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Limited possibilities for prezygotic barriers in the reproductive behaviour of sympatric *Ophthalmotilapia* species (Teleostei, Cichlidae)

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ABSTRACT

Since prezygotic rather than postzygotic barriers are believed to maintain the diversity of closely related sympatric cichlids, differences in phenotypic traits and reproductive behaviours are likely involved in maintaining species boundaries. Here, we focused on the reproductive behaviour of three *Ophthalmotilapia* species with distributions that only overlap on a small stretch of the shore line of Lake Tanganyika. Repeated introgression of mitochondrial DNA between these species was previously reported, which suggested they can hybridise. Our aim is to test the hypothesis that reproductive behaviour acts as a prezygotic barrier that prevents frequent hybridisation in sympatric *Ophthalmotilapia* species. We performed a quantitative analysis of twelve reproductions (four for *O. ventralis*, six for *O. nasuta*, one for *O. boops*, and one between a female *O. ventralis* and a male *O. nasuta*). Although similar ethograms were obtained for these reproductions, the *O. ventralis* and *O. boops* males displayed a behaviour that was never performed by *O. nasuta* males. This behaviour was displayed during courtship and we called it ‘invite’. In *O. ventralis*, we could show that it was associated with the emission of a single pulse sound. The comparison of *O. nasuta* and *O. ventralis* reproductive behaviours also revealed some quantitative differences: *O. ventralis* males showed the location of the bower more often to the female, whereas *O. ventralis* females followed the male more often. The similarity between the reproductive behaviours in *O. ventralis* and *O. nasuta* could explain the occurrence of the heterospecific spawning event recorded between an *O. nasuta* male and an *O. ventralis* female. Importantly, few eggs were laid and the maternal mouthbrooding that resulted from this heterospecific reproduction only lasted for two days, which suggested the abortion of egg development. Hence, in the absence of conspecifics, courtship and mating behaviours alone do not constitute perfect prezygotic barriers between these two species.

1. Introduction

Cichlid species flocks from the East African Great Lakes are classic examples of adaptive radiation (Day et al., 2008). With ca. 210 cichlid species grouped in 15 tribes, the cichlid assemblage of Lake Tanganyika (LT) is the least speciose of the three largest East African Lakes (Day et al., 2008). Yet, it is the oldest and the most diverse in terms of phylogeny (Salzburger et al., 2002a; Koblmüller et al., 2008), brood care, reproductive tactics, mate traits, and mating behaviour (Kuwamura, 1986; Sefc, 2011; Morita et al., 2014). For example, while the endemic cichlids from lakes Malawi and Victoria are all maternal mouthbrooders, LT cichlids show at least five types of parental care (Taborsky and Limberger, 1981; Kuwamura, 1986): biparental or maternal incubation in mouthbrooders, and maternal, biparental, or cooperative (i.e. parents and helpers) guarding in substrate brooders. Since prezygotic rather than postzygotic barriers are believed to maintain the diversity of closely related sympatric cichlids (Kornfield and Smith, 2000), differences in phenotypic traits and reproductive behaviours are likely involved in maintaining species boundaries. Yet, many instances of hybridisation have been found in Great Lake cichlids (Rüber et al., 2001; Salzburger et al., 2002b; Smith et al., 2003; Egger et al., 2007; Koblmüller et al., 2007; Keller et al., 2013), which may

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have played an important role in shaping the cichlid radiations (Seehausen, 2004; Sefc et al., 2017). Female mate choice, potentially based on male reproductive behaviour, has been suggested as the main barrier for hybridisation in cichlids (Kocher, 2004). So far, emphasis has been placed on the role of colour patterns (Kocher, 2004; Seehausen et al., 2008; Egger et al., 2010) and McElroy and Kornfield (1990) failed in the attempt to identify barriers to hybridisation in the reproductive behaviour of the mbuna species flock of Lake Malawi. However, they recognised that some differences may not have been quantified in their analysis and differences in the frequency with which courtship behaviours are performed were considered as sufficient to explain the reproductive isolation of two cichlids from Central America (Baylis, 1976). Additional efforts are needed to assess the role of reproductive behaviour in the evolutionary history of African cichlids (McElroy and Kornfield, 1990).

Here, we present a study on the genus *Ophthalmotilapia*, a member of the tribe Ectodini, containing four valid species (Hanssens et al., 1999): *Ophthalmotilapia ventralis*, *O. boops*, *O. nasuta*, and *O. heterodonta.* The status of the latter two species, which occur in allopatry, is still debated as the morphological differences between them fail to designate some populations to either of the two species (Hanssens et al., 1999; Nevado et al., 2011; Konings, 2014). *Ophthalmotilapia* can be distinguished from all other ectodine genera by the bifid spatulae at the distal end of the male pelvic fins (Liem, 1981; Hanssens et al., 1999). Because of their similarity in colour and shape to eggs, these spatulae are suggested to function as egg dummies during reproductive behaviour (Haesler et al., 2009; Immler and Taborsky, 2009).

Although *Ophthalmotilapia* has a circumlacustrine distribution, the distributions of the four species differ strongly (see Hanssens et al., 1999 for distribution maps). This could be a consequence of their different habitat preferences. *Ophthalmotilapia nasuta* prefers so-called intermediate habitats (i.e. sand with rocky patches) and can be found throughout the lake (Konings, 2014). The other three species have more restricted distributions and only occur at rocky shores (Konings, 2014). *Ophthalmotilapia ventralis* and *O. heterodonta* have non-overlapping distributions, with the former species occurring in the southern third and the latter in the northern and central parts of the lake. *Ophthalmotilapia boops* is only present along a small part of the south-eastern lake shore. This part of the lake forms the only stretch where three *Ophthalmotilapia* species occur in sympatry (Hanssens et al., 1999; Nevado et al., 2011). In this region, a comparison of mitochondrial markers and microsatellite patterns revealed hybridisation events mostly between *O. nasuta* males and females of *O. ventralis* and *O. boops* (Nevado et al., 2011)

All *Ophthalmotilapia* species are sexually dimorphic. Males attain a larger size than females and their bodies are generally deeper. Unlike females, mature males have prolonged pelvic fins, almost reaching to the origin of the caudal fin and terminating in bifid spatulae (Hanssens et al., 1999). They also show a spectacular colour pattern, while females are generally paler and uniformly coloured (Schupke, 1994). Territorial males manage a breeding site or bower that differs in size and shape depending on the species (Kuwamura, 1986; Morita et al., 2014); some build sand craters while others clean a small area on a rock, for example. The reproductive behaviour of *O. ventralis* has been briefly described by Haesler et al. (2009) and Immler and Taborsky (2009) as follows: (1) a territorial male approaches a female; (2) after a quick lateral display, the male swims to his bower with an exaggerated undulation of the tail; (3) the female follows the male and enters the bower; (4) the male places his genital papilla onto the bower and quivers (probably releasing sperm); (5) he subsequently presents the egg dummies where he has just quivered; (6) the female may take the egg dummies into her mouth; (7) the male leaves the bower and defends the territory; (8) the female lays an egg (up to three eggs in successive turns), takes it up in her mouth quickly and leaves the bower. According to Kuwamura (1986), females always lay one egg at a time. After the female leaves the bower, the male starts to court the female again and the pair can start another spawning bout (Immler and Taborsky, 2009). Some parts of the reproductive behaviour of *O. nasuta* and *O. boops* have also been mentioned in the literature (Brichard, 1989; Loose, 2007; Konings, 2014; Morita et al., 2014). In spite of small contradictions in the descriptions given by these authors (e.g. absence or presence of pelvic fin presentation), they generally considered the reproductive behaviour of *Ophthalmotilapia* species to be similar. The sole interspecific difference that was clearly identified was the type of the bower. The male *O. nasuta* builds a large ‘crater shape’ sand mount while males from the other species dig a small bower in the sand or clean a flat rock. To date, no quantitative comparison of the reproductive behaviour based on data collected in controlled conditions has been provided.

Although repeated unidirectional introgression of nuclear and mitochondrial DNA from congeners into *O. nasuta* occurred (Nevado et al., 2011), hybrid phenotypes are rare in the lake and species boundaries are maintained even where they are living in sympatry. Therefore, we aimed to test the hypothesis that reproductive behaviour acts as a prezygotic barrier that prevents frequent hybridisation in sympatric *Ophthalmotilapia* species. To this end, we compared the reproductive behaviour of *O. ventralis* and *O. nasuta* using successful reproductive events recorded under controlled conditions. Our prediction is that interspecific differences in reproductive behaviour or in the timing of these behaviours could cause the female not to follow, lay eggs in the bower, or take the sperm of a heterospecific male. In addition, sound recordings were performed to identify potential differences in acoustic cues used by these two species. Two additional reproductions were recorded and analysed, one of *O. boops* and one of a heterospecific combination of a female *O. ventralis* and a male *O. nasuta*. These data were qualitatively compared with those obtained for *O. nasuta* and *O. ventralis* as they are of great interest when discussing the importance of prezygotic barriers in *Ophthalmotilapia.*

2. Materials and methods

2.1. Fish collection and maintenance

We purchased wild-caught individuals of *Ophthalmotilapia* from two commercial suppliers: ‘Cichlidenstadl’ (Alerheim, Germany) and ‘Les Aquariums de Marbais’ (Marbais, Belgium). We bought four *O. ventralis* coming from ‘south of Moba’ (DR Congo), and five *O. ventralis*, thirteen *O. nasuta,* and four *O. boops* from the surroundings of Ulwile Island (Tanzania).

We kept the fishes in monospecific tanks (photoperiod: 12:12 h L:D; water temperature: 26 ± 1 °C; carbonate hardness: > 8 dKH) at the University of Liège (Belgium) and fed them once a day with ‘Tropical Spirulina forte’ mini-granules *ad libitum*. Three males and four or five females, all adults, were introduced simultaneously in monospecific tanks with a single water circulation system and a similar layer of sand from the Loire river on the bottom. Hiding places and flat rocks were also provided. This setup worked well only for *O. nasuta.* In the tanks of *O. ventralis* and *O. boops,* courting behaviours were also observed within weeks but the constant aggressiveness of the males towards the females seemed to prevent the latter from mating. Hence, the setup for *O. ventralis* and *O. boops* was changed to monospecific tanks with a single male and three to four females per tank and individual filtration systems. Again, hiding places and flat rocks were provided. Although it took several weeks before a first reproduction was observed, this setup gave better results. Later, this setup was also tested on *O. nasuta* to check if the number of males in the tank had a strong impact on their reproductive behaviour. After the conspecific reproductions were recorded, three females of *O. ventralis* were kept with one male of *O. nasuta.* The other fish were kept in conspecific setups for other studies. All experimental procedures were approved by the University of Liège Institutional Animal Care and Use Committee (protocol #1759).