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

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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.
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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*. 
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). Microsatellite-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 Pseudocrenilabrinai (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. trophiceps*, *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. trophiceps* 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*.**

<table>
<thead>
<tr>
<th>Morphs</th>
<th>Description (Colour)</th>
<th>Sex</th>
</tr>
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<tbody>
<tr>
<td><strong>Orange (O)</strong></td>
<td>Orange</td>
<td>Males and females</td>
</tr>
<tr>
<td><strong>Orange blotches (OB)</strong></td>
<td>Dark blue blotches on a pinkish, yellowish or orange body</td>
<td>Males and females</td>
</tr>
<tr>
<td><strong>Blue body (BB)</strong></td>
<td>Light blue with dark blue bars on body</td>
<td>Males</td>
</tr>
</tbody>
</table>
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 = \textit{M. callainos} sites, red = \textit{M. estherae} sites.
Chapter 1: General Introduction

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 km² (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 Palaeozoic 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 re-
colonization 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).
Chapter 1: General Introduction

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). Verzijden 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 (Plenderleith 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).
Chapter 2: Female Mate Choice

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 °C 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
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 chemical 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 conspicuous 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,
The aim of this study was to investigate whether males are able to recognise their conspecific females and thus maintain reproductive boundaries 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 compartment ($F_{2, 27} = 63.4, p < 0.001$; 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 visual 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 conspecific 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 to 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 nuptial 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 conspecific 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 suggest 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 courthship 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 recognition (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 acoustic 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 demonstrated that in the absence of conspecific males, females prefer males that have colour patterns similar to their conspecific males.

### 3.5 Conclusions

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: Acoustic 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).

<table>
<thead>
<tr>
<th>Species</th>
<th>Sound character</th>
<th>SL (r)</th>
<th>W (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. callainos</em></td>
<td>Call Duration (sec)</td>
<td>-0.02</td>
<td>-0.25</td>
</tr>
<tr>
<td></td>
<td>Peak Frequency (Hz)</td>
<td>-0.09</td>
<td>-0.18</td>
</tr>
<tr>
<td>Blue <em>M. estherae</em></td>
<td>Call Duration (sec)</td>
<td>-0.27</td>
<td>-0.42</td>
</tr>
<tr>
<td></td>
<td>Peak Frequency (Hz)</td>
<td>-0.55</td>
<td>-0.62</td>
</tr>
<tr>
<td>Red <em>M. estherae</em></td>
<td>Call Duration (sec)</td>
<td>0.37</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>Peak Frequency (Hz)</td>
<td>0.36</td>
<td>0.44</td>
</tr>
</tbody>
</table>
# Chapter 4: Acoustic Cues

**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 < 0.001, 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 < 0.001, 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*.

![Average peak frequency produced by M. callainos and red and blue M. estherae](image)

**Figure 4.2:** Average peak frequency produced by *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 (Verzijl 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 (Verzijl 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.
Chapter 5: Molecular Studies

5.1. Introduction

The highest diversity of cichlid species is found in Lake Malawi where ~1500 species are endemic (Genner et al., 2004; Kohlmbuller 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 \((\pi)\) 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*.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. estherae</em></td>
<td>0.006 ± 0.006</td>
<td>0.64</td>
</tr>
<tr>
<td><em>M. callainos</em></td>
<td>0.006 ± 0.006</td>
<td>0.05</td>
</tr>
<tr>
<td><em>M. estherae</em> vs <em>M. callainos</em></td>
<td>0.009 ± 0.042</td>
<td>0.98</td>
</tr>
</tbody>
</table>
Table 5.2: The distribution of Control region haplotypes of *M. estherae* and *M. callainos* in Minos Reef, Lake Malawi, Nkatha Bay and Chidunga.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Locality</th>
<th><em>M. estherae</em> (18)</th>
<th><em>M. callainos</em> (16)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hap 1</td>
<td>Minos</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Hap 2</td>
<td>Minos Reef</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Hap 3</td>
<td>Minos Reef</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Hap 4</td>
<td>Minos Reef, Lake Malawi, Nkatha Bay</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Hap 5</td>
<td>Nkatha, Chidunga</td>
<td>0</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Hap 6</td>
<td>Nkatha Bay</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Hap 7</td>
<td>Lake Malawi</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Hap 8</td>
<td>Lake Malawi</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Hap 9</td>
<td>Lake Malawi</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>18</strong></td>
<td><strong>16</strong></td>
<td><strong>34</strong></td>
</tr>
</tbody>
</table>
Table 5.3: Genetic diversity of *M. estherae* and *M. callainos*. \( N = \) sample size, \( \text{Nh} = \) haplotype number, \( S = \) number of polymorphic sites, \( h = \) haplotype diversity, \( \pi = \) nucleotide diversity, \( D = \) Tajima’s \( D \), \( F = \) Fu’s \( F_s \). \( p > 0.10 \).

<table>
<thead>
<tr>
<th>Population</th>
<th>( N )</th>
<th>( \text{Nh} )</th>
<th>( S )</th>
<th>( h )</th>
<th>( \pi )</th>
<th>( D )</th>
<th>( F_s )</th>
<th>( K )</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. estherae</em></td>
<td>18</td>
<td>4</td>
<td>8</td>
<td>0.647 ±0.095</td>
<td>0.006 ±0.001</td>
<td>0.753</td>
<td>0.800</td>
<td>2.830</td>
</tr>
<tr>
<td><em>M. callainos</em></td>
<td>16</td>
<td>8</td>
<td>15</td>
<td>0.758 ±0.110</td>
<td>0.006 ±0.002</td>
<td>-1.448</td>
<td>-1.620</td>
<td>2.850</td>
</tr>
</tbody>
</table>

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 Chidungu 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 *Oreochromis 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 = 0.359, F = 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*.

<table>
<thead>
<tr>
<th>Significantly different between species</th>
<th>Significantly different between sexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard length</td>
<td>Standard length</td>
</tr>
<tr>
<td>Orbit diameter</td>
<td>Orbit diameter</td>
</tr>
<tr>
<td>Pre-dorsal length</td>
<td>Pre-dorsal length</td>
</tr>
<tr>
<td>Pre-anal length</td>
<td>Head length</td>
</tr>
<tr>
<td>Pre-pelvic length</td>
<td>Inter-orbital width</td>
</tr>
<tr>
<td></td>
<td>Dorsal fin length</td>
</tr>
</tbody>
</table>
Figure 5.3: Canonical centroid plot of the first two discriminant functions of the morphometric measurements taken from \textit{M. estherae} and \textit{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*.

<table>
<thead>
<tr>
<th>Function</th>
<th>Eigenvalue</th>
<th>% of Variance</th>
<th>Cumulative %</th>
<th>Canonical Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.837&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.2</td>
<td>52.2</td>
<td>0.891</td>
</tr>
<tr>
<td>2</td>
<td>1.473&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.0</td>
<td>72.2</td>
<td>0.772</td>
</tr>
<tr>
<td>3</td>
<td>1.334&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.1</td>
<td>90.3</td>
<td>0.756</td>
</tr>
<tr>
<td>4</td>
<td>0.710&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.7</td>
<td>100.0</td>
<td>0.644</td>
</tr>
</tbody>
</table>
Table 5.6: Results of the Standardized Canonical Discriminant Function (CDFs) Coefficients of the morphometric measurements obtained from *M. estherae* and *M. callainos*.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SL</strong></td>
<td>0.024</td>
<td>0.142</td>
<td>0.734</td>
<td>-0.702</td>
</tr>
<tr>
<td><strong>FL</strong></td>
<td>0.288</td>
<td>-0.356</td>
<td>-0.257</td>
<td>-0.408</td>
</tr>
<tr>
<td><strong>TL</strong></td>
<td>0.248</td>
<td>0.485</td>
<td>0.563</td>
<td>-0.112</td>
</tr>
<tr>
<td><strong>BW</strong></td>
<td>0.188</td>
<td>-0.648</td>
<td>0.426</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>SNL</strong></td>
<td>-0.297</td>
<td>-0.179</td>
<td>0.070</td>
<td>0.381</td>
</tr>
<tr>
<td><strong>HL</strong></td>
<td>-0.481</td>
<td>-0.281</td>
<td>-0.672</td>
<td>-0.087</td>
</tr>
<tr>
<td><strong>OD</strong></td>
<td>1.007</td>
<td>0.120</td>
<td>0.919</td>
<td>0.416</td>
</tr>
<tr>
<td><strong>IO</strong></td>
<td>-0.502</td>
<td>0.198</td>
<td>0.068</td>
<td>0.523</td>
</tr>
<tr>
<td><strong>CPL</strong></td>
<td>-0.511</td>
<td>0.293</td>
<td>-0.526</td>
<td>0.848</td>
</tr>
<tr>
<td><strong>CPD</strong></td>
<td>-0.169</td>
<td>0.179</td>
<td>-0.514</td>
<td>0.460</td>
</tr>
<tr>
<td><strong>UCL</strong></td>
<td>0.037</td>
<td>0.432</td>
<td>-0.544</td>
<td>0.360</td>
</tr>
<tr>
<td><strong>MCL</strong></td>
<td>-0.050</td>
<td>0.200</td>
<td>-0.152</td>
<td>0.063</td>
</tr>
<tr>
<td><strong>DL</strong></td>
<td>-0.011</td>
<td>0.032</td>
<td>0.908</td>
<td>-0.340</td>
</tr>
</tbody>
</table>
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 (Tajima, 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).
Chapter 6: General Discussion

There is contradicting evidence regarding the cues used by African cichlid males for mate recognition. Some authors suggest 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


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References


References


References


References


References


References


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*.

<table>
<thead>
<tr>
<th>Experiment</th>
<th><em>M. callainos</em></th>
<th><em>M. estherae</em></th>
<th><em>M. callainos</em></th>
<th><em>M. estherae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>male</td>
<td>male</td>
<td>female</td>
<td>female</td>
</tr>
<tr>
<td>Visual cues</td>
<td>84.2 ± 8.04</td>
<td>81.2 ± 2.7</td>
<td>78.3 ± 1.8</td>
<td>75.7 ± 3.5</td>
</tr>
<tr>
<td>Visual and chemical cues</td>
<td>88.7 ± 2.1</td>
<td>80.3 ± 5.0</td>
<td>78.1 ± 2.4</td>
<td>79.3 ± 8.0</td>
</tr>
<tr>
<td>Chemical cues</td>
<td>82.5 ± 1.0</td>
<td>76.0 ± 2.8</td>
<td>75.4 ± 1.9</td>
<td>76.2 ± 4.2</td>
</tr>
</tbody>
</table>
Table 8.2: Average weight (g) and standard deviation of fish used in the trials for red *M. estherae* and *M. callainos*.

<table>
<thead>
<tr>
<th>Experiment</th>
<th><em>M. callainos</em> male</th>
<th><em>M. estherae</em> male</th>
<th><em>M. callainos</em> female</th>
<th><em>M. estherae</em> female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual cues</td>
<td>9.8 ± 2.6</td>
<td>8.4 ± 0.5</td>
<td>9.0 ± 1.5</td>
<td>7.9 ± 1.1</td>
</tr>
<tr>
<td>Visual and chemical cues</td>
<td>11.3 ± 0.6</td>
<td>9.3 ± 1.2</td>
<td>8.4 ± 0.5</td>
<td>8.2 ± 1.2</td>
</tr>
<tr>
<td>Chemical cues</td>
<td>9.5 ± 0.7</td>
<td>7.0 ± 0.0</td>
<td>8.6 ± 0.9</td>
<td>7.6 ± 1.5</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Experiment</th>
<th><em>M. callainos</em> male</th>
<th><em>M. estherae</em> male</th>
<th><em>M. callainos</em> female</th>
<th><em>M. estherae</em> female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual cues</td>
<td>88.1 ± 0.9</td>
<td>80.9 ± 5.4</td>
<td>78.8 ± 2.3</td>
<td>79.1 ± 2.9</td>
</tr>
<tr>
<td>Visual and chemical cues</td>
<td>87.7 ± 1.5</td>
<td>81.7 ± 6.0</td>
<td>79.2 ± 2.5</td>
<td>79.1 ± 3.7</td>
</tr>
<tr>
<td>Chemical cues</td>
<td>88.0 ± 2.0</td>
<td>78.3 ± 3.9</td>
<td>78.3 ± 3.5</td>
<td>81.0 ± 3.7</td>
</tr>
</tbody>
</table>
Table 8.4: Average weight (g) and standard deviation of fish used in the trials for blue M. estherae and M. callainos.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>M. callainos male</th>
<th>M. estherae male</th>
<th>M. callainos female</th>
<th>M. estherae female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual cues</td>
<td>11.7 ± 0.8</td>
<td>9.1 ± 1.8</td>
<td>8.7 ± 0.9</td>
<td>8.5 ± 1.0</td>
</tr>
<tr>
<td>Visual and chemical cues</td>
<td>11.7 ± 0.5</td>
<td>9.2 ± 1.6</td>
<td>8.8 ± 0.8</td>
<td>8.3 ± 1.0</td>
</tr>
<tr>
<td>Chemical cues</td>
<td>11.8 ± 1.3</td>
<td>88.0 ± 1.4</td>
<td>7.9 ± 0.6</td>
<td>8.8 ± 1.4</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Red M. estherae male</th>
<th>Blue M. estherae male</th>
<th>M. callainos female</th>
<th>M. estherae female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual cues</td>
<td>82.0 ± 9.2</td>
<td>78.5 ± 6.3</td>
<td>77.6 ± 2.5</td>
<td>77.6 ± 4.3</td>
</tr>
<tr>
<td>Visual and chemical cues</td>
<td>73.5 ± 0.7</td>
<td>79.5 ± 6.4</td>
<td>76.8 ± 1.8</td>
<td>75.2 ± 2.9</td>
</tr>
<tr>
<td>Chemical cues</td>
<td>83.0 ± 7.8</td>
<td>79.2 ± 3.1</td>
<td>78.2 ± 1.9</td>
<td>76.5 ± 3.3</td>
</tr>
</tbody>
</table>
Table 8.6: Average weight (g) and standard deviation of fish used in the trials of red *M. estherae* versus blue *M. estherae*.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Red <em>M. estherae</em> male</th>
<th>Blue <em>M. eshterae</em> male</th>
<th><em>M. callainos</em> female</th>
<th><em>M. estherae</em> female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual cues</td>
<td>10.0 ± 3.5</td>
<td>8.0 ± 1.2</td>
<td>8.4 ± 0.9</td>
<td>8.0 ± 0.7</td>
</tr>
<tr>
<td>Visual and chemical cues</td>
<td>7.0 ± 0</td>
<td>8.5 ± 2.1</td>
<td>7.6 ± 0.5</td>
<td>7.5 ± 1.5</td>
</tr>
<tr>
<td>Chemical cues</td>
<td>9.3 ± 2.7</td>
<td>7.8 ± 0.8</td>
<td>8.3 ± 0.9</td>
<td>8.0 ± 1.3</td>
</tr>
</tbody>
</table>
Chapter 9. Appendix 2

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

<table>
<thead>
<tr>
<th>Experiment</th>
<th><em>M. callainos male</em></th>
<th><em>M. callainos female</em></th>
<th><em>M. estherae</em> female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual cues</td>
<td>89.3 ± 0.6</td>
<td>82.5 ± 3.1</td>
<td>78.5 ± 2.7</td>
</tr>
<tr>
<td>Visual and chemical cues</td>
<td>82.5 ± 0.6</td>
<td>78.1 ± 2.4</td>
<td>78.5 ± 2.7</td>
</tr>
<tr>
<td>Chemical cues</td>
<td>92.7 ± 1.5</td>
<td>81.8 ± 2.9</td>
<td>81.8 ± 1.6</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Experiment</th>
<th><em>M. callainos male</em></th>
<th><em>M. callainos female</em></th>
<th><em>M. estherae</em> female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual cues</td>
<td>12.5 ± 1.0</td>
<td>9.2 ± 0.7</td>
<td>7.8 ± 0.7</td>
</tr>
<tr>
<td>Visual and chemical cues</td>
<td>12.3 ± 0.6</td>
<td>10.3 ± 0.8</td>
<td>8.2 ± 1.6</td>
</tr>
<tr>
<td>Chemical cues</td>
<td>13.3 ± 1.5</td>
<td>9.5 ± 1.8</td>
<td>8.8 ± 1.1</td>
</tr>
</tbody>
</table>

Table 9.2: Average weight (g) and standard deviation of fish used in the trials for *M. callainos* males.
### Table 9.3: Average total length (mm) and standard deviation of fish used in the trials for red *M. estherae* males.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Red <em>M. estherae</em> male</th>
<th><em>M. callainos</em> female</th>
<th><em>M. estherae</em> female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual cues</td>
<td>85.5 ± 6.2</td>
<td>81.8 ± 3.6</td>
<td>76.8 ± 2.1</td>
</tr>
<tr>
<td>Visual and chemical cues</td>
<td>80.7 ± 2.5</td>
<td>79.0 ± 1.0</td>
<td>79.4 ± 2.9</td>
</tr>
<tr>
<td>Chemical cues</td>
<td>80.0 ± 4.5</td>
<td>80.8 ± 1.2</td>
<td>81.7 ± 1.5</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Red <em>M. estherae</em> male</th>
<th><em>M. callainos</em> female</th>
<th><em>M. estherae</em> female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual cues</td>
<td>10.8 ± 2.6</td>
<td>10.3 ± 0.8</td>
<td>7.2 ± 0.4</td>
</tr>
<tr>
<td>Visual and chemical cues</td>
<td>9.7 ± 0.6</td>
<td>9.6 ± 0.6</td>
<td>8.0 ± 0.7</td>
</tr>
<tr>
<td>Chemical cues</td>
<td>9.0 ± 1.7</td>
<td>10.0 ± 1.5</td>
<td>9.3 ± 0.9</td>
</tr>
</tbody>
</table>
Table 9.5: Average total length (mm) and standard deviation of fish used in the trials for blue *M. estherae* males.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Blue <em>M. estherae</em> male</th>
<th><em>M. callainos</em> female</th>
<th><em>M. estherae</em> female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual cues</td>
<td>79.7 ± 0.6</td>
<td>82.3 ± 3.1</td>
<td>77.3 ± 3.1</td>
</tr>
<tr>
<td>Visual and chemical</td>
<td>83.3 ± 5.5</td>
<td>80.0 ± 1.1</td>
<td>77.9 ± 3.3</td>
</tr>
<tr>
<td>Chemical cues</td>
<td>82.1 ± 2.6</td>
<td>80.0 ± 2.1</td>
<td>79.0 ± 2.5</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Blue <em>M. estherae</em> male</th>
<th><em>M. callainos</em> female</th>
<th><em>M. estherae</em> female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual cues</td>
<td>9.0 ± 0</td>
<td>10.3 ± 0.8</td>
<td>8.2 ± 1.8</td>
</tr>
<tr>
<td>Visual and chemical</td>
<td>10.0 ± 1.8</td>
<td>9.0 ± 0.8</td>
<td>7.5 ± 0.6</td>
</tr>
<tr>
<td>Chemical cues</td>
<td>9.6 ± 0.8</td>
<td>9.2 ± 0.8</td>
<td>9.0 ± 1.9</td>
</tr>
</tbody>
</table>
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*.

<table>
<thead>
<tr>
<th>Morphometric</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard length (SL)</td>
<td>Measurement from tip of snout to the base of the caudal fin.</td>
</tr>
<tr>
<td>Fork length (FL)</td>
<td>Measurement from tip of snout to the shortest/central portion of the tail.</td>
</tr>
<tr>
<td>Total length (TL)</td>
<td>Measurement from tip of snout to the end of the longest caudal fin lobe.</td>
</tr>
<tr>
<td>Body width (BW)</td>
<td>Width of the body at widest point.</td>
</tr>
<tr>
<td>Snout length (SNL)</td>
<td>Distance from tip of snout to anterior bony orbit of the eye.</td>
</tr>
<tr>
<td>Head length (HL)</td>
<td>Distance from tip of snout to end of opercular membrane.</td>
</tr>
<tr>
<td>Orbit diameter (OD)</td>
<td>Greatest distance between eye orbit rims</td>
</tr>
<tr>
<td>Inter-orbital width (IO)</td>
<td>The distance across the top of the head between the eyes.</td>
</tr>
<tr>
<td>Caudal peduncle length (CPL)</td>
<td>Distance between the posterior end of the anal fin base and the base of the caudal fin rays.</td>
</tr>
<tr>
<td>Measurement</td>
<td>Description</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Caudal peduncle depth (CPD)</td>
<td>Least depth of caudal peduncle.</td>
</tr>
<tr>
<td>Upper-caudal lobe (UCL)</td>
<td>Distance from shortest portion of forked tail to end of upper caudal lobe.</td>
</tr>
<tr>
<td>Middle-caudal lobe (MCL)</td>
<td>Distance from origin of caudal rays to middle of the shortest/central portion of the caudal fin.</td>
</tr>
<tr>
<td>Dorsal fin length (DL)</td>
<td>Length of the dorsal fin base from anterior to posterior end.</td>
</tr>
<tr>
<td>Anal fin length (AL)</td>
<td>Length of the anal fin base from anterior to posterior end.</td>
</tr>
<tr>
<td>Pectoral fin length (PL)</td>
<td>Length of the pectoral fin from the anterior to the posterior end.</td>
</tr>
<tr>
<td>Pre-dorsal length (PDL)</td>
<td>Distance between the dorsal fin origin and tip of snout.</td>
</tr>
<tr>
<td>Pre-anal length (PAL)</td>
<td>Distance between the anal fin origin and the tip of snout.</td>
</tr>
<tr>
<td>Pre-pelvic length (PVL)</td>
<td>Distance between the pelvic fin origin and the tip of snout.</td>
</tr>
</tbody>
</table>