blue *M. estherae* males were recorded. Ten sounds were selected for analysis from each individual. Total length (TL) of the males was measured to the nearest millimetre (mm) with a Vernier digital calliper, and weighted (g) with a balance scale before each recording session.

4.2.3. Data analysis

Sounds were analysed using Raven Pro 1.4 Software (Cornell Lab of Ornithology). Delta time and peak frequency were measured and only clear sounds were considered for analysis. The data was tested for normality using Shapiro-Wilk's W test and homogeneity of variances tested using Levene's test. As data did not conform to a normal distribution, nonparametric Kruskal-Wallis tests were used to assess the differences in courtship sounds between and within the species. Correlation tests (Spearman's correlation) were used to test the effects of size and weight of the sounds produced by the fish. All statistics were carried out using Statistica (StaSoft Inc, 2005).

4.3 Results

4.3.1 Differences between all species

Mean TL for *M. callainos* males used for the experiments was 93 mm (± 1.5 mm) and weight was 13.6 g (± 0.7 mm). Mean TL for blue *M. estherae* males was 82 mm (± 2.3 mm) with 9.6 g (± 0.8 g) weight. Mean TL for red *M. estherae* was 80.4 mm (± 2.6 mm) and weight was 7.8 g (± 3.4 g). There was no significant relationship of either TL or weight and sound characteristics ($p > 0.05$) (Table 4.1). There was no significant difference in the peak frequency between all groups, p = 0.403, H (2, N = 220) = 6.420 (Figures 4.1 and 4.2). However, there was a significant difference in call duration between all groups, p < 0.001, H (2, N = 440) = 37.12 (Figure 4.1 and 4.3).

Table 4.1: Results of Spearman correlation tests showing the relationship between SL (mm), weight (g) and sound characteristics (duration and peak frequency) of the study species (p > 0.05 in all cases).

Species	**Sound character**	**SL (r)**	**W (r)**
M. callainos	Call Duration (sec)	-0.02	-0.25
	Peak Frequency (Hz)	-0.09	-0.18
Blue *M. estherae*	Call Duration (sec)	-0.27	-0.42
	Peak Frequency (Hz)	-0.55	-0.62
Red *M. estherae*	Call Duration (sec)	0.37	0.34
	Peak Frequency (Hz)	0.36	0.44

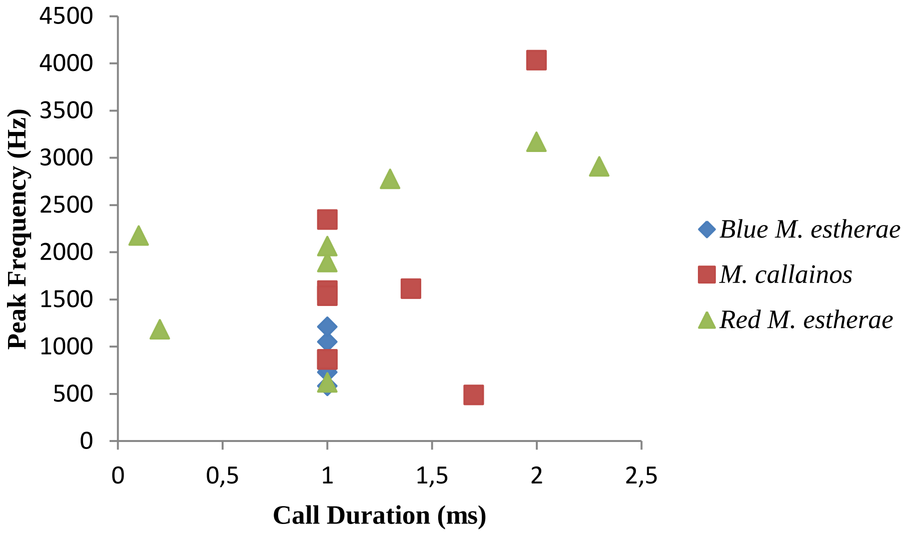

Figure 4.1: Average individual peak frequency (Hz) and call duration (ms) of *M. callainos* and red and blue *M. estherae*.

4.3.2 *Metriaclima callainos*

There was a significant difference in both peak frequency (p < 0.001, H (7, N = 80) = 24.16, Figures 4.1 and 4.2) and call duration (p < 001, H (7, N = 160) = 60. 98, Figures 4.1 and 4.3) among individuals of *M. callainos*.

4.3.3 Blue *M. estherae*

There was no significant difference in peak frequency for blue *M. estherae* males (p = 0.111, H (5, N = 60) = 8. 94, Figures 4.1 and 4.2). However, there was a significant difference in call duration among individuals of blue *M. estherae* (p < 0001, H (5, N = 120) = 45, 05, Figures 4.1 and 4.3).

4.3.4 Red *M. estherae*

There was a significant difference in both peak frequency ($p < 0.001$, H (7, N = 160) = 54.30, Figures 4.1 and 4.2) and call duration ($p = 0.013$, H (7, N = 80) = 17.73, Figures 4.1 and 4.3) of sounds produced by males of red *M. estherae*.

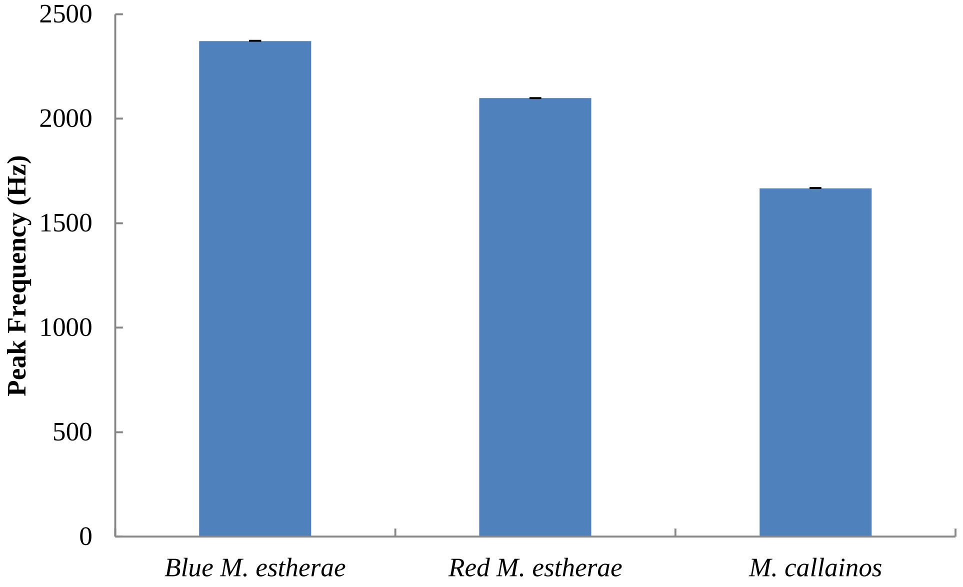

Figure 4.2: Average peak frequency produced by *M. callainos* and red and blue *M. estherae*. Error bars indicate standard deviation.

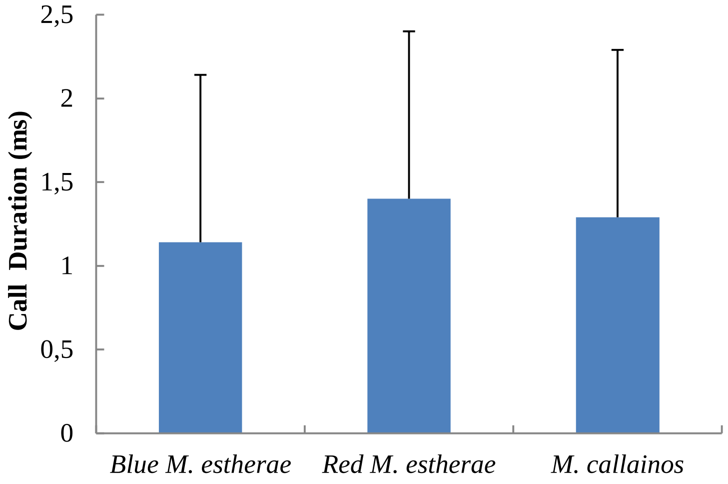

Figure 4.3: Average call duration of *M. callainos* and red and blue *M. estherae*. Error bars indicate standard deviation.

4.4 Discussion

In teleosts, courtship, spawning, defence, aggression and feeding are sometimes accompanied by acoustics (Ripley and Lobel, 2004). Acoustic signalling in cichlid fish is widespread and has been documented in over 20 species (Lobel, 1998; 2001, Verzijden *et al*., 2010; Danley *et al*., 2012). Studies show that there is a divergence in female preferences for male signals in cichlid fish and, this has resulted in behavioural isolation (Boughman, 2001; Panhuis *et al*., 2001; Masta and Maddison, 2002). Another study demonstrated that in amphibians, sexual selection could lead to divergence in male courtship calls and a strong female preference for mating calls between populations, resulting in behavioural isolation (Boul *et al*., 2007). Previous studies focused on visual cues as a mechanism of species recognition and speciation in African cichlids (Seehausen and van Alphen, 1998; Couldridge and Alexander, 2002). Recently, studies found non-visual cues such as olfactory (Plenderleith *et al*., 2005) and acoustic (Amorim, 2004; 2008) also play an important role in species recognition in African cichlids.

African cichlids use acoustics during antagonistic encounters, territorial defence and also during courtship (Amorim *et al*., 2004; Simões *et al*., 2008). A study by Danley *et al*. (2012), suggested that cichlids are able use acoustics to distinguish between conspecific and heterospecific mates. In the current study, acoustics cues were investigated in the context of courtship. The results of this study found a significant difference in acoustic cues between *M. callainos* and *M. estherae*. Previous studies also found significant differences in acoustic signals between different species of cichlids in Lake Malawi (Lobel, 2001; Amorim *et al.,* 2004; 2008). The results of this study also found a significant difference in acoustic cues between red and blue *M. estherae* males. This is in agreement with previous studies that also found a significant difference in acoustic signals between populations of the same species in cichlids of Lake Malawi (Danley *et al*., 2012) and Lake Victoria (Verzijden *et al*., 2010). Differences in sounds between individuals of the same species are thought to (1) play a role in sexual selection through male-male competition (2) play a role in mate choice and mate recognition (Amorim *et al*., 2008), and (3) may suggest that acoustic signals may be diverging between geographically isolated individuals (Danley *et al*., 2012). Elsewhere in the world, intra-population acoustic differences were observed in the frog species *Allobates femoralis* (Simões *et al*., 2008) and *Physalaemus petersi* (Boul *et al*., 2007). However, these studies found that the acoustic differences were due to body size, and this was also demonstrated in other fish species (Myrberg *et al*., 1993; Lobel and Mann, 1995). In the current study, the results of the correlation tests did not show any significant correlations between sound characteristics and body size (SL and weight) of the fish. A previous study of

Lake Malawi (Amorim *et al*., 2008) and Lake Victoria (Verziljil *et al*., 2010) cichlid fish also did not find any significant correlation between body size and acoustic cues.

Playback experiments show that female cichlids prefer to court with males that are associated with sound (Verzijden *et al*., 2010). This further suggests that sounds can influence mate choice and is thought to assist in assessing the quality of males (Simões *et al*., 2008). One study (Maruska *et al*., 2012) found that in the Lake Tanganyika cichlid fish *Astatotilapia burtoni*, the females always courted with males that produced sound. Their study found that gravid females of *A. burtoni* were 2 - 5 times more sensitive to low frequency sounds compared to mouth brooding females, and that gravid females responded more to males associated with courtship sound. This further suggests that courtship sound is used during mate choice.

Some previous studies show that sound production is always accompanied by courtship displays (Lobel, 1998; Ripley and Lobel, 2004). However, this was not the case during the current study. In other instances, sound was produced with no apparent courtship display. Maruska *et al.*, (2012) also demonstrated that the males of *A. burtoni* did not always produce sounds during body quivers. A previous research demonstrated that sound is not a by-product of body movements and that males are able to control when and where sound is produced (Spinks *et al*., 2016). Other research (Betucci *et al*., 2010) found that acoustic signals alone cannot trigger a response in Lake Malawi cichlids and also that behavioural response is associated with visual cues. This may suggest that multimodal cues are employed in mate selection.

4.5 Conclusions

The results of the current study demonstrate that there are acoustic differences between *M. estherae* and *M. callainos*. Intraspecific differences in acoustic cues were also demonstrated in the current study. Previous studies also found differences in acoustic cues in cichlid fish from Lake Malawi (Amorim *et al*., 2004; Simões *et al*., 2008; Danley *et al*., 2012), Lake Victoria (Verzijden *et al*., 2010) and Lake Tanganyika (Maruska *et al*., 2012). Acoustic differences are also found in other fish species (Myrberg *et al*., 1993; Lobel and Mann, 1995), insects (Gray and Cade, 2000; Izzo and Gray, 2004) and in amphibians (Boul *et al*., 2006) and are thought to promote mate recognition. The results of the current study suggest that acoustic cues may be used for species recognition and can potentially influence reproductive isolation in cichlid fishes.

Chapter 5. Genetic and morphological differences between *Metriaclima estherae* and *M. callainos*

Abstract

Cichlid fish are one of the most specious fishes in the world, with over 2000 species endemic in East African Lakes. Allopatric speciation and isolation of peripheral lagoons due to fluctuations of water levels is thought to have contributed to the radiation of these fishes. However, studies also suggest that sympatric speciation through the aid of sexual selection has driven rapid speciation in these fishes. *Metriaclima estherae* and *M. callainos* belong to the *Pseudotropheus zebra* species complex of Lake Malawi. This species complex is one of the most species rich complexes; it is the most colour diversity and contains species that are similar in both morphology and behaviour. *Metriaclima estherae* and *M. callainos* are morphologically similar. The aim of this study was to investigate the genetic and morphological differences between *M. estherae* and *M. callainos*. Genetic analyses were conducted using mitochondrial control region FISH L15926 and H00650 and multivariate methods were used to assess morphological variation. The results demonstrate that *M. estherae* and *M. callainos* are distinct enough to be considered separate species, although there is not complete genetic differentiation between the two species.

5.1. Introduction

The highest diversity of cichlid species is found in Lake Malawi where ~1500 species are endemic (Genner *et al*., 2004; Koblmbuller *et al*., 2008). The cichlid fish of Lake Malawi are thought to have evolved ~ 700 000 years ago into high species diversity and abundances (Meyer, 1993; Stiassny and Meyer, 1999; Turner, 1999). The radiation of these species was formed in intralacustrine conditions from a single common ancestor (Meyer *et al*., 1990; Moran *et al*., 1994) because of subdivision (Coulter, 1991) or isolation of peripheral lagoons due to fluctuations of water levels (Greenwood, 1965). However, more recent studies suggest that sympatric speciation through the aid of sexual selection is the main mechanism through which the speciation of haplochromine cichlids of Lake Malawi occurred (Turner and Burrows, 1995; Higashi *et al*., 1999). Further the process of sympatric speciation often occurs rapidly compared to allopatric speciation (Dieckmann and Doebeli, 1999), which could explain the extremely young age of Lake Malawi cichlids (Barluenga and Meyer, 2004).

Lake Malawi only has five species belonging to the talipine tribe and the rest to the haplochromine tribe (Turner *et al*., 2001). The haplochromine tribe of Lake Malawi has ~700 species (Kocher, 2004; Danley *et al*., 2012) and can further be divided into two major clades: the rock-dwelling (also known as the mbuna) and the sand dwelling clade (Albertson *et al*., 1999). Morphological, ecological and molecular studies suggest that the mbuna clade of Lake Malawi forms a monophyletic group (Meyer, 1993; Moran *et al*., 1994). The species of this clade have similar body shape and trophic morphology, but their males differ in nuptial colour (Allender *et al*., 2003; Stauffer *et al*., 2013). Colour polymorphism is common within populations of haplochromine cichlids and it closely resembles differences between species (Seehausen *et al*., 1999).

Metriaclima estherae and *M. callainos* are mbuna cichlids endemic to Lake Malawi. *Metriaclima estherae* was collected from Minos Reef and *M. callainos* was collected from Chidunga Rocks. *Metriaclima estherae* has orange females and their males have red and blue colour morphs (Kasembe, 2017). The blue colour morphs of *M. estherae* males have faint vertical bars (Kasembe, 2017) while both sexes of *M. callainos* are blue and do not have bars (Stauffer and Hert, 1992). The blue morph of *M. estherae* bears a striking resemblance to *M. callainos* and both species can easily be confused with each other. As the study species are closely related and have similar morphology and colour (Stauffer and Hert, 1992), these two taxa provide an excellent model to demonstrate reproductive isolation and species recognition among closely related taxa.

Mate choice and species recognition between the study species was investigated in Chapter 2 and 3 of the current thesis. The results found that *M. estherae* does not mate assortatively while *M. callainos* recognize and choose conspecific mates by using visual and chemical cues. Much of the mbuna taxonomy is known; however, there is still debate about their status as biological species (Genner and Turner, 2005). Furthermore, the species diagnosis of some mbuna has not been validated due to little morphological and genetic differences (Genner and Turner, 2005). The aim of this study was to examine the extent of genetic differentiation and thereby confirm that these two species are indeed separate species. This will also help to eliminate any morphological confusion between these two species. Molecular techniques and morphological analyses were employed to validate the taxonomy of the study species, using mitochondrial DNA markers.

5.2. Materials and methods

5.2.1. Housing

Details about fish collection and housing are described in Chapter 2, Section 2.1.1.

5.2.2. DNA extraction

DNA was extracted from *M. estherae* (N = 48) and *M. callainos* (N = 36) individuals. The DNA was collected from small clippings of caudal fins since this would have limited impact on fish mobility (Schebesta *et al*., 2006). The DNA was extracted using the Promega Wizard DNA Extraction Kit (Madison, New York) following the methods of the manufacturer. DNA was further diluted by mixing 1µL of DNA with 20µL of Millipore water and stored in a -20 ºC freezer for further analysis.

5.2.3. Polymerase chain reaction (PCR)

The following primers were amplified: Cytochrome oxidase I (COI) mitochondrial gene LCO1490 (5′GGTCAACAAATCATAAAGATATTGG-3′) and HCO 2198 (5′TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al*., 1994) and Control region (CR) FISH L15926 (5’GAGCGCCGGTCTTGTAAKCC) and H00650 (5’-TGATAGTAAAGTCAGGACCAAGC) (Shaw *et al*., 2000). The primers were amplified by mixing 8µL of purified water, 12.5µL of enzyme KM507 (KAPA Biotech), 1µL primers and 3.5µL of DNA template. The 25µL PCR volume was run in a BioRad PCR machine, with the following program: 3 min at 94 ºC, 40 cycles of 3 s of 93 ºC, 45 s at 60 ºC, 30 s at 72 ºC and a holding temperature of 15 ºC. All PCR products were visualized on 1 % agarose gel stained with ethidium bromide to observe the quality and quantity of DNA. The PCR samples were purified using Illustra™, GE Healthcare commercial kit following the manufactures protocol. Sequencing was performed in an automated sequencer (AB 3100, Applied Biosystems) at Central Analytical Facilities (CAF), Stellenbosch University, South Africa. All sequences were blasted in GenBank (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) to authenticate the sequences. Only N = 18 *M. estherae* and N = 5 *M. callainos* specimens were successfully amplified. The following 11 additional sequences for *M. callainos* CR were therefore obtained from GenBank for analysis, Accession number: HQ335186, KC208908.1, KC208910.1, KC208912.1, KC208915.1, KC208916.1, KC208917.1, KC208909.1, AY911810, AY911811 and AF213620.1.

5.2.4. Morphological analysis

Morphometric differences between *M. estherae* and *M. callainos* were analysed following the procedure of Allen (1985 and 1987) by measuring parts of the fish with a digital calliper to the nearest 0.01 mm (see Appendix 3 , Table 10.1).

5.3. Statistical analysis

5.3.1. Sequence alignment

A 704 base pair (bp) DNA fragment was produced. All sequences were edited using BioEdit Sequence Alignment Editor v. 7.2.5 (Hall, 1999) and aligned with Mega X v. 10.0.1 (Kumar *et al.*, 2016). To examine the current levels of variation and genetic diversity of *M. estherae* and *M. callainos*, pairwise differences were generated by using PAUP* v. 4.0 beta 10 (Swofford, 2002). Population differentiation among the localities was calculated using Arlequin v. 3.5.1.2 (Excoffier and Lischer, 2010). DNAsp v. 5.10.1 (Librado and Rozas,

2009) was used to review the Haplotype (*h*) and nucleotide diversity (π) were calculated for each species. Tajima's *D* test (Tajima, 1989) and Fu's F_s test (Fu, 1997) were used to calculate population changes and gene selection. To determine the genealogical relationships among haplotypes/alleles and to visualize their geographic distributions, a median joining tree was generated using Network v. 5.0.0.3 (Fluxus Technologies).

The phylogenetic relationships between the species was estimated using a maximum parsimony (MP) tree, by using outgroup rooting and the heuristic search algorithm in PAUP* v. 4.0 beta 10 (Swofford, 2002). A consensus tree was produced from equally parsimonious trees using the 50 % majority rule. To evaluate confidence of the nodes a bootstrap analysis were performed in PAUP* v. 4.0 beta 10 with 1000 replications. Bootstrap values above 75 % were considered well supported while bootstrap values below 75 % were considered poorly supported (Felsenstein, 1985). A non mbuna cichlid, *Oreochromis niloticus* was selected as outgroup (Accession number: AF328849.1). Lake Malawi cichlids are very closely related due to their rapid expansion; hence an outgroup slightly more distant was chosen to effectively eliminate phylogenetic noise due to the high similarity between Lake Malawi cichlids.

5.3.2. Morphometric analysis

Multivariate Analysis of Variance (MANOVA) and Discriminate Function Analysis (DFA) were used to test for morphometric differences between the species and sexes. Statistics were performed using IBM SPSS software v. 24.0.

5.4. Results

5.4.1. Sequence divergence

Average sequence divergence within *M. estherae* was 0.006 ± 0.006 and 0.64 %. Average sequence divergence was 0.011 ± 0.112 and 0.05 % for *M. callainos* (Table 5.1). The data contained a total of 9 haplotype (Table 5.2). Haplotype 4 and 5 were shared amongst some localities. Haplotype 4 (H4) was shared between Nkatha Bay, Lake Malawi and Minos Reef and H5 was shared between Nkatha Bay and Chindunga Rocks (Table 5.2). Haplotype diversity for *M. estherae* was 0.647 (±0.095) and nucleotide diversity was 0.006 (±0.001). Haplotype diversity (*h*) for *M. callainos* was 0.758 (±0.110) and nucleotide diversity (π) was 0.006 (±0.002) (Table 5.3). The Tajima's D and Fu's Fs were also low for *M. estherae* and negative for *M. callainos* and were non-significant for both species, $p > 0.10$ (Table 5.3).

Table 5.1: Average pairwise differentiation, with standard deviation, within and between *M. estherae* and *M. callainos*.

	Mean ± SD	**%**
M. estherae	0.006 ± 0.006	0.64
M. callainos	0.006 ± 0.006	0.05
M. estherae* vs *M. callainos	0.009 ± 0.042	0.98

Table 5.2: The distribution of Control region haplotypes of *M. estherae* and *M. callainos* in Minos Reef, Lake Malawi, Nkatha Bay and Chidunga.

Haplotype	**Locality**	***M. estherae* (18)**	***M. callainos* (16)**	**Total**
Hap 1	Minos	4	0	4
Hap 2	Minos Reef	10	0	10
Hap 3	Minos Reef	1	0	1
Hap 4	Minos Reef, Lake Malawi, Nkatha Bay	3	1	4
Hap 5	Nkatha, Chidunga	0	8	8
Hap 6	Nkatha Bay	0	1	1
Hap 7	Lake Malawi	0	2	2
Hap 8	Lake Malawi	0	2	2
Hap 9	Lake Malawi	0	2	2
Total		**18**	**16**	**34**

Table 5.3: Genetic diversity of *M. estherae* and *M. callainos*. N = sample size, Nh = haplotype number, S = number of polymorphic sites, h = haplotype diversity, π = nucleotide diversity, D = Tajima's D, F = Fu's Fs. p > 0.10.

Population	**N**	**Nh**	**S**	**h**	**π**	**D**	**Fs**	**K**
M. estherae	18	4	8	0.647 ±0.095	0.006 ±0.001	0.753	0.800	2.830
M. callainos	16	8	15	0.758 ±0.110	0.006 ±0.002	-1.448	-1.620	2.850

5.4.2. Population structure

The haplotype network tree shows that in some localities, *M. estherae* (Minos Reef) share similar genes with *M. callainos* from other parts of Lake Malawi and *M. callainos* from Nkhatha Bay. *Metriaclima callainos* from Chidunga Rocks in some cases shares the same genes with *M. callainos* from Nkhatha Bay (Figure 5.1).

The phylogenetic tree showed that the data consists of three clades. Clade 1 consists of *M. estherae* plus one *M. callainos* sample from an unknown location in Lake Malawi. Clade 2 consists of *M. estherae* only. The third clade consists of a mixture of both species, although mostly *M. callainos* (Figure 5.2).

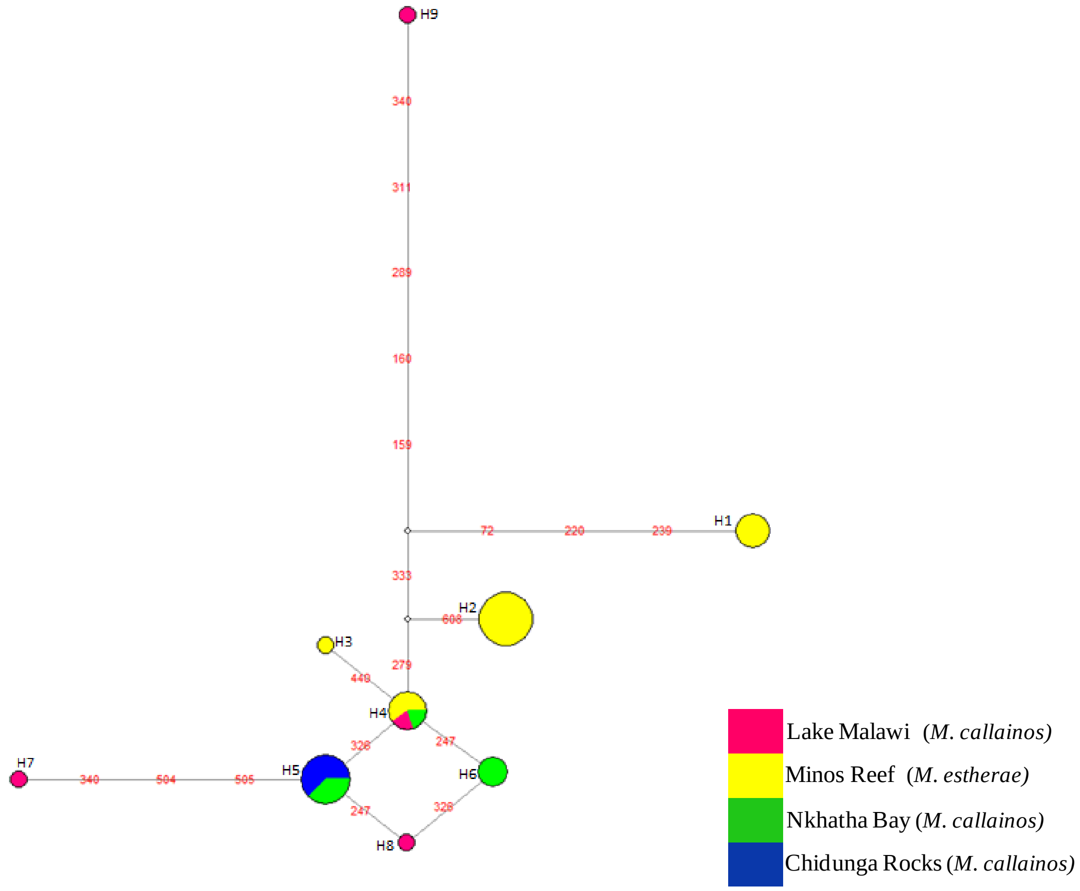

Figure 5.1: Median-joining haplotype network of *M. estherae* and *M. callainos* based on mtDNA control region from four localities from Lake Malawi. Each circle represents a single haplotype and its size is proportional to the number of individuals within that haplotype. The colour codes represent the geographical origin of the haplotypes.

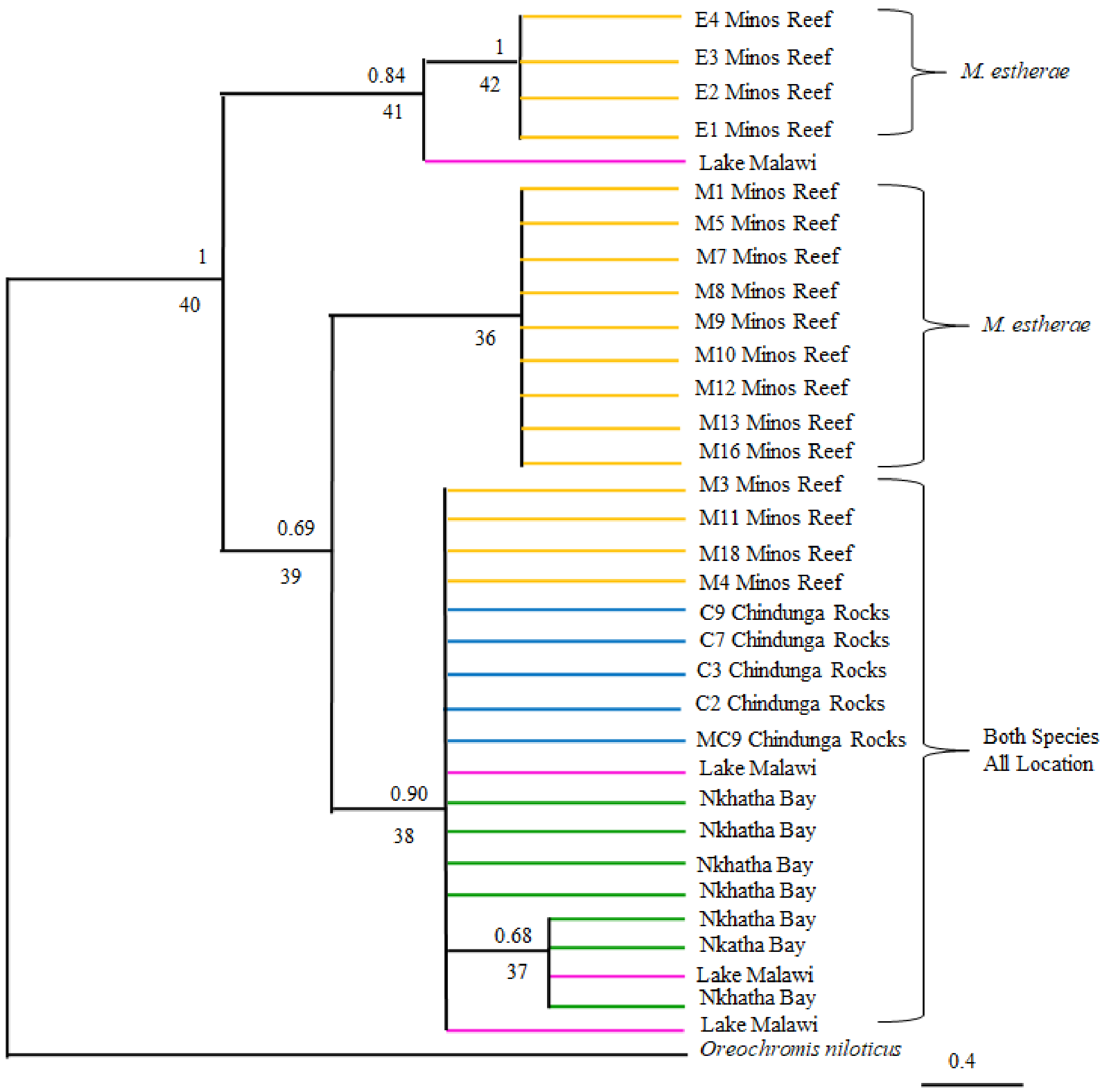

Figure 5.2: Phylogenetic tree of *M. estherae* and *M. callainos* using Control region and rooted by *Orechromis niloticus.* Bootstrap support (Bayesian and Maximum parsimony) are shown on the branches, the tree forms three major clades indicated by the lines.

5.4.3. Morphometric analysis

Overall, the multivariate analysis showed that morphometric measurements were significantly different between *M. estherae* and *M. callainos* (Wilks' Lambda = 0.262, F = 4.075, p < 0.001) and between sexes (Wilks' Lambda = 0.359F = 2.583, p = 0.013) (Figure 5.3). There was a significant difference between the blue and red male *M. estherae* and the female *M. estherae* (Figure 5.3). No significant difference was observed between the interaction of species and sex (Wilks' Lambda = 0.502, F = 1.431, p = 0.198). The morphometric characteristics that were significantly different (p < 0.05) between the species and sexes are presented in Table 5.4. The results of the canonical discriminant function analysis show that Functions 1 and 2 contributed a combined 72.2 % of the total variation in the data (Table 5.5). Morphometric characters that contributed the most to the first Discriminant Function were Orbit diameter (1.007), Pre anal length (0.574), Caudal peduncle length (-0.511) and Inter orbital diameter (-0.502), and to the second Discriminant Function were Pre anal length (-0.746), Body width (-0.648), Anal fin length (0.583) and Total length (0.485) (Table 5.6). The first discriminant function separates out the two species, with blue *M. estherae* closer to *M. callainos* on this axis. The second discriminant function separates males and females and also separates the two male colour morphs of *M. estherae.*

Table 5.4: Morphometric characteristics that were significantly different between species and sexes ($p < 0.05$) of *M. estherae* and *M. callainos*.

Significantly different between species	Significantly different between sexes
Standard length	Standard length
Orbit diameter	Orbit diameter
Pre-dorsal length	Pre-dorsal length
Pre-anal length	Head length
Pre-pelvic length	Inter-orbital width
	Dorsal fin length

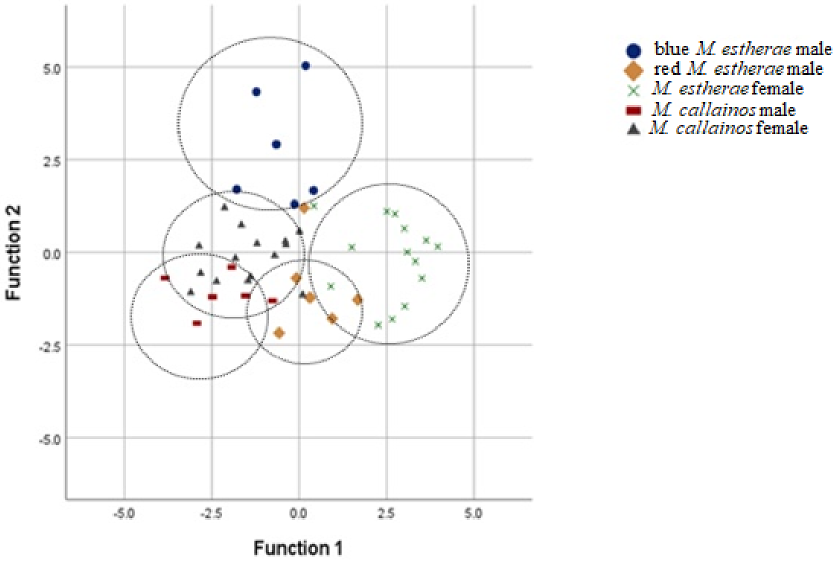

Figure 5.3: Canonical centroid plot of the first two discriminant functions of the morphometric measurements taken from *M. estherae* and *M. callainos*.

Table 5.5: The first four Canonical Discriminant Functions from the Discriminant Function Analysis obtained from morphometric variables of *M. estherae* and *M. callainos*.

Eigenvalues

Function	**Eigenvalue**	**% of Variance**	**Cumulative %**	**Canonical Correlation**
1	3.837[a]	52.2	52.2	0.891
2	1.473[a]	20.0	72.2	0.772
3	1.334[a]	18.1	90.3	0.756
4	0.710[a]	9.7	100.0	0.644

Table 5.6: Results of the Standardized Canonical Discriminant Function (CDFs) Coefficients of the morphometric measurements obtained from *M. estherae* and *M. callainos*.

	1	**2**	**3**	**4**
SL	0.024	0.142	0.734	-0.702
FL	0.288	-0.356	-0.257	-0.408
TL	0.248	0.485	0.563	-0.112
BW	0.188	-0.648	0.426	0.000
SNL	-0.297	-0.179	0.070	0.381
HL	-0.481	-0.281	-0.672	-0.087
OD	1.007	0.120	0.919	0.416
IO	-0.502	0.198	0.068	0.523
CPL	-0.511	0.293	-0.526	0.848
CPD	-0.169	0.179	-0.514	0.460
UCL	0.037	0.432	-0.544	0.360
MCL	-0.050	0.200	-0.152	0.063
DL	-0.011	0.032	0.908	-0.340

AL	0.065	0.583	0.297	0.207
PL	-0.253	0.029	-0.130	0.010
PDL	0.318	0.100	-0.237	-0.558
PAL	0.574	0.746	-0.171	-0.867
PVL	-0.316	-0.314	-0.349	1.044

5.5. Discussion

Lake Malawi contains the highest diversity of cichlid species (Turner *et al*., 2001). Previously, it was suggested that the rapid speciation of Lake Malawi cichlids was due to factors such as mouth brooding characteristics (Fryer and Iles, 1972), habitat complexity (Temple, 1969), lake fluctuations (Fryer, 1959), predation (Greenwood, 1965), mutation (Fryer and Iles, 1972), hybridization (Capron de Caprona and Fritzsch, 1984) and trophic polymorphism (Sage and Selander, 1975). However, some studies suggest that sexual selection may have played a role in the speciation of these fishes (Turner and Burrows, 1995; Higashi *et al*., 1999). Molecular studies have also discovered that the radiation of these fishes have occurred by intralacustrine speciation from a single common ancestor, which is demonstrated by monophyly within the lake (Meyer *et al*., 1990; Moran *et al*., 1994). The study species, *M. estherae* and *M. callainos*, belong to the mbuna tribe of Lake Malawi (Kasembe, 2017). Most species within the mbuna tribe, especially those belonging to the subgenus *Pseudotropheus zebra* complex, are morphologically and behaviourally similar (Ribbink *et al.*, 1983). The aim of the current study was to investigate the genetic relationship and morphological variation between these two species.

Initially, mitochondrial DNA COI and CR were amplified in the current study. Although COI was successfully amplified, there was no variation in the sequences produced. As a result, statistical analysis could not be further performed for COI and only results for CR are presented. In addition, a previous study demonstrated that the control region is the best marker to address splits among closely related taxa (Durtner *et al*., 2005). Bowers *et al*., (1994) investigated intra and inter-specific genetic variation between mbuna cichlids using CR. Their results found a mean sequence divergence of 0.45 % for *Melanochromis auratus*, 1.13 % for *M. heterochromis* and a sequence divergence of 1.1 % between the two species (Bowers *et al.,* 1994). The low sequence divergence between the two species was attributed to recent isolation between the two species (less than 10000 years) or population bottlenecks following colonization events. In the present study, *M. estherae* demonstrated an average sequence divergence of 0.64 % and *M. callainos* 0.05 %. An average sequence divergence of 0.98 % was observed between *M. estherae* and *M. callainos*. Moran *et al*. (1994) also found a sequence divergence of 1.0 % between 40 species of Lake Malawi. In contrast, the latter study found a sequence divergence of 1.7 % within the non-mbuna haplochromine cichlids of Lake Malawi. The results of the current study thus show that there is genetic variation between *M. estherae* and *M. callainos*, as the values of the sequences are similar to previous studies. However, the values of sequence divergence within the Malawi haplochromine cichlids are low when compared to other non-cichlid fish species. For example, Bermingham and Avise (1986) found a sequence divergence of 4.0 % within the freshwater fish *Lepomis punctatus*.

The Tajima’s D and Fu’s F test are indicators of demographic changes such as population decline, bottlenecks, founder events or population expansion (Tajimas, 1989; Fu, 1997). In the current study, positive Tajima’s D and Fu’s F values were observed for *M. estherae*. This is thought to be an indication of balancing selection or a mixture of isolated populations (Table 5.3) (Rand, 1996). The negative Tajima's D and Fu's F values for *M. callainos* may suggest an excess of low frequency haplotype and population expansion following a severe reduction in population size (Table 5.3) (Tajima, 1989). The results of the current study further demonstrate that the study species form three clades and at some localities these species exchange genes (Figures 5.1 and 5.2). This suggests that hybridization occurs in these species. Indeed, mate choice studies (Chapter 2 and 3 of the current thesis) demonstrate that both sexes of *M. estherae* and *M. callainos* can potentially interbreed.

In this study, *M. estherae* had higher sequence divergence compared to *M. callainos*. Genetic barriers such as colour (Fryer, 1959), water depths (Marsh and Ribbink, 1981) and habitat discontinuation (McKaye and Gray, 1984) are probably limiting gene flow in *M. callainos*. Reinthal and Meyer (1997) also found little interspecific sequence divergence between the populations of the *Pseudotropheus* (*Tropheops*) complex. A previous study focused on gene dispersal of four populations of the widely distributed *Pseudotropheus zebra* in Lake Malawi, found a significant differentiation at some allozymes loci (Mackaye *et al*., 1984). These findings were attributed to barriers that prohibited genetic dispersal (Mackaye *et al*., 1984). Lake Malawi alternates between sandy, muddy and rocky habitats, with numerous offshore islands and reefs (Genner and Turner, 2005). The mbuna species of Lake Malawi are confined within rocky substrates (Genner and Turner, 2005). These rocky habitats of Lake Malawi are generally small and patchily distributed, with the longest continuous stretch of rock recorded being 8.5 km long (Genner and Turner, 2005). These rocky habitats are thought to restrict migration rates and gene dispersal (Mackaye *et al*., 1984). On the other hand, van Oppen *et al*. (1997) found a significant genetic difference among the populations of four rocky species found in muddy or sandy bays of ~1 km wide. Additionally, Markert *et al*.

(1999) showed that Lake Malawi cichlid populations separated by deep water tend to be more genetically diverse and those found in rocky and sandy habitats tend to have less genetic differentiation. Another study found that fine-scale genetic sub-structuring exists in some mbuna species (Arnegard *et al*., 1999). This implies that the mbuna species may represent thousands of genetically divergent subunits (van Oppen *et al*., 1997). The results of a study (Chapters 2 and 3 of the current thesis) found that both sexes of *M. estherae* species have not established pre-zygotic barriers and are potentially capable of hybridizing with other species. This was further confirmed in the results of the current chapter that these two species do exchange genes at some localities (Figures 5.1 and 5.2). Previous studies also indicate that these species do hybridise in natural (van Oppen *et al*., 1998) and laboratory conditions (Knight *et al*., 1998).

There is no formal morphometric diagnosis for the haplochromine cichlids (Genner and Turner, 2005). Research demonstrates that they share similar characteristics, such as a large number of small scales on the nape and chest region, abrupt transition from large flank scales to small chest scales, a reduction of the left ovary and possession of true ocelli (Fryer, 1959; Oliver, 1984). The morphometric of Lake Malawi cichlid species are especially problematic because many distinct species are morphologically similar (e.g. number of lateral line scales, number of cheek scales, horizontal eye diameter, head length) (Gerner and Turner, 2005) and their morphological characters may be subjected to convergence (Kotcher *et al*., 1993). Some authors suggest that colour and not morphometric differences are important for species diagnosis for mbuna cichlids (Fryer, 1959; Bowers and Stauffer, 1993; Stauffer *et al*., 1995; 1997). The results of the morphometric analysis conducted here demonstrated that there are significant morphological differences between *M. estherae* and *M. callainos* and also between sexes and colour morphs within the same species.

Overall, the results of the study show that *M. estherae* and *M. callainos* exhibit genetical and morphological divergence, but that this divergence is incomplete. There is also low genetic differentiation between the two species, possibly due to hybridizing, or due to being recently diverged.

Chapter 6. General Discussion

The role of sexual selection in the evolution of African cichlids has been extensively studied. However, many of those studies focused on visual cues as the main driver of speciation (Seehausen and Alphen, 1998; Knight and Turner, 1999; Couldridge and Alexander, 2002; Knight and Turner, 2004; Maan *et al*., 2004), with little consideration that visual cues alone do not always provide sufficient evidence regarding the role of sexual selection in the divergence of African cichlids (Knight and Turner, 1999). All modes of communication must be considered in order to understand how mating decisions can influence sexual selection and reproductive isolation in closely related cichlid species (Verzijden *et al*., 2010). In the current study, visual, chemical and acoustics cues were examined to investigate species recognition and reproductive isolation within and between two closely related species of African cichlids that differ in their colouration.

The results of this study demonstrate that *M. callainos* females use visual cues for mate recognition (Chapter 2), whereas both visual and chemical cues are important for species recognition for *M. callainos* males (Chapter 3). However, *M. callainos* females did not prefer blue males when given a choice between the males of red and blue *M. estherae*. The reason for this is not clear since previous studies showed that in the absence of conspecific males, females choose males with similar colours (Couldridge and Alexander, 2002; Knight and Turner, 2004)

Both males and females of *M. estherae* did not recognise conspecific mates (Chapter 2 and Chapter 3, respectively). The failure of *M. estherae* males to recognise conspecific females is not clear since *M. estherae* females only have an orange morph, which can be easily identified from the blue morph of *M. callainos* females. However, a previous study demonstrated that some males of African cichlids simply cannot recognise conspecific females even when the females are differently coloured (Knight and Turner, 1999). *Metriaclima estherae* females could have failed to prefer conspecifics due to the resemblance of the males of the two species. Alternatively, as the males of *M. estherae* come in two contrasting colour morphs, there may be selection against a strong colour preference in this species.

When taking into account the results of the current study and of other previous studies (Knight *et al.*, 1998; Knight and Turner, 1999; Couldridge and Alexander, 2002; Maan and Sefc, 2013; Selz *et al.*, 2013; Tyers and Turner, 2013; Selz *et al.*, 2014), it seems that visual (colour) cues are the main cue used for reproductive isolation for the majority of mbuna cichlids, although colour is not equally important for all species. It appears that the role of chemical cues for species recognition is less important than that of visual cues. Previously, Fryer (1959) suggested that visual cues, not molecular characteristics or morphometric, is important for species diagnosis in mbuna cichlids. This lends further support for the importance of visual (colour) cues in the process of speciation in African cichlids. Indeed, research shows that cichlids have trichromatic vision, implying that visual capabilities are well developed in these fishes (van der Meer and Bowmaker, 1995; Escobar-Camacho *et al.*, 2017).

There is contradicting evidence regarding the cues used by African cichlid males for mate recognition. Some authors suggests that visual cues are used for species recognition by males (Knight and Turner, 1999), while others suggest that males use chemical cues instead (Venesky *et al*., 2005). The results of the current study suggest that both visual and chemical cues are important for mate recognition by males. More experiments are required to assess the role of male African cichlids in species recognition and reproductive isolation.

The results of Chapter 4 show that there are inter- and intra-specific acoustic differences between *M. estherae* and *M. callainos*. This suggests that acoustic cues can also be used for species recognition and reproductive isolation in mbuna cichlids. Studies demonstrate that in cichlids fishes, acoustic signals are used during antagonistic encounters, territorial defence and during courtship (Amorim *et al*., 2004; Simões *et al*., 2008). A study further demonstrated that some species of African cichlids are able to produce sound as early as 45 days old (Bertucci *et al*., 2012). Research further suggests that acoustic signals in cichlids may provide information about mate location and readiness to spawn (Lobel *et al*., 1998).

Previous research that focused on playback experiments show that the females of African cichlid fishes prefers to court with males that are associated with sound (Verzijden *et al*., 2010). Future studies should conduct playback experiments for *M. estherae* and *M. callainos* to observe how sound influences their mate choice.

The findings of the current study indicate that there are morphological differences between *M. estherae* and *M. callainos* and between the sexes of these species (Chapter 5). The results also confirmed that *M. estherae* and *M. callainos* demonstrate genetic variation and at some localities these species do exchange genes (Chapter 5, Figures 5.1 and 5.2). Several studies investigating the genetic relationship of mbuna cichlids using mtDNA and microsatellites did not get tangible results due to small sequence variation between species (Bowers *et al*., 1994; Moran and Kornfield, 1993; Kornfield and Parker, 1997; Smith and Kornfield, 2002; Rico *et al.*, 2003). Allozyme polymorphism based on variation in the sequences of protein-coding nuclear DNA also did not give adequate information about mbuna relationships (Verheyen and van Rompaey, 1986; Kuusipalo, 2000). Future studies should focus on investigating whole genome sequences to obtain clear genetic relationships of the mbuna species.

The in the current study, we hypothesise that multiple sensory cues are required for species recognition and reproductive isolation in African cichlid species. Our findings suggest that visual cues alone may be sufficient for species recognition by females in some species of African cichlids, but not others. Chemical cues are not essential for species recognition and reproductive isolation for females, but are probably important for males. We also show that even extremely closely related species differ in their abilities to discriminate conspecifics and may rely on different factors to maintain isolation.

Chapter 7. References

Albertson, R.C., Markert, J.A., Danley P.D, and Kocher, T.D. 1999. Phylogeny of a rapidly evolving clade: the cichlid fishes of Lake Malawi, East Africa. *Proceedings of the National Academy of Sciences of the United States of America* **96:** 5107-5110.

Allen, G. R. 1987. Synopsis of the circum tropical fish genus *Lutjanus* (Lutjanidae). In: Tropical snappers and groupers: biology and fisheries management. J. J. Polovina and Ralston S. (eds.). Westview Press Inc., Boulder, Colorado, pp 33-88.

Allender, C. J., Seehausen, O., Knight, M. E., Turner, G. F. and Maclean, N. 2003. Divergent selection during speciation of Lake Malawi cichlid fish inferred from parallel radiations in nuptial coloration. *Proceedings of the National Academy of Sciences of the United States of America* **100:** 14074-14079.

Amorim, M. C. P, Simoes, J. M., Fonseca, P.J. and Turner, G.F. 2008. Species differences in courtship acoustic signals among five Lake Malawi cichlid species (*Pseudotropheus* spp.). *Journal of fish Biology* **72:** 1355-1368.

Amorim, M.C.P. and Almada, V.C. 2005. The outcome of male-male encounters affects subsequent sound production during courtship in the cichlid fish *Oreochromis mossambicus*. *Animal Behaviour* **69:** 595-601.

Amorim, M.C.P., Knight, M.E., Stratoudakis, Y. and Turner, G.F. 2004. Differences in sounds made by courting males of three closely related Lake Malawi cichlid species. *Journal of Fish Biology* **65:** 1358-1371.

Andersson, M. 1982. Female choice selects for extreme tail length in a widow bird. *Nature* **299:** 818-820.

Andersson, M. 1994. Sexual selection. Princeton, NJ: Princeton University Press.

Arnegard, M. E., Markert, J. A. Danley, Jr. P. D., Stauffer, J. R. Ambali, A. and Kocher, T. D. 1999. Population structure and colour variation in the lithophilous cichlid *Labeotropheus fuelleborni* Ahl along a recently formed archipelago of rocky habitat patches in southern Lake Malawi. *Proceedings of the Royal Society of London: Series B* **266:** 119-130.

Barel, E.D.N., Van Oijen, M.J.O., Witte, F. and Witte-Maas, E.L.M. 1977. An introduction to the taxonomy and morphology of the Haplochromine Cichlidae from Lake Victoria. Part A. Text. *Netherlands Journal of Zoology* **27:** 333-389.

Baric, S., Salzburger, W. and Sturmbauer, C. 2002. Phylogeography and evolution of the Tanganyikan cichlid genus *Tropheus* based upon mitochondrial DNA sequences. *Journal of Molecular Evolution* **55:** 1-15.

Barlow, G.W. 2002. How behavioural studies contribute to the species problem: a piscine perspective. *Fish and Fisheries* **3**:197-212.

Barluenga, M. and Meyer, A. 2004. The Midas cichlid species complex: incipient sympatric speciation in Nicaraguan cichlid fishes? *Molecular Ecology* **3:** 2061-2076.

Barton, N.B. and Charlesworth, B. 1984. Founder effects, genetic revolutions, and speciation. *Annual Review of Ecology and Systematics* **15:** 133-164.

Beeching, S.C. and Hopp, A.B. 1999. Male mate preference and size-assortative pairing in the convict cichlid. *Journal of Fish Biology* **55:** 1001-1008.

Bermingham, E. and Avise, J. C. 1986. Molecular zoogeography of freshwater fishes in the South- Eastern United States. *Genetics* **113:** 939-965.

Bertucci, F., Beauchaud, M., Attia, J. and Mathevon, N. 2010. Sound modulates males Aggressiveness in a cichlid fish. *Ethology* **116:** 1179-1188.

Bertucci, F., Scaion, D., Beauchaud, M., Attia, J. and Mathevon, N. 2012. Ontogenesis of agonistic vocalizations in the cichlid fish M*etriaclima zebra*. *Ethology* **335**: 529-534.

Blais, J., Plenderleith, M., Rico, C., Taylor, M.I., Seehausen, O, van Oosterhout, C. and Turner, G. 2009. Assortative mating among Lake Malawi cichlid fish populations is not simply predictable from male nuptial colour. *BMC Evolutionary Biology* **9:** 1-12.

Blows, M.W. and Allan, R. A. 1998. Levels of mate recognition within and between two *Drosophila* species and their hybrids. *The American Naturalist* **152:** 826–837

Boake, C. R.B., De Angelis, M.P. and Andreadis, D.K. 1997. Is sexual selection and species recognition a continuum? Mating behaviour of the stalk-eyed fly *Drosophila heteroneura*. *Proceedings of the National Academy of Sciences of the United States of America* **94:** 442-445.

Boake, C.R.B. 2002. Sexual signalling and speciation, a micro evolutionary perspective. *Genetica* **116:** 205-214.

Boughman, J.W. 2001. Divergent sexual selection enhances reproductive isolation in sticklebacks. *Nature* **411:** 944-948.

Boul, K.E., Funk, W.C., Darst, C.R., Cannatella, D.C. and Ryan, M.J. 2007. Sexual selection drives speciation in an Amazonian frog. *Proceedings of the Royal Society of London* **274:** 399-406.

Bowers, N., Stauffer, J.R. and Kocher, T.D. 1994. Intra- and interspecific mitochondrial DNA sequence variation within two species of rock-dwelling cichlids (Teleostei: Cichlidae) from Lake Malawi, Africa. *Molecular Phylogenetics and Evolution* **3:**75-82.

Bowers, N.J. and Stauffer, J.R. 1993. A new species of rock- dwelling cichlid (Pisces: Cichlidae) from Lake Malawi, Africa, with comments on *Melanochromis vermivorus* Trewavas. *Copeia* **1993:** 715-722.

Byers, B.E. and Kroodsma, D.E. 2009. Female mate choice and songbird song repertoires. *Animal Behaviour* **77:** 13-22.

Candolin, U. 2003. The use of multiple cues in mate choice. *Biological Review of the Cambridge Philosophical Society* **78:** 575-595.

Capron de Caprona, D. and Ryan, M. 1990. Conspecific mate recognition in swardtails, *Xophophorus nigrensis* and *X. pygmaeus* (Poeciliidae): olfactory and visual cues. *Animal Behaviour* **39:** 290-296.

Carleton, K.L., Spady, T.C. and Kocher, T.D. 2006. Visual communication in East African cichlid fishes: Diversity in a phylogenetic context. In: Communication in fishes, vol. 1. F. Ladich, S.P. Collin, P. Moller and B.G. Kapoor (eds.). Science Publishers, Enfield, pp 485-515.

Carson, H. L. 2003. Mate choice theory and the mode of selection in sexual populations. *Proceedings of the National Academy of Sciences of the United States of America* **100:** 6584-6587.

Catchpole, C.K. and Slater, P.J.B. 2008. Bird song: biological themes and variations. Cambridge (UK): Cambridge University Press.

Chenoweth, S.F. and Blows, M.W. 2006. Dissecting the complex genetic basis of mate choice. *Nature Review Genetics* **7:** 681-692.

Cohen, A.S. 1995. Paleoecological approaches to the conservation biology of benthos in ancient lakes: a case study from Lake Tanganyika. *Journal of the North American Benthological Society* **14:** 654-668.

Cohen, A.S., Stone, J.R. and Beuning, K.R.M. 2007. Ecological consequences of early Late Pleistocene mega droughts in tropical Africa. *Proceedings of the National Academy of Sciences of the United States of* America **104:** 16416-16421.

Conde´, B. and Gery, J. 1999. Maylandia Meyer et Foerster, 1984, un nom ge´ne´riquedisponible (Teleostei, Perciformes, Cichlidae). *Revue Francaised' Aquariologie Herpetologie* 2**6:** 21-22.

Couldridge, V.C. K. and Alexander, G.J. 2001. Does time spent near a male predict female mate choice in a Malawian cichlid? *Journal of Fish Biology* **59:** 667-672.

Couldridge, V.C.K. and Alexander, G.J. 2002. Colour patterns and species recognition in four closely related species of Lake Malawi cichlid. *Behavioural Ecology* **13:** 59-64.

Coulter, G.W. 1991. Lake Tanganyika and its Life. Oxford University Press, Oxford.

Coyne, J.A. and Orr, H.A. 2004. Speciation. Sunderland, MA: Sinauer Associates.

Crapon de Caprona, M.D. and Fritzsch, B. 1984. Interspecific fertile hybrids of Haplochromine Cichlidae (Teleostei) and their possible importance for speciation. *The Netherlands Journal of Zoology* **34:** 503-538.

Danley, P.D., Husemann, M. and Chetta, J. 2012. Acoustic diversity in Lake Malawi's rock-dwelling cichlids. *Environmental Biology of Fishes*: **93:** 23-30.

Darwall, W.R.T., Smith, K.G., Allen, D.J., Holland, R.A., Harrison, I.J. and Brooks, E.G.E. 2011. The diversity of Life in African Freshwaters: *Under Water, Under Threat. An analysis of the status and distribution of freshwater species throughout mainland Africa*. Cambridge, United Kingdom and Gland, Switzerland: IUCN.

Darwin, C. 1871. The descent of man and selection in relation to sex. London, UK: Murray.

Delvaux, D. 1995. Age of Lake Malawi (Nyasa) and water level fluctuations. Mus R Afr Centr Tervuren (Belg.) Dept Geol Min Rapp Ann **1996:** 99-108.

Dieckmann, U. and Doebelli, M. 1999. On the origin of species by sympatric speciation. *Nature* **400:** 354-357.

Dominey, W.D. 1984. Effects of sexual selection and life history on speciation: species flocks in African cichlids and Hawaiian *Drosophila*. In: Evolution of fish species flocks. A. A. Echelle and I. Kornfield (eds.). University of Maine Press, Orono, pp 231-249.

Dunz, A. R. and Schliewen, U. K. 2013. Molecular phylogeny and revised classification of the haplotilapiine cichlid fishes formerly referred to as ‘‘Tilapia’’. *Molecular Phylogenetics and Evolution* **68:** 64-80.

Egger, B., Mattersdorfer, K. and Sefc, M.C. 2010. Variable discrimination and asymmetric preferences in laboratory tests of reproductive isolation between cichlid colour morphs. *Journal of Evolutionary Biology* **23:** 433-439.

Egger, B., Obermüller, B., Eigner, E., Sturmbauer, C. and Sefc, K.M. 2008. Assortative mating preferences between colour morphs of the endemic Lake Tanganyika cichlid genus *Tropheus*. *Hydrobiologia* **615:** 37-48.

Endler, J.A. 1977. GeographicVariation, Speciation, and Clines. Princeton University Press, Princeton, NJ.

Escobar-Camacho, D. and Carleton, K.L. 2015. Sensory modalities in cichlid fish behaviour. *Current Opinion in Behavioral Sciences* **6:** 115-124.

Escobar-Camacho, D., Marshall, J.and Carleton, K.L. 2017. Behavioural colour vision in a cichlid fish: *Metriaclima benetos*. *Journal of Experimental Biology* **220:** 2887-2899.

Estramil, N., Bouton, N., Verzijden, M.N., Hofker, K., Riebel, K. and Slabbekoorn, H. 2013. Cichlids respond to conspecific sound but females exhibit no phonotaxis without the presence of live males. *Ecology of Freshwater Fish* **23:** 305-312.

Excoffier, L., Lischer, H. E. L. 2010. Arlequin suite version 3.5. A new series of programs to perform population genetics analysis under Linux and Windows. *Molecular Ecology Resources* **10:** 564-567.

Fisher, R.A. 1930. The Genetical Theory of Natural Selection. Clarendon Press, Oxford.

Folmer, O., Black, M., Hoeh, W., Lutz, R. and Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3:** 294-297.

Forsgren, E. 1992. Predation risk affects mate choice in a gobiid fish. *The American Naturalist* **140:** 1041-1049.

Fryer, G. 1959. The trophic inter-relationships and ecology of some littoral communities of Lake Nyasa with special references to the fishes and a discussion of the evolution of a group of rock-frequenting cichlidae. *Proceedings of the Zoological Society of London* **132:** 153-281.

Fryer, G. and Iles, T.D. 1972. The Cichlid Fishes of the Great Lakes of Africa. T.F.H. Publications, Neptune City, New Jersey.

Fu, Y.X. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147:** 915-925.

Gashagaza, M.M. 1991. Diversity of breeding habits in lamprologini cichlids in Lake Tanganyika. *Physiology and Ecology Japan* **28:** 29-65.

Geerts, M. 2002. Stripes do not a zebra make. *Maylandia* versus *Metriaclima*. *Cichlid News* **11:** 21-26.

Genner, M. J., Seehausen, O., Cleary, D. F. R., Knight, M. E., Michel, E. and Turner, G. F. 2004. How does the taxonomic status of allopatric populations influence species richness within African cichlid fish assemblages? *Journal of Biogeography* **31:** 93-102.

Genner, M.J. and Turner, G.F. 2005. “The mbuna cichlids of Lake Malawi: a model for rapidspeciation and adaptive radiation.” *Fish and Fisheries* **6:** 1-34.

Gibelli, J., Aubin-Horth, N. and Dubois, F. 2018. Are some individuals generally more behaviourally plastic than others? An experiment with sailfin mollies. PeerJ 6: 5454.

Glenfell, S. 1993. Lake Malawi National Park Management and Development Plan. Department of National Parks and Wildlife, Lilongwe, Malawi, pp. 64.

Goudswaard, P. C. and Ligtvoet, W. 1988. Recent developments in the fishery for haplochromines (Pisces: Cichlidae) and the Nile Perch, *Latesniloticus* (L) (Pisces: Centropomidae) in Lake Victoria. Fisheries Report 388. FAO, Rome.

Gozlan, R.E., Burnard, D., Britton, J.R. and Andreou, D. 2014. Evidence of female preference for hidden sex signals in distant fish species. *Behavioral Ecology* **25:** 53-57.

Gray, D.A. and Cade, W.H. 2000. Sexual selection and speciation in field crickets. *Proceedings of the National Academy of Sciences* **97:** 14449-14454.

Greenwood, P.H. 1965. The cichlid fishes of Lake Nabugabo, Uganda. *Bulletin of the American Museum of Natural History* **12:** 315-357.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editors and analysis program for windows 95/98/ *Nucleic Acids Symposium Series* **41:** 95-98.

Harrison, I.J. and Stiassny, M.L.J. 1999. The quiet crisis: a preliminary listing of the freshwater fishes of the World that are extinct or “missing in action”. In: Extinctions in Near Time: Causes, Contexts, and Consequences. R. MacPhee (eds.). Kluwer Academic/Plenum Publishers, New York, pp 271-332.

Hebets, E.A and Papaj, D.R. 2005. Complex signal function: developing a framework of testable hypotheses. *Behavioral Ecology and Sociobiology* **57:** 197-214.

Hebets, E.A. 2011. Current status and future directions of research in complex signaling. *Current Zoology* **57:** 1-5.

Hecky, F., Seehausen, O., Wnink, J.H., Kishe-Machumu, M.A., Rensing, M. and Goldchmidt, T. 2013. Cichlids species diversity in naturally and anthropogenically turbid habitats of Lake Victoria, East Africa. *Aquatic Science* **75:** 169-183.

Hecky, R.E., Bugenyi, F.W.B., Ochumba, P., Talling, J.F., Mugidde R., Gophen, M. and Kaufman, L. 1994. Deoxygenation of the deep water of Lake Victoria, East Africa. *Limnology and Oceanography* **39:** 1476-1481.

Hert, E. 1991. Female choice based on egg-spots in *Pseudotropheus aurora* Burgess 1976, a rock-dwelling cichlid of Lake Malawi, Africa. *Journal of Fish Biology* **38:**951-953.

Higashi, M., Takimoto, G. and Yamamura, N. 1999. Sympatric speciation by sexual selection. *Nature* **402:** 523-526.

Hohenlohe, P.A. and Arnold, S.J. 2010. Dimensionality of mate choice, sexual isolation, and speciation. *Proceedings of the National Academy of Science of the United States of America* **107:** 16583-16588.

Houde, A.E. 1987. Male choice based upon naturally occurring colour pattern variation in a guppy population. *Evolution* **41:** 1-10.

Hough, J. 1989. Malawi's National Parks and Game Reserves. Wildlife Society of Malawi, Blantyre, Malawi.

Huelsenbeck, J. P. and Ronquist, F. 2001. MR BAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17:** 754-755.

Izzo, A.S. and Gray, D.A. 2004. Cricket Song in Sympatry: Species Specificity of Song without Reproductive Character Displacement in *Gryllus rubens*. *Annals of the Entomological Society of America* **97:** 831-837.

Jiggins, C.D., Naisbit, R.E., Coe, R.L. and Mallet, J. 2001. Reproductive isolation caused by colour pattern mimicry. *Nature* **411**: 302-305.

Johnson, T. C.,.Scholz, C. A. Talbot, M. R. KeltsK, Ricketts, R. D. Ngobi, G. Beuning, K. Ssemmanda, I. and McGill, J. W. 1996. Late Pleistocene dessication of Lake Victoria and rapid evolution of cichlid fishes. *Science* **273:** 1091-1093.

Johnson, T.C. and Ng'ang'a P. 1990. Reflections on a rift lake. In: KatzBJ (ed) Lacustrine basin exploration: case studies and modern analogs. American Association of Petroleum Geologists.

Johnstone, R.A. 1997. The tactics of mutual mate choice and competitive search. *Behavioural Ecology and Sociobiology* **40:** 51-59.

Jonsson, B. and Jonsson, N. 2001. Polymorphism and speciation in Arctic charr. *Journal of Fish Biology* **58:** 605-638.

Jordan, R., Kellogg, K., Juanes, F. and Stauffer, J. 2003. Evaluation of Female Mate Choice Cues in a Group of Lake Malawi Mbuna (Cichlidae). *Copeia* **2003:** 181-186.

Jordan, R., Mellor, D., Wilt, L., Gershenson, D. and Howe D. 2010. Male interactions in a group of Malawi cichlids. *Ethology Ecology and Evolution* **22:** 359-364.

Kasembe, J. 2017. *Metriaclima callainos*. The IUCN Red List of Threatened Species 2017: e.T61134A120799517.

Kaufman, L. 1992. Catastrophic change in species-rich freshwater ecosystems: the lessons ofLake Victoria. *BioScience* **42:** 846-85.

Kellogg, K.A., Markert, J.A., Stauffer, J.R. and Kocher, T.D. 1995. Microsatellite variation demonstrates multiple paternity in lekking cichlid fishes from Lake Malawi, Africa. *Proceedings of the Royal Society of London: Series B. Biological Sciences* **260:** 79-84.

Klappert, K., Mazzi, D, Hoikkala, A. and Ritchie, M.G. 2007. Male courtship song and female preference variation between phylogeographically distinct populations of *Drosophila montana*. *Evolution* **61:** 1481-1488.

Knight, M.E. and Turner, G.F. 1999. Reproductive isolation among closely related Lake Malawi cichlids: can males recognize conspecific females by visual cues? *Animal Behaviour* **58:** 761-768.

Knight, M.E. and Turner, G.F. 2004. Laboratory mating trials indicate incipient speciation by sexual selection among populations of the cichlid fish *Pseudotropheus zebra* from Lake Malawi. *Proceedings of the Royal Society of London: Series B: Biological Sciences* **271:** 675-680.

Knight, M.E., Turner, G.F. Rico, C. van Oppen, M.J.H. and Hewitt, G.M. 1998. Microsatellite paternity analysis on captive Lake Malawi cichlids supports reproductive isolation by direct mate choice. *Molecular Ecology* **7:** 1605-1610.

Kocher, T. 2004. Adaptive evolution and explosive speciation: the cichlid fish model. *Nature Reviews Genetics* **5:** 288-298.

Kocher, T.D., Conroy, J.A., McKaye, K.R. and Stauffer, J.R. 1993. Similar morphologies of cichlid fishes in Lakes Tanganyika and Malawi are due to convergence. *Molecular Phylogenetics and Evolution* **2:** 158-165.

Kodric-Brown, A. and Strecker, U. 2001. Responses of *Cyprinodon maya* and *C. labiosus* females to visual and olfactory cues of conspecific and heterospecific males. *Biological Journal of the Linnean Society* **74:** 541-548.

Kodric-Brown, A. and West, R.J.D. 2014. Asymmetries in premating isolating mechanisms in a sympatric species flock of pupfish (*Cyprinodon*). *Behavioral Ecology* **25:** 69-75.

Kolding, J., van Zwieten, P., Mkumbo, O., Silsbe, G. and Hecky, R.E. 2008. Are the Lake Victoria fisheries threatened by exploitation or eutrophication? Towards an ecosystem based approach to management. In: The Ecosystem Approach to Fisheries. G. Bianchi and H.R. (eds.). CAB International and FAO, pp. 309 - 355.

Konings, A. 1990. Cichlids and All the Other Fishes of Lake Malawi. Tropical Fish Hobbyist Publications, Neptune City, NJ, pp 495.

Konings, A. 1995. Malawi Cichlids in their Natural Habitat, 2nd edn. Cichlid Press, Germany.

Konings, A. 2001. Malawi Cichlids in their Natural Habitat, 3rd edn El Paso: Cichlid Press. USA.

Konings, A. 2007. Malawi cichlids in their natural habitat, 4th edn. El Paso: Cichlid Press USA.

Konings, A. 2016. Malawi Cichlids in their natural habitat, 5th edn. El Paso: Cichlid Press, USA.

Kornfield, I. and Parker, A. 1997. Molecular systematic of a rapidly evolving species flock: the mbuna of Lake Malawi and the search for the phylogenetic signal. In: Molecular

Systematics of Fishes. T.D. Kocher and C.A. Stepien (eds.). Academic Press, London, pp. 25-37.

Kornfield, I.L. and Smith, P.F. 2000. African cichlid fishes: model systems for evolutionary biology. *Annual Review of Ecology and Systematics* **31:** 163-196.

Kullander, S. O., 1998. A phylogeny and classification of the South American Cichlidae (Teleostei: Perciformes). In: Phylogeny and Classification of Neotropical Fishes. L. R. Malabarba, R. E. Reis, R. P. Vari, Z. M. S. de Lucena and C. A. S. de Lucena (eds.). Edipucrs, Porto Alegre, pp 461-498.

Kuusipalo, L. 2000. Evolution of the scale morphology of Malawi cichlid fishes. *Advances in Ecological Research* **31:** 377-398.

Lackey, A.C.R., Martin, M.D. and Tinghitella, R. M. 2018. Male competition and speciation: expanding our framework for speciation by sexual selection. *Current Zoology* 2018 **64:** 69-73.

Ladiges, W. 1968. Die bedutung okologischer Faktoren fur die differenzierung der Cichliden desTanganjika und des Njassa-Sees. *International Review of Hydrobiology* **53:** 339-52.

Lamml, M. and Kramer, B. 2007. Allopatric differentiation in the acoustic communication of a weakly electric fish from southern Africa, *Marcusenius macrolepidotus* (Mormyridae, Teleostei). *Behavioral Ecology and Sociobiology* **61:** 385-399.

Lande, R. 1981. Models of speciation by sexual selection on polygenic traits. *Proceedings of the National Academy of Sciences of the United States of America* **78:** 3721-3725.

Lande, R., Seehausen, O. and van Alphen J.J.M. 2001. Mechanisms of rapid sympatric speciation by sex reversal and sexual selection in cichlid fish. *Genetica* **112-113:** 435-443.

Lewis, D., Reinthal, P. and Trendall, J. 1986. A guide to the fishes of Lake Malawi National Park. Gland, Switzerland: WWF.

Librado, P. and Rozas, J. 2009. DnaSP version 5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25:** 1451-1452.

Liem, K.F. 1973. Evolutionary strategies and morphological innovations: cichlid pharyngeal jaws. *Systematic Zoology* **22:** 425-441.

Llopart, A., Elwyn, S., Lachaise, D. and Coyne, J.A. 2002. Genetics of a difference in pigmentation between *Drosophila yakuba* and *Drosophila santomea*. *Evolution* **56:** 2262-2277.

Lobel, P. S. and Mann, D. 1995. Spawning sounds of the damselfish, *Dascyllus albisella* (Pomacentridae), and relationship to male size. *Bioacoustics* **6:** 187-198.

Lobel, P.S. 2001. Acoustic behaviour of cichlid fishes. *African Journal of Aquatic Science* **9:**167-186.

Lobel, PS. 1998. Possible species specific courtship sounds by two sympatric cichlid fishes in Lake Malawi, Africa. *Environmental Biology of Fishes* **52:** 443-452.

Lugli, A., Pavan, G. and Torricelli, P. 2004. The response of the male freshwater goby to natural and synthetic male courtship sound playback following exposure to different female sexual sitimuli. *Ethology Ecology and Evolution* **16:** 55-70.

Lugli, M. 1997. Response of male goby, *Padogobius martensii*, to aggressive sound playback following pre-experimental visual stimulation. *Behaviour* **134:** 1175-1188.

Maan, M.E. and Seehausen, O. 2011. Ecology, sexual selection and speciation. *Ecology Letters* **14:** 591-602.

Maan, M.E. and Sefc, K.M. 2013. Colour variation in cichlid fish: developmental mechanisms, selective pressures and evolutionary consequences. *Seminars in Cell and Developmental Biology* **24:** 516-528.

Maan, M.E., Seehausen, O. and van Alphen, J.J.M. 2010. Female mating preferences and male coloration covary with water transparency in a Lake Victoria cichlid fish. *Biological Journal of the Linnean Society* **99:** 398-406.

Maan, M.E., Seehausen, O., Soderberg, L., Johson, L., Ripmeester, E.A.P., Mrosso, H.D.J., Taylor, M.I., van Dooren, T.J.M. and van Alphen, J.M. 2004. Intraspecific sexual selection on a speciation trait, male colouration, in the Lake Victoria cichlid *Pundamilia nyererei*. *Proceedings of the Royal Society Landon: Series B* **271:** 2445-2452.

Magurran, A. E. 2001. Sexual conflict and evolution in Trinidadian guppies. *Genetica* **112:** 463-474.

Markert, J. A., M. E. Arnegard, P. D. Danley, and Kocher,T. D. 1999. Biogeography and population genetics of the Lake Malawi cichlid *Melanochromis auratus*: habitat transience, philopatry and speciation. *Molecular Ecology* **8:** 1013-1026.

Markert, J.A., Danley, P.D. and Arnegard, M.E. 2001. New markers for new species: microsatellite loci and the East African cichlids. *Trends in Ecology and Evolution* **16:** 100-107.

Martens K. 1997. Speciation in ancient lakes. *Trends in Ecology and Evolution* **12:** 177-181.

Maruska, K.P., Ung, U.S. and Fernald, R.D. 2012. The African cichlid fish *Astatotilapia burtoni* uses acoustic communication for reproduction: sound production, hearing, and behavioural significance. *PLoS ONE* **7:** 1-13

Masta, S.E. and Maddison, W.P. 2002. Sexual selection driving diversification in jumping spiders. *Proceedings of the National Academy of Sciences of the United States of America* **99:** 4442-4447.

McElroy, D. M. and Kornfield, I. 1990. Sexual selection, reproductive behaviour, and speciation in the mbuna species flock of Lake Malawi (Pisces: Cichlidae). *Environmental Biology of Fishes* **28:** 273-284.

McKaye, M.C., Kocher, T.D., Reinthal, P., Harrison, H. and Kornfield, I. 1984. Genetic evidence for allopatric and sympatric differentiation among colour morphs of a Lake Malawi cichlid fish. *Evolution* **38:** 215-19.

McLennan, D.A. 2004. Male brook sticklebacks' (*Culaea inconstans*) response to olfactory cues. *Behaviour* **141:** 1411-1424.

McLennan, D.A. and Ryan, M.J. 1999. Interspecific recognition and discrimination based upon olfactory cues in northern swordtails. *Evolution* **53:** 880-888.

McMillan, W. O., Weigt, L. A. and Palumbi, S. R. 1999. Colour pattern evolution, assortative mating, and genetic differentiation in brightly coloured butterfly fishes (Chaetodontidae). *Evolution* **53:** 247-260.

McMillan, W.O., Jiggins, C.D. and Mallet, J. 1997. What initiates speciation in passion vine butterflies? *Proceedings of the National Academy of Sciences of the United States of America* **94:** 8628-8633.

Meyer A. 1993. Phylogenetic relationships and evolutionary processes in east African cichlid fishes. *Trends in Ecology and Evolution* **8:** 279-284.

Meyer, A. 1990. Ecological and evolutionary consequences of the trophic polymorphism in *Cichlasoma citrinellum* (Pisces: Cichlidae). *Biological Journal of the Linnean Society* **39:** 279-299.

Meyer, A., Kocher, T.D., Basasibwaki, P. and Wilson, A.C. 1990. Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequences. *Nature* **347:** 550-553.

Meyer, M.K. and Foerster, W. 1984. Un nouveau *Pseudotropheus* du lac Malawi avec des remarques sur le complexe *Pseudotropheus-Melanochromis*. R*evue Francaise d'Aquariologie Herpetologie* **10:** 107-112.

Moran, P. and Kornfield, I. 1993. Retention of ancestral polymorphism in the mbuna species flocks (Teloestei: Cichlidae) of Lake Malawi. *Molecular Biology and Evolution* **10:** 1015-1029.

Moran, P., Kornfield, I. and Reinthal, P. 1994. Molecular systematics and radiation of the haplochromine cichlids (Teleostei: Perciformes) of Lake Malawi. *Copeia* **1994:** 274-288.

Myrberg, A. A., Ha, S. J. and Shamblott, H. 1993. The sounds of bicolour damselfish (*Pomacentrus partitus*): predictors of body size and a spectral basis for individual recognition and assessment. *The Journal of the Acoustical Society of America* **94:** 3067-3070.

Myrberg, A.A. and Riggio, R.J. 1985. Acoustically mediated individual recognition by a coral reef fish (*Pomacentrus partitus*). *Animal Behaviour* **33:** 411-416.

Nelissen, M.H.J. 1985. Structure of the dominance hierarchy and dominance determining ‘group factors’ in *Melanochromis auratus* (Pisces, Cichlidae). *Behaviour* **94:** 85-107.

Nuttall, D. B. and Keenleyside, M. H. A. 1993. Mate choice by the male convict cichlid (*Cichlasoma nigrofasciatum*; Pisces, Cichlidae). *Ethology* **95:** 247-256.

Oliver, M.K. 1984. Systematics of African cichlid fishes: determination of the most primitive taxon, and studies on the haplochromines of Lake Malawi (Teleostei: Cichlidae). PhD dissertation, Yale University, New Haven, 326 pp.

Oliver, M.K. and Arnegard, M.E. 2010. A new genus for *Melanochromis labrosus*, a problematic Lake Malawi cichlid with hypertrophied lips (Teleostei: Cichlidae). *Ichthyological Exploration of Freshwaters* **21:** 209-232.

Oliver, M.L. and Loiselle, P.V. 1972. A new genus and species of cichlid of the mbuna group (Pisces: Cichlidae) from Lake Malaŵi. Revue de Zoologieet de Botannique Africaines **855:** 309-320.

Ortiz-Barrientos D, Counterman BA and Noor MA. 2004. The genetics of speciation by reinforcement. *PLoS Biology* **2:** 2256-2263.

Owen, R.B., Crossley, R., Johnson, T.C., Tweddle, D. and Komfield, I. 1990. Major low levels of Lake Malawi and their implications for speciation rates in cichlid fishes. *Proceedings of the Royal Society of London: Series B. Biological Sciences* **240:** 519-553.

Owens, I., Bennet, P. and Harvey, P. 1999. Species richness among birds: body size, life history, sexual selection, or ecology? *Proceedings of the Royal Society of London: Series B. Biological Sciences* **266:** 933-939.

Owens, I.P.F. and Thompson, D.B.A. 1994. Sex differences, sex ratios and sex roles. *Proceedings of the Royal Society of London: Series B. Biological Sciences* **258:** 93-99.

Panhuis, T.M., Butlin, R., Zuk, M. and Tregenza, T. 2001. Sexual selection and speciation. *Trends in Ecology and Evolution* **16:** 364-371.

Parker, A. and Kornfield, I. 1996. Polygynandry in *Pseudotropheus zebra*, a cichlid fish from Lake Malawi. *Environmental Biology and Fisheries* **47:** 345-352.

Partan, S. and Marler, P. 1999. Behaviour communication goes multimodal. *Science* **83:** 1272-1273.

Partan, S.R. and Marler, P. 2005. Issues in the classification of multimodal communication signals. *The American Naturalist* **166:** 231-245.

Pauers, M.J. and McKinnon, J.S. 2012. Sexual selection on color and behaviour within and between cichlid populations: implications for speciation. *Current Zoology* **58:** 475-483.

Pauers, M.J., Ehlinger, T.J. and McKinnon, J.S. 2010. Female and male visually based mate preferences are consistent with reproductive isolation between populations of the Lake Malawi endemic *Labeotropheus fuelleborni*. *Current Zoology* **56:** 65-72.

Penn, D.J. 2002. The scent of genetic compatibility: sexual selection and the major his to compatibility complex. *Ethology* **108:** 1-21.

Phillips, C. T. and Johnston, C. E. 2008. Sound production and associated behaviours in *Cyprinella galactura*. *Enviromental Biology of Fishes* **82:** 265-275.

Pisanski, K., Marsh-Rollo S. E. and Balshine, S. 2014. Courting and fighting quietly: a lack of acoustic signals in a cooperative Tanganyikan cichlid fish. *Hydrobiologia* **748:** 87-97.

Plenderleith, M., van Oosterhout, C., Robinson, R.L. and Turner, G.F. 2005. Female preference for conspecific males based on olfactory cues in a Lake Malawi cichlid fish. *Biology Letters* **1:** 411-414.

Poll, M., 1986. Classification des cichlidae du lac Tanganyika: tribus, genres etespeces. Academie Royale de Belgique, Memoires de la Classe des Sciences. 8-2 ser, tome XLV, fasc **2:** 1-163.

Price T. 1998. Sexual selection and natural selection in bird speciation. *Philosophical Transactions of the Royal Society* B **353:** 251-260.

Regan, C.T. 1922. The cichlid fishes of Lake Nyasa. *Proceedings of the Zoological Society of London* **1921:** 675-727.

Reinthal, P. and Meyer, A. 1997. Molecular phylogenetic tests of speciation models in African cichlid fishes. In: Molecular Phylogenetics of Adaptive Radiations. T. Givinish and K. Sytsma (eds.). Cambridge University Press, Boston, pp. 375-90.

Reynolds, R.G. and Fitzpatrick, B.J. 2007. Assortative mating in poison-dart frogs based on an ecologically important trait. *Evolution* **61:** 2253-2259.

Ribbink, A.J. 2001. Lake Malawi/Niassa/Nyasa ecogion-based conservation programme: Biophysical reconnaissance. Harare, Zimbabwe. WWF Southern African Regional Programme Office.

Ribbink, A.J., 1994. Alternative perspectives on some controversial aspects of cichlid fish speciation. 101-125. In: Speciation in ancient Lakes. K. Martens, B. Goddeeris and G. Coulter (eds). Ergebnisse der Limnologie, Heft pp. 44.

Ribbink, A.J., Marsh, B.J., Marsh, A.C.,Ribbink, A.C. and Sharp, B.J. 1983. A preliminary survey of the cichlid fishes of the rocky habitats of Lake Malawi. *South African Journal of Zoology* **18:** 155-309.

Rico, C., Bouteillon, P., van Oppen, M.J.H., Knight, M.E., Hewitt, G.M. and Turner, G.F. 2003. No evidence for parallel sympatric speciation in cichlid species of the genus *Pseudotropheus* from north-western Lake Malawi. *Journal of Evolutionary Biology* **16:** 37-46.

Ripley, J.L., and Lobel, P.S. 2004. Correlation of acoustic and visual signals in the cichlid fish, *Tramitichromis intermedius*. *Environmental Biology of Fishes* **71:** 389-394.

Ritchie, M.G. 2007. Sexual selection and speciation. *Annual Review of Ecology, Evolution, and Systematics* **38:** 79-102.

Rodriguez, R.L., Ramaswamy, K. and Cocroft, R.B. 2006. Evidence that female preferences have shaped male signal evolution in a clade of specialized plant-feeding insects. *Proceedings of the Royal Society of London: Series B* **273:** 2585-2593.

Rollo, A. and Higgs, D. 2008. Differential acoustic response specificity and directionality in the round goby, *Neogobius melanostomus*. *Animal Behaviour* **75:** 1903-1912.

Rollo, A., Andraso, G., Janssen, J. and Higgs, D. 2007. Attraction and localization of round goby (*Neogobius melanostomus*) to conspecific calls. *Behaviour* **144:** 1-21.

Ronquist, F. and Huelsenbeck, J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572-1574.

Rosenzweig, M.L. 1978. Competitive speciation. *Biological Journal of the Linnean Society* **10:** 275-289.

Rowe, C. 1999. Receiver psychology and the evolution of multi-component signals. *Animal Behaviour* **58:** 921-931.

Ryan, M.J. and Rand, A.S. 1993. Species recognition and sexual selection as a unitary problem in animal communication. *Evolution* **47:** 647-657.

Ryan, M.J. 1991. Sexual selection and communication in frogs. *Trends in Ecology and Evolution* **6:** 351-355.

Sage, D. and Selander, R.K. 1975. Trophic radiation through polymorphism in cichlid fishes. *Proceedings of the National Academy of Sciences of the United States of America* **72:** 4669-4673.

Salzburger, W., Niederstätter, H., Brandstätter, A., Berger, B., Parson, W., Snoeks, J. and Sturmbauer, C. 2006. Colour-assortative mating among populations of *Tropheus moorii*, a cichlid fish from Lake Tanganyika, East Africa. *Proceedings of the Royal Society of London: Series B* **273:** 257-266.

Sato, T. and Gashagaza, M.M. 1997. Shell brooding cichlid fishes of Lake Tanganyika: their habits and mating systems. In Fish Communities in Lake Tanganyika, H. Kawanabe, M. Hori, M. Nagoshi (eds.). Sakyoku, Kyoto University Press, Japan, pp. 221-40.

Schebesta, M., Ching-Ling L., Engel, F.B. and Keating M. T. 2006. Transcriptional Profiling of Caudal Fin Regeneration in Zebra fish. *The Scientific World* **1:** 38-54.

Schliewen, U.K. and Stiassny, M.L.J. 2003. *EtiaNguti*, a new genus and species of cichlid fish from the River Mamfue, Upper Cross River basin in Cameroon, West-Central Africa. *Ichthyological Exploration of Freshwaters* **14:** 61-71.

Scholz, C.A. and Rosendahl, B.R. 1988. Low lake stands in Lakes Malawi and Tanganyika, East Africa, delineated with multi fold seismic data. *Science* **240:** 1645-1648

Schwarzer, J., Misof, B., Tautz, D. and Schliewen, U.K. 2009. The root of the East African cichlid radiations. BMC *Evolutionary Biology* **9:** 186.

Seehausen, O, Terai, Y, Magalhaes, I.S., Carleton, K.L., Mrosso, H.D.J. and Miyagi, R. 2008. Speciation through sensory drive in cichlid fish. *Nature* **455:** 620-625.

Seehausen, O, van Alphen, J.J.M. and Witte F. 1997. Cichlid fish diversity threatened by eutrophication that curbs sexual selection. *Science* **277:** 1808-1811.

Seehausen, O. 1997. Distribution of and reproductive isolation among colour morphs of a rock-dwelling Lake Victoria cichlid (*Haplochromis nyererei*). *Ecology of Freshwater Fish* **5:** 59-66.

Seehausen, O. and van Alphen, J.J.M. 1998. The effect of male coloration on female mate choice in closely related Lake Victoria cichlids (*Haplochromis nyererei* complex). *Behavioural Ecology and Sociobiology* **42:** 1-8.

Seehausen, O. and van Alphen, J.J.M. 1999. Can sympatric speciation by disruptive sexual selection explain evolution of cichlid diversity in Lake Victoria? *Ecology Letters* **2:** 262-271.

Seehausen, O., van Alphen, J. J. A. and Lande, R. 1999. Colour polymorphism and sex ratio distortion in a cichlid fish as an incipient stage in sympatric speciation by sexual selection. *Ecology Letters* **2:** 367-378.

Sefc, K. M. 2011. Mating and parental care in Lake Tanganyika's cihlids. *International Journal of Evolutionary Biology* ***2011*****:** 470875.

Selz, O. M., Pierotti, M. E. R., Maan, M. E., Schmid, C. and Seehausen, O. 2014. Female preference for male color is necessary and sufficient for assortative mating in 2 cichlid sister species. *Behavioural Ecology* **25:** 612-626.

Selz, O.M., Thommen, R., Maan, M.E. and Seehausen, O. 2013. Behavioural isolation may facilitate homoploid hybrid speciation in cichlid fish. *Journal of Evolutionary Biology* **27:** 275-289.

Shaw, P.W., Turner, G.F., Idid, M.R., Robinson, R.L. and Carvalho, G.R. 2000. Genetic population structure indicates sympatric speciation of Lake Malawi pelagic cichlids. *Proceedings of the Royal Society of London: Series B. Biological Sciences* **267:** 2273-2280.

Silberglied, R.E. and Taylor, O.R. 1973. Ultraviolet differences between the sulphur butterflies, *Colias eurytheme* and *C. philodice*, and a possible isolating mechanism. *Nature, London* **241:** 406-408.

Simões, J. M., Duarte, I. G., Fonseca, P. J., Turner, G. F. and Amorim, M. C. 2008. Courtship and agonistic sounds by the cichlid fish *Pseudotropheus zebra*. *The Journal of the Acoustical Society of America* **124:** 1332-1338.

Slatkin, M. 1982. Pleiotropy and parapatric speciation. *Evolution* **36:** 263-270.

Smith, P.F. and Kornfield, I. 2002. Phylogeography of Lake Malawi cichlids of the genus *Pseudotropheus*: Significance of allopatric colour variation. *Proceedings of the Royal Society of London: Series B. Biological Sciences* **269:** 2495-2502.

Smith, T.B., Mila, B., Grether, G.F., Slabbekoorn, H., Sepil, I., Buermann, W., Saatchi, S. and Pollinger, J.P. 2008. Evolutionary consequences of human disturbance in a rain forest bird species from Central Africa. *Molecular Ecology* **17:** 58-71

Snoeks, J. Harrison, I.J. and Stiassny, M.L.J. 2011. The status and distribution of freshwater fishes. In: The Diversity of Life in African Freshwaters: Under Water, Under Threat: An analysis of the status and distribution of freshwater species throughout mainland Africa. W.R.T Darwall, K.G. Smith, D.J. Allen, R.A. Holland, I.J. Harrison, and E.G.E. Brooks (eds.). Cambridge, United Kingdom and Gland, Switzerland: IUCN, pp 42-71

Snowberg, L.K. and Benkman, C.W. 2007. The role of marker traits in the assortative mating within red crossbills, *Loxia curvirostra* complex. *Journal of Evolutionary Biology* **20:** 1924-1932.

Sobel, J.M., Chen, G.F., Watt, L.R. and Schemske, D.W. 2010. The biology of speciation. *Evolution* **64:** 295-315.

Sparks, J.S. and Smith, W.L. 2004. Phylogeny and biogeography of cichlid fishes (Teleostei: Perciformes: Cichlidae). *Cladistics* **20:** 501-517

Spinks, R.K., Muschick, M., Salzburger, W. and Gante, H.F. 2016. Singing above the chorus: cooperative Princess cichlid fish (*Neolamprologus pulcher*) has high pitch. *Hydrobiologia* **791:** 115-125.

StatSoft, Inc. 2005. STATISTICA (data analysis software system), version 7. http://www.statsoft.com.

Stauffer, J. R., Bowers, N.J., McKaye, K. R. and Kocher, T. D. 1995. Evolutionary significant units among cichlid fishes: The role of behavioural studies. *American Fisheries Society Symposium Series* **17:** 227-244.

Stauffer, J.R. Jr., and Hert, E. 1992. *Pseudotropheus callainos*, a new species of mbuna (Cichlidae), with analyses of changes associated with two intra-lacustrine transplantations in Lake Malawi, Africa. *Ichthyological Exploration of Freshwaters* **3:** 253-264.

Stauffer, J.R. Jr., Black, K. and Konings, A.F. 2013. Descriptions of five new species of *Metriaclima* (Teleostei: Cichlidae) from Lake Malawi, Africa. *Zootaxa* **3647:** 101-136.

Stauffer, J.R. Jr., Bowers, N.J., Kellogg, K.A. and McKaye, K.R. 1997. A revision of the blue-black *Pseudotropheus zebra* (Teleostei: Cichlidae) complex from Lake Malawi, Africa, with a description of a new genus and ten new species. *Proceedings of the Academy of Natural Sciences of Philadelphia* **148:** 189-230.

Stauffer, J.R. Jr., Konings, A.F. and Ryan, T.M. 2016. Redescription of *Pseudotropheus livingstonii* and *Pseudotropheu selegans* from Lake Malawi, Africa. *Zootaxa* **4154:** 169-178.

Stiassny, M.L.J. and Meyer, A. 1999. Cichlids of the Rift lakes. *Scientific American* **280:** 64-69.

Swofford, D.L., 2002. PAUP* Phylogenetic Analysis using Parsimony and Other Methods v. 4.0b10.

Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123:** 585-595.

Taylor, M.I., Meardon, F., Turner, G., Seehausen, O., Mrosso, H.D.J. and Rico, C. 2002. Characterization of tetranucleotide microsatellite loci in a Lake Victorian, Haplochromine cichlid fish: a *Pundamilia pundamilia-Pundamilia nyererei hybrid*. *Molecular Ecology Notes* **2:** 443-445.

Taylor, M.I., Turner, G.F., Robinson, R.L. and Stauffer, J.R. 1998. Sexual selection, parasites and bower height skew in a bower-building cichlid fish. *Animal Behaviour* **56:** 379-84.

Temple, P.H. 1969. Some biological implications of a revised geological history for Lake Victoria. *Biological Journal of the Linnean Society* **1:** 363- 71.

Thieme, M.L., Abell, R., Stiassny, M.L.J., Skelton, P, Lehner, B.,Teugels, G.G., Dinerstein, E., KamdemToham, A., Burgess, N. and Olson, D. 2005. Freshwater ecoregions of Africa and Madagascar: A conservation assessment. Island Press, Washington DC, USA.

Tinghitella, R.M., Lackey, A.C.R., Martin, M., Dijkstra, P.D., Drury, J.P., Heathcote, R., Keagy, J., Scordato, E.S.C. and Tyersi, A.M. 2018. On the role of male competition in speciation: a review and research agenda. *Behavioral Ecology* **29:** 783-797.

Trewavas, E. 1935. A synopsis of the cichlid fishes of Lake Nyasa. *Annals and Magazine of Natural History Series* **16:** 65-118.

Trewavas, E. 1983. Tilapiine Fishes of the Genera *Sarotherodon*, *Oreochromis* and *Danakilia*. British Museum (Natural History), London.

Trewavas, E. 1984. Nouvelexamen des genres etsousgenres du complexe *Pseudotropheus-Melanochromis* dulac Malawi (Pisces, Perciformes, Cichlidae). *Revue Francaised'Aquariologie Herpetologie* **10:** 97-106.

Turner, G. F. 1996. Offshore cichlids of Lake Malawi. Lauenau, Germany: Cichlid Press.

Turner, G. F., and M. T. Burrows. 1995. A model of sympatric speciation. *Proceeding of the Royal Society of London: Series B* **260:** 287-292.

Turner, G. F., Seehausen, O., Knight, M. E., Allender, C. J. and Robinson, R. L. 2001. How many cichlid fishes are there in African lakes? *Molecular Ecology* **10:** 793-806.

Turner, G.F. 1999. Explosive speciation of African cichlid fishes. In: Evolution of Biological Diversity. A.E. Magurran and R.M. May (ed.). Oxford University Press, Oxford, UK, pp. 217-229.

Turner, G.F., Seehausen, O., Knight, M.E., Allender, C.J. and Robinson, R.L. 2001. How many species of cichlid fishes are there in African lakes? *Molecular Ecology* **10:** 793-806.

Tweddle, D., Eccles, D.H., Frith, C.B., Fryer, G. and Jackson, P.B.N.1998. Cichlid spawning structures-bowers or nests? *Environmental Biology of Fishes* **51:** 107-109.

Tyers, A.M. and Turner, G.F. 2013. Signal and preference divergence among populations of the non-endemic basal Lake Malawi cichlid fish *Astatotilapia calliptera* (Perciformes: Cichlidae). *Biological Journal of the Linnean Society* **110**: 180-188.

van der Meer, H. J. and Bowmaker, J. K.. 1995. Interspecific variation of photoreceptors in four co-existing haplochromine cichlid fishes. *Brain Behaviour and Evolution* **45:** 232-240.

van Oppen, M. J. H., Rico, C., Deutsch, J. C., Turner, G. F. and Hewitt, G. M. 1997. Isolation and characterisation of microsatellite loci in the cichlid fish *Pseudotropheus zebra*. *Molecular Ecology* **6:** 387-388.

van Oppen, M.J.H., Turner, G.F., Rico, C., Robinson, R.L., Deutsch, J.C., Genner, M.J. and Hewitt, G.M. 1998. Assortative mating among rock-dwelling cichlid fishes supports high estimates of species richness from Lake Malawi. *Molecular Ecology* **7:** 991-1001.

Van Staaden, M.J. and Smith, A.R. 2011. Cutting the Gordian knot: Complex signalling in African cichlids is more than multimodal. *Current Zoology* **57:** 237-252.

Venesky, M.D., Andraso, G.M. and Ropski, S.J. 2005. Behaviour of male Kenyi cichlids *Pseudotropheus lombardoi* in response to visual and olfactory cues from females. *BIOS* **76:** 77-83.

Verheyen, E. and van Rompaey, J. 1986. Genetics and speciation in African lacustrine cichlids. *Annales Musee Royal de l'Afrique Centrale Tervuren, Sciences Zoologiques* **251:** 95-101.

Verzijden, M.N., van Heusden, J., Bouton, N., White, F., ten Cate, C. and Slabbekoorn, H. 2010. Sounds of male Lake Victoria cichlids vary within and between species and affect female mate preferences. *Behavioural Ecology* **21:** 548-555.

Waage, J.K. 1975. Reproductive isolation and the potentialfor character displacement in thedamselflies, *Calopteryx maculata* and *C. aequabilis* (Odonata: Calopterygidae). *Systematic Zoology* **24:** 24-36.

Wagner, C.E., Harmon, L.J. and Seehausen, O. 2012. Ecological opportunity and sexual selection together predict adaptive radiation. *Nature* **487:** 366-369.

Wells, M. M. and Henry, C. S. 1998. Songs, reproductive isolation and speciation in cryptic species of insects. In: Endless Forms. Species and Speciation. D.J. Howard and S. H. Berlocher (ed.). Oxford University Press, New York, pp. 217-233.

Werner, N.Y. and Lotem, A. 2003. Choosy males in a haplochromine cichlid: First experimental evidence for male mate choice in a lekking species. *Animal Behaviour* **66:** 293-298.

Witte, F., Goldschmidt, T., Wanick, J., van Oijen, M., Goudswaard, K, Witte-Maas, E., and Bouton, N. 1992. The destruction of an endemic species flock: qualitative data on the decline of the haplochromine cichlids of Lake Victoria. *Environmental Biology of Fishes* **34:** 1-28.

Witte, F., Wanink, J.H., Kishe-Machumu, M., Mkumbo, O.C., Goudswaard, P.C. and Seehausen, O. 2007. Differential decline and recovery of haplochromine trophic groups in the Mwanza Gulf of Lake Victoria. *Aquatic Ecosystem Health and Management* **10:** 416-433.

Witte-Maas, E. and Witte, F. 1985. *Haplochromis nyererei*, a new cichlid fish from Lake Victoria named in honour of Mwalimu Julius Nyerere, president of Tanzania. Brill; Leiden: 1985.

Yanagisawa, Y., Ochi, H. and Gashagaza, M.M. 1997. Habitat use in cichlid fishes for breeding. In: Fish Communities in Lake Tanganyika. H. Kawanabe, M. Hori and M. Nagoshi (ed.). Kyoto University Press, Kyoto, pp. 149-173.

Chapter 8. Appendix 1

Female mate choice and species recognition between two closely related cichlid fish of Lake Malawi *Metriaclima estherae* and *M. callainos*

Table 8.1: Average total length (mm) and standard deviation of fish used in the trials for red *M. estherae* and *M. callainos*.

Experiment	***M. callainos* male**	***M. eshterae* male**	***M. callainos* female**	***M. estherae* female**
Visual cues	84.2 ± 8.04	81.2 ± 2.7	78.3 ± 1.8	75.7 ± 3.5
Visual and chemical cues	88.7 ± 2.1	80.3 ± 5.0	78.1 ± 2.4	79.3 ± 8.0
Chemical cues	82.5 ± 1.0	76.0 ± 2.8	75.4 ±1.9	76.2 ± 4.2

Table 8.2: Average weight (g) and standard deviation of fish used in the trials for red *M. estherae* and *M. callainos*.

Experiment	***M. callainos* male**	***M. eshterae* male**	***M. callainos* female**	***M. estherae* female**
Visual cues	9.8 ± 2.6	8.4 ± 0.5	9.0 ± 1.5	7.9 ± 1.1
Visual and chemical cues	11.3 ± 0.6	9.3 ± 1.2	8.4 ± 0.5	8.2 ± 1.2
Chemical cues	9.5 ± 0.7	7.0 ± 0	8.6 ± 0.9	7.6 ± 1.5

Table 8.3: Average total length (mm) and standard deviation of fish used in the trials for blue *M. estherae* and *M. callainos*.

Experiment	***M. callainos* male**	***M. eshterae* male**	***M. callainos* female**	***M. estherae* female**
Visual cues	88.1 ± 0.9	80.9 ± 5.4	78.8 ± 2.3	79.1 ± 2.9
Visual and chemical cues	87.7 ± 1.5	81.7 ± 6.0	79.2 ± 2.5	79.1 ± 3.7
Chemical cues	88.0 ± 2.0	78.3 ± 3.9	78.3 ± 3.5	81.0 ± 3.7

Table 8.4: Average weight (g) and standard deviation of fish used in the trials for blue *M. estherae* and *M. callainos*.

Experiment	***M .callainos* male**	***M. estherae* male**	***M. callainos* female**	***M. estherae* female**
Visual cues	11.7 ± 0.8	9.1 ± 1.8	8.7 ±0.9	8.5 ± 1.0
Visual and chemical cues	11.7 ± 0.5	9.2 ± 1.6	8.8 ± 0.8	8.3 ± 1.0
Chemical cues	11.8 ± 1.3	88.0 ± 1.4	7.9 ± 0.6	8.8 ± 1.4

Table 8.5: Average total length (mm) and standard deviation of fish used in the trials of red *M. estherae* and blue *M. estherae*.

Experiment	**Red *M. estherae* male**	**Blue *M. eshterae* male**	***M. callainos* female**	***M. estherae* female**
Visual cues	82.0 ± 9.2	78.5 ± 6.3	77.6 ± 2.5	77.6 ± 4.3
Visual and chemical cues	73.5 ± 0.7	79.5 ± 6.4	76.8 ± 1.8	75.2 ± 2.9
Chemical cues	83.0 ± 7.8	79.2 ± 3.1	78.2 ± 1.9	76.5 ± 3.3

Table 8.6: Average weight (g) and standard deviation of fish used in the trials of red *M. estherae* versus blue *M. estherae*.

Experiment	**Red *M. estherae* male**	**Blue *M. eshterae* male**	***M. callainos* female**	***M. estherae* female**
Visual cues	10.0 ± 3.5	8.0 ± 1.2	8.4 ± 0.9	8.0 ± 0.7
Visual and chemical cues	7.0 ± 0	8.5 ± 2.1	7.6 ± 0.5	7.5 ± 1.5
Chemical cues	9.3 ± 2.7	7.8 ± 0.8	8.3 ± 0.9	8.0 ± 1.3

Chapter 9. Appendix 2

The role of males in maintaining reproductive isolation and species recognition in *Metriaclima estherae* and *M. callainos*

Table 9.1: Average total length (mm) and standard deviation of fish used in the trials for *M. callainos* males.

Experiment	***M. callainos* male**	***M. callainos* female**	***M. estherae* female**
Visual cues	89.3 ± 0.6	82.5 ± 3.1	78.5 ± 2.7
Visual and chemical cues	82.5 ± 0.6	78.1 ± 2.4	78.5 ± 2.7
Chemical cues	92.7 ± 1.5	81.8 ± 2.9	81.8 ± 1.6

Table 9.2: Average weight (g) and standard deviation of fish used in the trials for *M. callainos* males.

Experiment	***M. callainos* male**	***M. callainos* female**	***M. estherae* female**
Visual cues	12.5 ± 1.0	9.2 ± 0.7	7.8 ± 0.7
Visual and chemical cues	12.3 ± 0.6	10.3 ± 0.8	8.2 ± 1.6
Chemical cues	13.3 ± 1.5	9.5 ± 1.8	8.8 ± 1.1

Table 9.3: Average total length (mm) and standard deviation of fish used in the trials for red *M. estherae* males.

Experiment	**Red *M. estherae* male**	***M. callainos* female**	***M. estherae* female**
Visual cues	85.5 ± 6.2	81.8 ± 3.6	76.8 ± 2.1
Visual and chemical cues	80.7 ± 2.5	79.0 ± 1.0	79.4 ± 2.9
Chemical cues	80.0 ± 4.5	80.8 ± 1.2	81.7 ± 1.5

Table 9.4: Average weight (g) and standard deviation of fish used in the trials for red *M. estherae* males.

Experiment	***Red M. estherae* male**	***M. callainos* female**	***M. estherae* female**
Visual cues	10.8 ± 2.6	10.3 ± 0.8	7.2 ± 0.4
Visual and chemical cues	9.7 ± 0.6	9.6 ± 0.6	8.0 ± 0.7
Chemical cues	9.0 ± 1.7	10.0 ± 1.5	9.3 ± 0.9

Table 9.5: Average total length (mm) and standard deviation of fish used in the trials for blue *M. estherae* males.

Experiment	**Blue *M. estherae* male**	***M. callainos* female**	***M. estherae* female**
Visual cues	79.7 ± 0.6	82.3 ± 3.1	77.3 ± 3.1
Visual and chemical cues	83.3 ± 5.5	80.0 ± 1.1	77.9 ± 3.3
Chemical cues	82.1 ± 2.6	80.0 ± 2.1	79.0 ± 2.5

Table 9.6: Average weight (g) and standard deviation of fish used in the trials for blue *M. estherae* males.

Experiment	**Blue *M. estherae* male**	***M. callainos* female**	***M. estherae* female**
Visual cues	9.0 ± 0	10.3 ± 0.8	8.2 ± 1.8
Visual and chemical cues	10.0 ± 1.8	9.0 ± 0.8	7.5 ± 0.6
Chemical cues	9.6 ± 0.8	9.2 ± 0.8	9.0 ± 1.9

Chapter 10. Appendix 3

Genetic and morphological differences between *Metriaclima estherae* and *M. callainos*

Table 10.1: Characters and description of the morphological measurements taken from *M. estherae* and *M. callainos*.

Morphometric	**Description**
Standard length (SL)	Measurement from tip of snout to the base of the caudal fin.
Fork length (FL)	Measurement from tip of snout to the shortest/central portion of the tail.
Total length (TL)	Measurement from tip of snout to the end of the longest caudal fin lobe.
Body width (BW)	Width of the body at widest point.
Snout length (SNL)	Distance from tip of snout to anterior bony orbit of the eye.
Head length (HL)	Distance from tip of snout to end of opercular membrane.
Orbit diameter (OD)	Greatest distance between eye orbit rims
Inter-orbital width (IO)	The distance across the top of the head between the eyes.
Caudal peduncle length (CPL)	Distance between the posterior end of the anal fin base and the base of the caudal fin rays.

Caudal peduncle depth (CPD)	Least depth of caudal peduncle.
Upper-caudal lobe (UCL)	Distance from shortest portion of forked tail to end of upper caudal lobe.
Middle-caudal lobe (MCL)	Distance from origin of caudal rays to middle of the shortest/central portion of the caudal fin.
Dorsal fin length (DL)	Length of the dorsal fin base from anterior to posterior end.
Anal fin length (AL)	Length of the anal fin base from anterior to posterior end.
Pectoral fin length (PL)	Length of the pectoral fin from the anterior to the posterior end.
Pre-dorsal length (PDL)	Distance between the dorsal fin origin and tip of snout.
Pre-anal length (PAL)	Distance between the anal fin origin and the tip of snout.
Pre-pelvic length (PVL)	Distance between the pelvic fin origin and the tip of snout.