

A direct assay of female choice in cichlids: all the eggs in one basket

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A novel testing apparatus is presented which affords the researcher maximum control over the testing environment, but allows for the scoring of actual spawning events. Female *Metriaclima zebra* and *Metriaclima benetos* chose the appropriate conspecific mate in every mating trial performed. This apparatus provides support for a critical assumption of many cichlid speciation models: that female cichlids use visual cues to recognize conspecifics. These results demonstrate that females are able to identify conspecific mates using only visual cues, and provide further support for the importance of sexual selection in the speciation of these fishes.

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INTRODUCTION

Assessment of mate recognition, especially as it applies to female choice, is critical to the understanding of speciation. Behavioural isolation is often an important barrier restricting gene flow between sympatric species (Dobzhansky, 1940; Ptacek, 2000; Kirkpatrick & Ravigné, 2002). Divergence of mate recognition systems is a characteristic common to many species-rich lineages (Boughman, 2002; Streelman & Danley, 2003). While Coyne & Orr (2004) caution that current barriers to gene flow may not have been the original cause of population divergence, identifying the specific traits used in species discrimination is a necessary prerequisite to identifying the forces involved in the evolution of reproductive isolation. Enumerating the traits used during conspecific mate recognition is difficult (Halliday, 1983) and requires robust assays of female choice.

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Barlow (2002) identified three classes of experiments used to quantify female mate choice in fishes: free access, female only access and restrained access. In each case females are presented with two or more males that vary for some trait (two-stimulus test; Wagner, 1998). During free access experiments (referred to as 'free contact' by Turner *et al.*, 2001) all subjects are allowed to directly interact with each other. This is the most 'natural' type of setting and most field observations and manipulations would fall into this experimental class. Female only access (partial partition method; Turner *et al.*, 2001) can be used when the female is smaller than the male. Size selective barriers limit male to male interaction, but still allow the female unfettered access to multiple males. In restrained access (closed chamber; Turner *et al.*, 2001) experiments, subjects are confined behind transparent barriers, which limits the cues available to the female.

While female choice has been effectively quantified using each methodology, they each have specific disadvantages. Both free access and female-only access methods allow for the most direct measure of mate choice (spawning and number of eggs), but their use as a research tool is limited because they do not control the types of cues available to the female (auditory, olfactory, visual and mechanical). Additionally, costly genotyping of the embryos is necessary if spawning is not witnessed (Kellogg, 1997; Knight *et al.*, 1998). In contrast, restrained access experiments offer maximum control over the testing variables because the test subjects are isolated in individual compartments. Under these conditions the investigator is able to prevent male to male interaction and to manipulate the types of cues available to each subject. The physical barriers between test subjects, however, typically force the investigator to use indirect measures to quantify mate choice. Due to its expedience, time spent in association with a subject is often used as a measure of mate choice (Forsgren, 1992; Ptacek, 1998; Brooks & Endler, 2001; Gabor & Ryan, 2001; Ludlow *et al.*, 2001; Morris *et al.*, 2003; Dosen & Montgomerie, 2004; Wong *et al.*, 2004). Unfortunately, using an indirect measure can lead to the misinterpretation of female choice when the organism's motivational state is not considered (Barlow, 1989). Specifically, fear response (Barlow, 2002), the female's reproductive state (Clement *et al.*, 2005) and aggression (Danley, 2001) can obscure female choice. Clement *et al.* (2005) found that while gravid female *Haplochromis burtoni* Günther tend to associate with territorial males, non-gravid females show no preference. The use of indirect measures of mate choice can lead to errors of interpretation when the organism's natural history is not carefully considered (Scott & Foster, 2000).

The cichlids of East Africa make an excellent model for studying the role of mate choice in speciation. Intersexual selection has probably played a key role in the explosive speciation of Lake Malawi's 500+ species of haplochromine cichlids (Dominey, 1984). Rock dwelling cichlids (*mbuna*) of Lake Malawi are often distinguished on the basis of male colour pattern and frequently show strong sexual dimorphism (Fryer & Iles, 1972; Ribbink *et al.*, 1983; Deutsch, 1997; Stauffer *et al.*, 1997; Turner *et al.*, 2001). Even though several congeners are typically found together in each mbuna community, few hybrids have been observed in the wild. Interspecific and even intergeneric fertile hybrids are easily produced in the laboratory by restricting female choice (Crapon de

Caprona & Fritsch, 1984; Albertson *et al.*, 2003), suggesting that pre-mating behaviours maintain reproductive isolation. The importance of behavioural isolation and the large variance in reproductive success among males on a lek, suggest that female choice may be an important mechanism of selection (Hert, 1989; Deutsch, 1997; Seehausen *et al.*, 1999). Several recent models propose that intersexual selection on male colour pattern has played an important role in speciation (Turner & Burrows, 1995; Deutsch, 1997; van Doorn *et al.*, 1998; Higashi *et al.*, 1999; Kondrashov & Kondrashov, 1999; Seehausen *et al.*, 1999; Takimoto *et al.*, 2000; Lande *et al.*, 2001).

Models that depend on female choice to initiate divergence are predicated on the assumption that females recognize conspecifics using visual cues. Unfortunately, attempts to quantify female choice in cichlids have produced conflicting results. Orientation toward, or time spent in proximity to the subject is often used as a measure of cichlid mate choice (Falter & Charlier, 1989; Beeching & Hopp, 1999; Couldridge & Alexander, 2001, 2002; Jordan *et al.*, 2003; Werner & Lotem, 2003). Couldridge & Alexander (2001) suggested that time spent could be used to quantify mate choice in a restricted access experiment of cave spawning *Metriaclima zebra* (Boulenger) 'red dorsal'. The effectiveness of 'time spent', however, was corroborated by an unnatural 'pit digging' behaviour. Seehausen *et al.* (1998) found that scoring 'relative responsiveness' in a restrained access assay yielded indeterminate results with a population of *Pundamilia nyererei* (Witte-Maas & Witte) that demonstrated completely assortative mating during free access experiments. Additionally, Danley (2001) found that female *Metriaclima benetos* (Stauffer, Bowers, Kellogg & McKaye) were more likely to associate with heterospecific *M. zebra* males, possibly because of an unusual aggressive response toward heterospecific males. Measuring time spent by a female cichlid may be analogous to scoring 'visiting' behaviour in the field, which often includes heterospecific visits (Seehausen *et al.*, 1998) and is a poor indicator of spawning success (McKaye *et al.*, 1990; Kellogg *et al.*, 2000).

In this study, a novel experimental technique was used to quantify female mate choice among members of the *M. zebra* complex from Lake Malawi. This 'egg catcher' design uses a restrained access assay to eliminate male to male interactions, which can inhibit female choice (Morris *et al.*, 1992; Kodric-Brown, 1993).

METHODS AND MATERIALS

STUDY ANIMALS

Species of the cichlid genus *Metriaclima* live in rocky habitats throughout Lake Malawi, Africa. While *M. zebra* is found at many localities around the lake, *M. benetos* is endemic to Mazinzi Reef (Stauffer *et al.*, 1997). On this reef, the two species coexist on similar habitats and their territories often interdigitate. *Metriaclima zebra* and *M. benetos* are ecologically, morphologically and behaviourally similar, but can be distinguished easily on the basis of adult colour pattern.

Territorial *M. zebra* males are bright blue with five to seven black vertical body bars, a black bar in their pelvic fin and a prominent black interorbital bar [Fig. 1(a)]. Territorial *M. benetos* are also bright blue, but lack the conspicuous melanistic markings observed in *M. zebra* [Fig. 1(b)]. Females of both species are drab olive brown [Fig. 1(c), (d)]. Female *M. zebra* also exhibit many of the same melanistic patterns observed in male *M. zebra*, however, these markings are much less conspicuous in the females than territorial males.



FIG. 1. Colour patterns of (a), (c) *Metriaclima zebra* and (b), (d) *Metriaclima benetos*. While territorial males [(a), (b)] of both species possess a blue background, only *M. zebra* displays melanistic patterns, which include five to seven black vertical bars, a black bar on the pelvic fins and a black 'mask.' Females [(b), (d)] of both species are a drab olive to brown colour with a pattern of weak vertical bars.

Morphologically, *M. zebra* and *M. benetos* exhibit minor differences in horizontal eye diameter, caudal peduncle length and number of dorsal spines (Stauffer *et al.*, 1997). The animals used in these studies were first and second generation offspring of wild-caught animals collected at Mazinzi Reef in the south-east arm of Lake Malawi.

Spawning behaviour is stereotyped and appears to be identical among mbuna species (McElroy & Kornfield, 1990). Most mbuna cichlids are promiscuous. Females often visit and spawn with several males (Kellogg *et al.*, 1995; Parker & Kornfield, 1996). Male mbuna compete for permanent territories, from which they solicit females through a pattern of courtship displays. When a female approaches a territorial male, he will respond with a series of lateral displays and quivers. Males will then lead the female toward the location within his territory used for spawning (*e.g.* cave and flat rock surface). If the female follows, they enter the spawning area where she will deposit her eggs and immediately circle to pick them up into her mouth. During spawning the female nudges the male's anal fin and he releases sperm to fertilize the eggs held within her buccal cavity (Fryer & Iles, 1972; Holzberg, 1978; McElroy and Kornfield, 1990).

EGG-CATCHER ASSAY

The experimental design was modified from Nelson (1995). Males were housed in separate, but adjacent 37.8 l aquariums (Fig. 2). The test female was placed in a 193.5 l aquarium adjacent to the males. While the female had visual access to both males, the males were unable to see one another, preventing male to male interactions. Clay flowerpots were cut in half and glued to the aquarium walls to construct spawning caves, which allowed males and females to remain in visual contact during spawning. The bottom of the cave was constructed of plastic grating with 1 cm² openings spaced 1 mm apart. A dish was placed under the grating to collect any eggs laid during spawning. The combination of grating and dish prevented the female from picking the eggs up in her mouth, so that the number of eggs laid with each male could be accurately determined.

Males were matched for size (difference in standard length, L_S , <2 mm) and randomly assigned to stimulus tanks. Males were allowed to acclimatize for 48 h prior to the

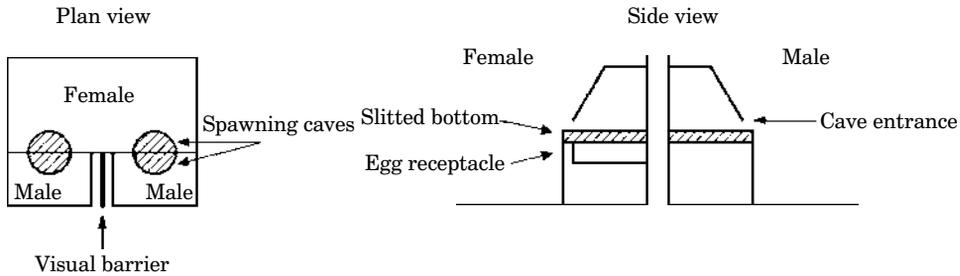


FIG. 2. Test arena for the egg catcher assay of mate choice (plan and side views). While double pane glass prevented visual, tactile and olfactory communication between males and females, the split caves allowed visual interaction between male and female subjects throughout the spawning sequence. Eggs laid within the cave fell through the slit cave floor and into the egg receptacle. Male success was scored as the presence of eggs in the cave within his territory.

introduction of the female. A female *M. zebra* or *M. benetos* was introduced to a neutral shelter in the centre of the testing aquarium in the morning and left until eggs were deposited. Receptacles were checked twice daily for eggs, and each trial continued until eggs were found. The presence of eggs was considered to be an indication of the female's choice of a specific male. After the trial, the female was removed and the testing chamber was drained and bleached to remove any pheromonal cues, which might promote female copying behaviour. The next pair of heterospecific males were arranged in alternate stimulus tanks to avoid side bias. Sixteen males of each species were used to test female preference. Due to a shortage of stock, four males of each species were reused, but were never paired with the same heterospecific male. The females used in these trials had no previous contact with any of the specific males presented, however, they were raised with other conspecifics. Ten females of each species were tested. The number of observed spawns each male received was compared to random mating using Fisher's exact test.

RESULTS

Trials ran a mean of 33.8 days (35.3 ± 8.84 for *M. benetos* and 32.0 ± 10.42 for *M. zebra*; mean \pm S.E.), which is similar to the duration of a female only access trial with the same species (mean = 25 days, range = 8–44 days, Kellogg, 1997) and with *Astatotilapia elegans* Trewaras (32–35 days, Hert, 1989). Eggs were never found in more than one dish during any of the trials, and no heterospecific spawning events were observed (Table I). Females deposited

TABLE I. Results of the egg catcher assay. Females of *Metriaclima zebra* and *Metriaclima benetos* were presented with a simultaneous choice between a heterospecific and conspecific male. Male reproductive success was indicated by the presence of eggs within his territory. Using only visual cues, females demonstrated complete assortative mate choice by depositing eggs in the caves of conspecifics (Fisher's exact test, $P < 0.05$)

Female	Number of spawns with male		Number of eggs deposited with male	
	<i>M. zebra</i>	<i>M. benetos</i>	<i>M. zebra</i>	<i>M. benetos</i>
<i>M. zebra</i>	10	0	152	0
<i>M. benetos</i>	0	10	0	166

eggs in the cave nearest the conspecific male in all 20 trials (10 for each species). These results demonstrated complete assortative mating (Fishers's exact test, $P = 0.039$ for both species).

Occasionally, a female was found holding some of the eggs in her buccal cavity. Females were never observed to be holding eggs, unless some eggs had also been deposited in a dish. Since the possibility that mouth-brooded eggs had been spawned elsewhere in the testing arena could not be discounted, these eggs were not scored. The mean number of eggs recovered from each species was nearly identical (20.25 ± 7.17 for *M. benetos* and 19.88 ± 5.74 for *M. zebra*; mean \pm s.e.) with a total range of 2–70 eggs per spawn (3–70 eggs per spawn for *M. benetos* and 2–61 eggs per spawn for *M. zebra*). This is very similar to the brood sizes reported for other populations of *M. zebra* in female only access trials (mean = 20.6 embryos; range = 2–56 embryos; Knight & Turner, 2004) and *A. elegans* (mean \pm s.d. = 21.8 ± 7.8 embryos; Hert, 1989). The size of broods collected in the 'egg catcher' apparatus was also similar to those observed in captive (mean \pm s.e. = 24.5 ± 11.5 embryos; Holzberg, 1978) and wild populations of *M. zebra* (mean \pm s.d. = 26.0 ± 6.7 embryos; Marsh *et al.*, 1986).

DISCUSSION

Attempts to quantify mate choice often rely on indirect measurements during restrained access experiments where male and female fishes are physically separated (Wagner, 1998). While physical barriers between test subjects are necessary to control the cues used by the individuals making a choice, it also forces the investigator to use indirect measures to quantify that choice. Studies in guppies *Poecilia reticulata* Peters (Kodric-Brown, 1993), sand gobies *Pomatoschistus minutus* (Pallas) (Forsgren, 1992), and Pacific blue-eyes (Wong, 2004) have demonstrated that association time can be an effective measure of eventual copulation success. All of these studies, however evaluated intraspecific variation as opposed to conspecific recognition. Gabor (1999) has demonstrated that female sailfin mollies *Poecilia latipinna* (Lesueur) may associate with larger individuals for reasons other than mate choice. In several species (sticklebacks: Hay & McPhail, 1975; darters: Fuller, 2003; cichlids: Seehausen, 1997; Danley 2001) association time fails to demonstrate the assortative mating observed in the wild or during free access experiments. It may be misleading to equate time spent with mate choice, without corroboration of some subsequent spawning behaviour or confirmation of the subject's reproductive state.

Barlow (2002) suggested that the inclusion of specific courtship behaviours would help to distinguish mating activity from a fear response. The use of unambiguous spawning behaviour has been used effectively to quantify mate preference in several species of fishes (Nagel & Schluter, 1998; Amundsen & Forsgren, 2003; Fuller, 2003). Unfortunately, the typical courtship behaviour of a haplochromine female is to follow and circle with the displaying male (Fryer & Iles, 1972; Holzberg, 1978; McElroy & Kornfield, 1990), which is difficult to score in a restricted access assay. Behaviours such as quivering, lateral displays, biting, head shaking and approaching have been used to quantify female mate

preference (Falter & Charlier, 1989; Barlow & Siri, 1997; Seehausen & van Alphen, 1998; Jordan *et al.*, 2003). Many of these behaviours are used in both courtship and aggression (Baerends & Baerends-van Roon, 1950), so it is sometimes difficult to distinguish the motivation driving the individual's behaviour (Danley, 2001). The motivational uncertainty inherent in indirect behavioural measures makes it necessary to use direct measurements of mate choice whenever possible. The 'egg catcher' assay combines the control of a 'restrained access' experiment with the power of a direct measure of mate choice.

The egg catcher assay unambiguously demonstrated that conspecific mate choice is possible when only visual cues are available to the female. As far as is known, this is the first study to use a direct measure of conspecific mate recognition using only visual cues in cichlids. Previous studies have demonstrated that species (Kellogg, 1997; Knight *et al.*, 1998; van Oppen *et al.*, 1998) and even populations (Knight & Turner, 2004) of mbuna mate assortatively when all cues are available. Further Couldridge & Alexander (2002) and Jordan *et al.* (2003) found that females tend to associate with conspecifics when presented with visual cues alone. Seehausen *et al.* (1997) suggested that visual cues are so important to conspecific recognition that a change in the normal photic environment results in the loss of species cohesiveness. Seehausen & van Alphen (1998) were able to disrupt conspecific mate choice between the blue and red morphs of *P. nyererei* when females viewed males under monochromatic light. In a similar experiment female *M. zebra* (blue barred), *M. benetos* (solid blue) and *Metriaclima barlowi* McKaye & Stauffer Jr (gold body with blue fins) were still able to recognize conspecifics under monochromatic light (Jordan *et al.*, 2003). While both *P. nyererei* morphs share a common melanistic pattern, the three *Metriaclima* species display differing patterns of colour contrast, which would not have been obscured by the change in lighting.

While this study demonstrates that visual cues are sufficient for mate choice, it does not preclude the possibility that multiple signals or sensory modalities are utilized during mate assessment (Hankison & Morris, 2003). A hierarchy of cues may be used during intraspecific mate choice. Females could be basing their choice on differences in male colour pattern, morphology, behaviour repertoire, or some sub-set and combination of each. Genus specific differences in courtship 'dances' have been reported for sympatric species of sand-dwelling haplochromine cichlids (Stauffer *et al.*, 1993), but McElroy & Kornfield (1990) failed to see any species-specific courtship behaviours among mbuna cichlids. Specific differences in the temporal elements of courtship sounds have been identified between sympatric genera of Lake Malawi sand-dwellers (Lobel, 1998) and species of mbuna (Amorim *et al.*, 2004). While there is sexual dimorphism in the enzyme activity of muscles that tend to be associated with sound production (Rice & Lobel, 2002), the effect of these auditory signals on mate choice remains to be tested. The use of olfactory cues have been demonstrated in other cichlid species (Chien, 1973; Crapron de Caprona, 1980), but Jordan *et al.* (2003) reported that female mbuna failed to respond differently when exposed to water containing conspecific *v.* heterospecific olfactory cues. The 'egg-catcher' design allows new approaches to identify both the specific cues used in inter- and intraspecific mate choice and the synergistic effects of various signal modalities.

The complete assortative mating demonstrated in this experiment could also be consistent with male choice of the females based on visual cues. If males are able to recognize conspecific females, then they may be selectively displaying to conspecific females. Knight & Turner (1999) found that male members of the genus *Metriaclima* were able to differentiate between hetero- and conspecific females only when the female colour patterns were different (e.g. orange blotched *v.* barred). If female colour patterns were similar, or identical, then the males were unable to distinguish hetero- from conspecifics. Seehausen & van Alphen (1998) found that while female *P. nyererei* displayed a preference for conspecific males, female colour form had no effect on male courtship. This is consistent with the observation of males displaying to heterospecific females during this study. Assessment of male courtship behaviours during the initial introduction of the female could be used to delineate between male and female choice (Nelson, 1995).

Female preference for male colour patterns is a necessary prerequisite for many models of cichlid speciation *via* female mate choice (Turner & Burrows, 1995; Deutsch, 1997; van Doorn *et al.*, 1998; Higashi *et al.*, 1999; Kondrashov & Kondrashov, 1999; Seehausen *et al.*, 1999; Takimoto *et al.*, 2000; Lande *et al.*, 2001). If this pattern of assortative mating by visual cues is consistent among the mbuna, then these models could provide a mechanism for the origin and maintenance of the incredible diversity of male colour patterns found within Lake Malawi.

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