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).

Table 1
Overview of data for the *Ophthalmotilapia* reproductions recorded.

Species	Reproduction(s)	Male(s)	Females	Hydrophone	Tank(s)
Ophthalmotilapia ventralis (OV)	4	1	3	3	2 × 260
Ophthalmotilapia nasuta (ON 1)	3	1	4	1	1 × 540
Ophthalmotilapia nasuta (ON 3)	3	3*	4 to 5	3	1 × 810
Ophthalmotilapia boops (OB)	1	1	3	0	1 × 800
O. nasuta and *O. ventralis* (ONOV)	1	1 (*O. vent*)	3 (*O. nas*)	1	1 × 260

Reproduction(s): number of reproduction(s) fully recorded; Male(s): number of male(s) in the tank; Females: number of females in the tank; Hydrophone: number of reproduction(s) also recorded by a hydrophone. Tank(s): number of tanks × volume (l). *O. nas*: *Ophthalmotilapia nasuta*; O. *vent*: *Ophthalmotilapia ventralis*; * two dominant and one subordinate males.

2.2. Video recordings and analyses of the reproductive behaviour

Every reproduction consisted of multiple spawning bouts, which means that most of the behaviours (e.g. female follows the male to the bower) were recorded multiple times during each reproduction: four reproductions for *O. ventralis* (OV), six reproductions for *O. nasuta* [three with one male and four females (ON 1) and three with three males (two dominants and one subordinate) and four to five females (ON 3)], one reproduction for *O. boops* (OB), and one heterospecific reproduction between an *O. ventralis* female from 'Moba' and an *O. nasuta* male (ONOV). We analysed every reproduction from 20 min before the first egg was laid to 10 min after the last egg was laid (Table 1). We never recorded the same mating female twice but it did happen that the same male was recorded more than once (three ON males for the six ON reproductions and two OV males for the four OV reproductions).

We defined nineteen behaviours (Table 2) using the nomenclature and descriptions by Baerends and Baerends-Van Roon (1950a). We encoded these behaviours as point (i.e. expansion in time is not recorded because it is very short) or state (i.e. expansion in time is recorded) events in the Boris v 2.72 open source software (Friard and Gamba, 2016). We encoded every behaviour from 20 min before the first egg was laid to 10 min after the last egg was laid. Moreover, when

it was feasible, we tracked inviting behaviours and pelvic fin presentations up to one hour before the first egg was laid (e.g. not feasible when mating started early in the morning shortly after the light was turned on). This protocol allowed the production of ethograms (Fig. 1) and quantitative analyses in STATISTICA 12 (Statsoft, Tulsa, U.S.A).

2.3. Sound recordings and analyses

We placed a hydrophone [HTI Min-96, −164.4 dB re. 1 V μPa−1; bandwidth 2 Hz and 30 kHz (Long Beach, MS, USA), or a Brüel and Kjær 8101 hydrophone connected to a calibrated Brüel and Kjær 2610 amplifier, sensitivity −184 dB re. 1 V μPa−1; bandwidth 0.1 Hz to 200 kHz (Nærum, Denmark)] at half the height of the water column, over or just next to the bower. We connected this hydrophone to a Tascam DR-05 recorder (TEAC, Wiesbaden, Germany) at a sampling rate of 44.1 kHz. Sound recording devices were not always turned on because of the necessity to turn the aeration and the pumps off. Therefore, sounds were not recorded during some reproductions (Table 1). We processed and analysed the signal in Adobe Audition 2.0 (Adobe Systems Incorporated, San Jose, CA, USA). To prevent signal distortion by resonant frequencies, the signal was down-sampled at 10,000 Hz and low-pass filtered at 2,500 Hz (see Akamatsu et al., 2002). We also high-pass filtered the signal at 60 Hz to reduce the impact of low frequency noise (e.g. building vibrations and electrical noise). Lastly, we estimated the pulse duration (ms) and, in some cases, pulse period (ms) from the waveform, and the peak frequencies from power spectra.

Since the sounds emitted by *Ophthalmotilapia* individuals were very weak (i.e. low signal to noise ratio), we could only analyse the sounds emitted close to the hydrophone (source at max. two body lengths from the hydrophone). However, it seems likely that sounds linked to every type of behaviour would have been detected because each of them was performed several times in the vicinity of the hydrophone.

2.4. Statistical analysis

We first performed principal component analyses (PCAs) to have an overview of the possible interspecific differences in the reproductive behaviour. For these PCAs, we considered every reproduction event (four OV, three ON 1, three ON 3, one OB, and one ONOV) as an individual and the prevailing behaviours (i.e. behaviours that were consistently observed for at least one species) as the variables. For state variables, data types were percentages of the total time and scores (i.e.
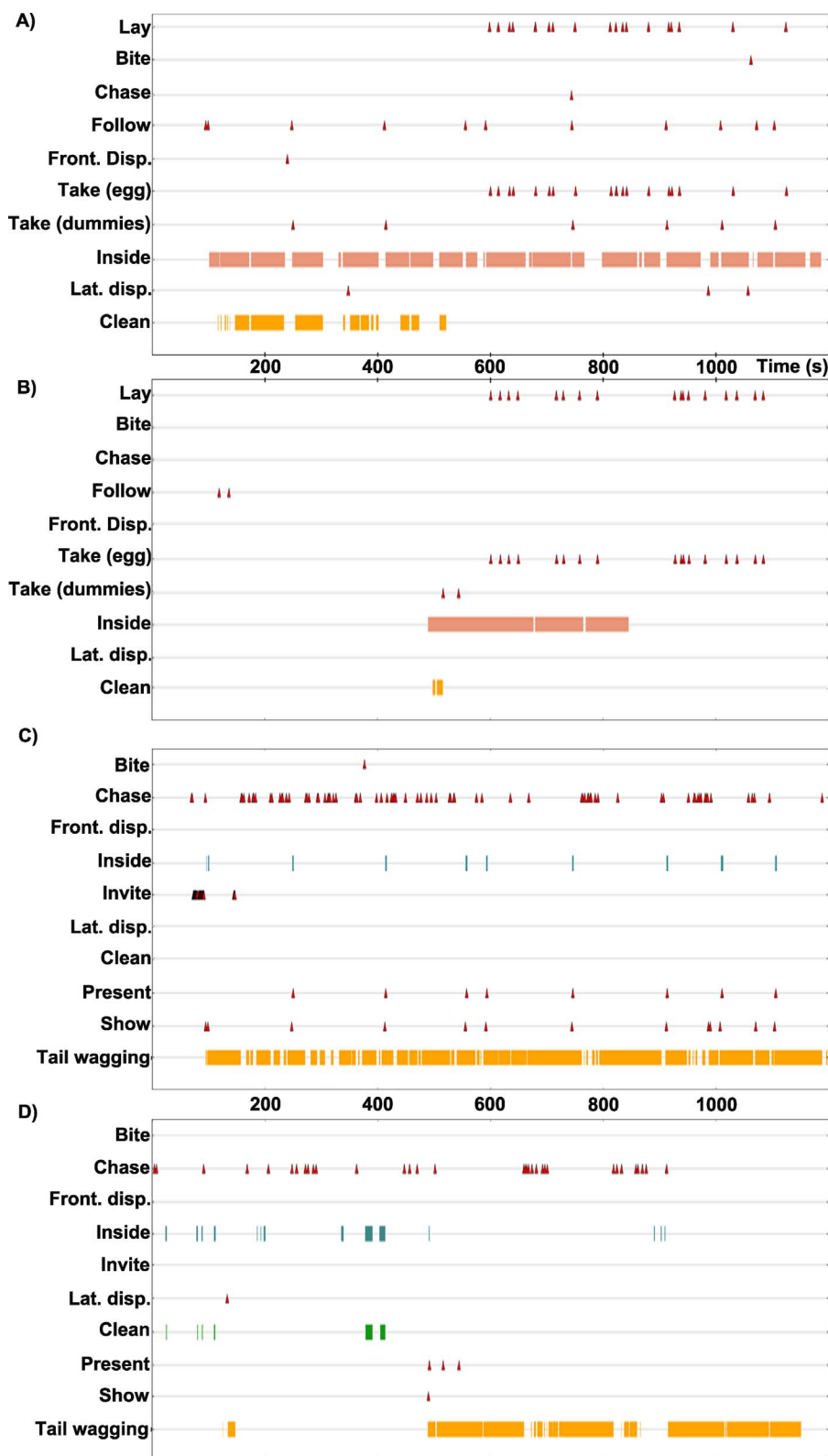
Table 2
Behaviours used for the quantitative analysis of *Ophthalmotilapia* reproductions.

	Nomenclature	Description
PE	Bite	Fish bites another fish
PE	Chase	Fish swims very fast towards another fish
SE	Clean	Fish removes dirt/sand to clean/build the pit
PE	Follow	Fish swims behind another fish
PE	Frontal display	Fish spreads its pectorals, swells its throat, head high
SE	Inside	Fish is inside the nest (less than one body length from the pit floor)
PE	Invite	Fish performs a fast and unilateral contraction of its body musculature, generally head down, close to another fish
PE	Lateral display	Fish presents his flank, swells its throat and leans slightly
PE	Lay	Fish lies an egg
PE	Lead	Fish swims at the front of another fish and guides it to the nest
SE	Pelvics flickering	Fish alternatively moves its pelvic fins up and down (fast movements)
PE	Present	Fish presents his pelvic fins to the mouth of a second fish, generally egg dummies are on the pit floor
PE	Present over the back	Fish abducts the pelvics slightly swimming just over the female. The latter being inside the nest
PE	Show	Fish stops at the entrance of the nest and tail waggle.
PE	Sound	Fish emits an acoustic signal of interest
SE	Tail wagging/ swimming on the spot	Fish shakes the caudal part of its body (caudal fin and caudal part of the dorsal fin) with head slightly up or down + Fish performs alternate forward movements of the pectoral.
PE	Take	Fish takes or tries to take something in mouth (modifiers: eggs or egg dummies)
PE	Uncommon	Any uncommon behaviour that was recorded in rare occasions

PE: point event; SE: state event.

Fig. 1. Ethograms of *Ophthalmotilapia ventralis* and *O. nasuta* spawning bouts. Behaviour recorded from 10 min before to 10 min after the first egg was laid. A mating female *O. ventralis* (A) and *O. nasuta* (B), and a mating male *O. ventralis* (C) and *O. nasuta* (D) were selected to illustrate the first spawning bouts of the reproduction in this genus.

number of times a behaviour occurred), whereas for point variables, these were only scores. Data for males and females were analysed separately.

After data were tested for normality (Kolmogorov-Smirnov test for normality), we performed multi- (T^2 Hotelling) and uni-variate (Student t or Mann-Whitney U) tests on the variables listed in Tables 3 and 4 to compare the reproductive behaviour of ON 1 and OV 1. We excluded behaviours that were completely absent in one species (no 'invite', 'frontal display' and 'bite' recorded for ON 1). Results from univariate tests were adjusted using the sequential Bonferroni correction (Rice, 1989). For state variables like 'inside' and 'tail wagging', the Boris software provided scores and total duration. Here, we showed the percentage of the total time of the analysis for the variables 'inside' and 'tail wagging'. However, the results were similar when we used scores

Table 3
Prevailing behaviours listed for the *Ophthalmotilapia* mating males.

	OV 1 (N = 4) Mean ± SD	ON 1 (N = 3) Mean ± SD	ON 3 (N = 3) Mean ± SD	OB 1 (N = 1)	ONOV (N = 1)
Bite (n)	3.8 ± 4.8	0 ± 0	7.3 ± 8.6	0	2
Chase (n)	164.8 ± 100.7	52.3 ± 28.4	90 ± 74.5	63	233
Clean (n)	12.5 ± 10.5	21.7 ± 15.2	11.3 ± 14.5	37	0
Frontal display (n)	0.3 ± 0.5	0 ± 0	0 ± 0	0	0
Inside (n)	51.8 ± 41.2	32 ± 19.1	54.7 ± 59.2	45	1
Inside (%)	4.5 ± 2.4	6.7 ± 1.7	6.1 ± 3.8	10.6	< 0.1
Invite (n)	40.3 ± 28.8	0 ± 0	0 ± 0	20	0
Lateral display (n)	0.3 ± 0.5	1.3 ± 0.6	0.7 ± 1.2	4	6
Present (n)	12.5 ± 3.9	5.3 ± 4.7	19.3 ± 10	8	22
Show (n)	17.3 ± 1.3	1.3 ± 1.5	20.7 ± 8.3	20	2
Tail wagging (n)	76.3 ± 45.1	54.3 ± 26.6	119.3 ± 59.1	27	178
Tail wagging (%)	38.6 ± 25.4	42.5 ± 24.6	61.2 ± 22.9	46.7	36.3

n: occurrence of the behaviour. N: number of reproductions. OV 1: *Ophthalmotilapia ventralis* reproductions with a single male in the tank. ON 1: *Ophthalmotilapia nasuta* reproductions with a single male in the tank. ON 3: *Ophthalmotilapia nasuta* reproductions with three males in the tank. OB 1: *Ophthalmotilapia boops* reproductions with a single male in the tank. ONOV: Reproduction between a male *O. nasuta* and a female *O. ventralis*. s: duration in seconds. %: 100 × total duration of the behaviour/total duration of the analysis. SD: standard deviation.

instead.

3. Results

3.1. Ethogram of the reproduction behaviour in the genus Ophthalmotilapia

We analysed 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*). The mean time recorded between the laying of the first and the last egg was approximately 25 min (mean ± SD: 24.7 ± 16.4 min). Therefore, the mean time analysed was 55 min (see Section 2.2).

The reproductive behaviour in *Ophthalmotilapia* (*O. ventralis*, *O. nasuta*, and *O. boops*) is composed of several units called spawning bouts. These bouts are stereotypic and can be summarized in eight major steps (Fig. 2):

1) Male **chases (a)** and **cleans (b)**: the territorial male digs or builds a bower, cleans its bower, and chases (i.e. swims very fast towards another fish) all male and female intruders off its territory.
2) Female **follows**: the mating female follows a male (generally after being chased) swimming back to its territory. At that time, the male starts to wag its tail (**tail wagging**). In *O. ventralis* and *O. boops*, a step called **'invite'** (Fig. 3) often preceded step 2 during the first spawning bout of a reproduction. A male that 'invites' performs a fast and unilateral contraction of its body musculature, generally head down, close to a female (Fig. 3). This behaviour was generally repeated several times and associated with the emission of a short sound (at least in *O. ventralis*). Interestingly, we never observed the

'invite' in *O. nasuta* during our experiments.
3) Male **shows** the bower: the male swims to its bower and stops at the entrance until the female arrives. Throughout this step, the male always wags its tail, with its head slightly up.
4) Male **presents** pelvic fins: the male enters the bower, spreads its pelvic fins (perpendicular to the body), and places its bifid spatulae near the floor of the bower, using them as egg dummies.
5) Female tries to **take egg dummies**: female enters the bower (**inside: start**), swims towards the egg dummies, and opens her mouth.
6) Female **lays egg(s)**: the female lays an egg (sometimes 2 or 3 eggs one after another) in the bower. Male is tail wagging and chasing interacting fish of both sexes.
7) Female **takes egg(s)**: the female takes the egg(s) in her mouth. Male is tail wagging and chasing interacting fish. Female generally repeats (6) and (7) several times before she proceeds to the next step.
8) Female **leaves** (**inside: stop**): female leaves the bower with eggs and/or sperm in her buccal cavity.

In the three species, some steps can be skipped during a spawning bout (e.g. steps 6 and 7 are skipped during the first bout while steps 3 to 5 are skipped during the second bout). It means that some spawning bouts lack egg laying or pelvic fin presentation (presumably when sperm is released).

The females laid between 7 and 28 eggs. Every female left the nest at least once (Table 3) between the release of the first and last egg and the mean time interval between the laying of two consecutive eggs varied from seconds to minutes (Table 4). For the three *O. nasuta* reproductions recorded in the tank that contained more than one male (i.e. two dominant males and a subordinate male, ON 3), each female

Table 4
Prevailing behaviours listed for the *Ophthalmotilapia* mating females.

	OV 1 (N = 4) Mean ± SD	ON 1 (N = 3) Mean ± SD	ON 3 (N = 3) Mean ± SD	OB 1 (N = 1)	ONOV (N = 1)
Bite (n)	0.5 ± 0.6	0 ± 0	0.3 ± 0.6	0	0
Chase (n)	2.5 ± 3.1	3.7 ± 6.4	17 ± 5.6	0	1
Clean (n)	13 ± 10.9	8 ± 11.4	21.3 ± 22	3	2
Eggs interval (s)	81.5 ± 73.7	53 ± 26.1	116 ± 108.2	20	389
Follow (n)	14.5 ± 2.9	3.7 ± 3.1	26.7 ± 11.7	15	10
Front. Disp. (n)	0.3 ± 0.5	0 ± 0	0 ± 0	1	0
Inside (n)	34.5 ± 10.1	13.3 ± 5.7	49.3 ± 26.3	19	99
Inside (%)	41.5 ± 19.4	38.9 ± 25.3	37.8 ± 29.2	34.6	41.1
Lay (n)	22.5 ± 3.3	21.3 ± 5.9	13.3 ± 0.6	17	7
Lat disp. (n)	7 ± 5.9	0.3 ± 0.6	4 ± 5.2	1	0
Take egg (n)	21.3 ± 4	21.7 ± 6.4	12.3 ± 1.2	14	7
Take dummies (n)	8 ± 2.9	2.3 ± 0.6	12 ± 12.5	7	11

n: occurrence of the behaviour. N: number of reproductions. OV 1: *Ophthalmotilapia ventralis* reproductions with a single male in the tank. ON 1: *Ophthalmotilapia nasuta* reproductions with a single male in the tank. ON 3: *Ophthalmotilapia nasuta* reproductions with three males in the tank. OB 1: *Ophthalmotilapia boops* reproductions with a single male in the tank. ONOV: Reproduction between a male *O. nasuta* and a female *O. ventralis*. s: duration in seconds. %: 100 × total duration of the behaviour/total duration of the analysis. SD: standard deviation.
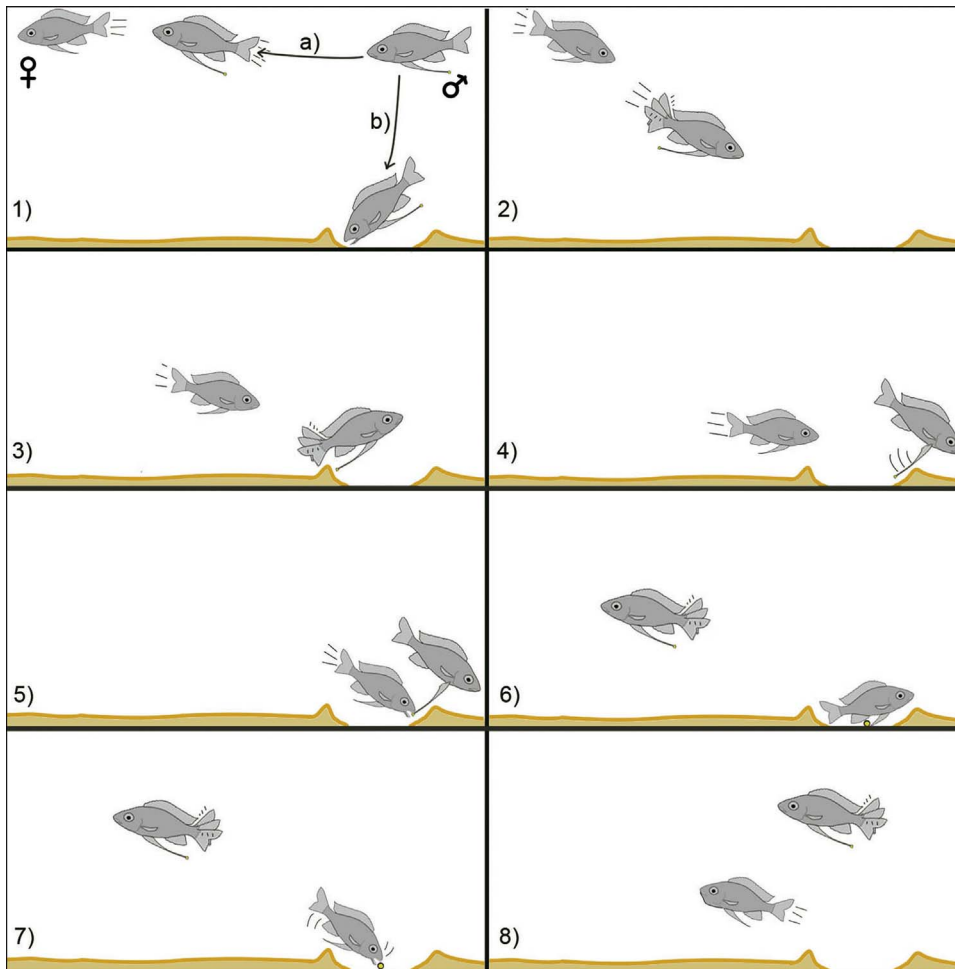
Fig. 2. The typical spawning bout of *Ophthalmotilapia ventralis*, *O. nasuta*, and *O. boops*. (1) The male chases the female and prepares the bower. (2) The female follows the male to his bower. (3) The male shows the bower to the female. (4) The male presents its pelvic fins. (5) The female enters the bower, swims towards the egg dummies, and opens the mouth. (6) The female lays an egg in the bower. (7) The female takes the egg in mouth. (8) The female leaves the bower. Stages 6 and 7 are generally repeated before 8. In stages 2, 3 and 6-8, the territorial male is generally tail wagging.

laid all her eggs in the bower of only one dominant male. We did, however, record reproductions for the two dominant males. In two instances the females visited the bowers of both dominant males before they laid their eggs.

Tail wagging was indisputably the most common courtship behaviour performed by the male and we observed it before, during, and after the female visited the bower. Mating males wagged their tails 14.1% to 81.8% of the analysed period. Females also sometimes wagged their tails, but this was rare (21.1 ± 27.2 times per reproductive event or 0.9 ± 1.5% of the analysis duration). Female tail wagging and pelvic fin flickering (18.8 ± 19.7 times per reproductive event or 1.2 ± 1.3% of the analysis duration) were mainly observed just before an egg was released. The mating male often stopped tail wagging to chase another interacting fish or to present its 'egg

dummies' to the female. In 60.4% of the cases, the pelvic fin presentation was followed by the female swimming with her mouth open towards the 'egg dummies'. Although it was not possible to see sperm emission on film or during direct observations, it most likely happened at this point. With a mean of 115.2 ± 87 chases (also towards males for ON 3 reproductions), the mating male invested a lot of energy in territory defence during the courtship and mating process. However, agonistic interactions other than chases (i.e. bites, lateral displays, and frontal displays) were not frequent during courtship and mating (Tables 3 and 4).

3.2. Inter- and intraspecific differences

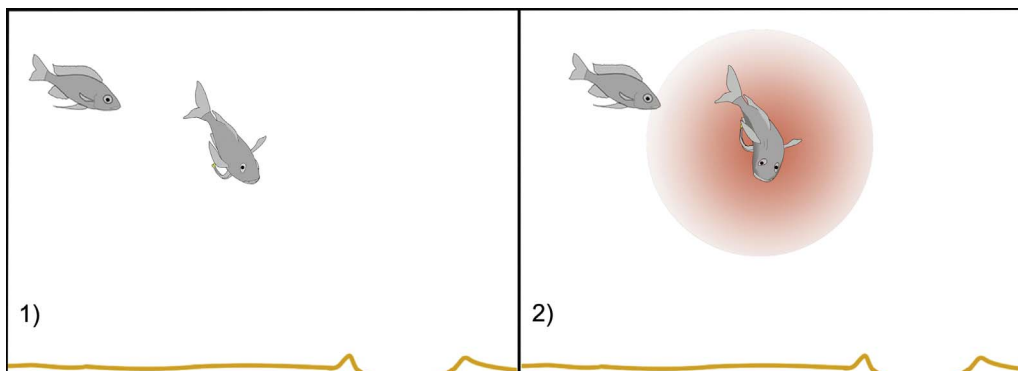Using the screen plot and the Kaiser-Guttman rule, the first four PCs

Fig. 3. Schematic representation of the courtship behaviour call 'invite' in *Ophthalmotilapia ventralis* and *O. boops.* (A) The male positions himself in front of the female (often head down). (B) He performs a fast-lateral movement of anterior part of his body. This movement results in the emission of an acoustic signal, the intensity of which drops on short distance (red sphere). This behaviour was always performed very close to the female.
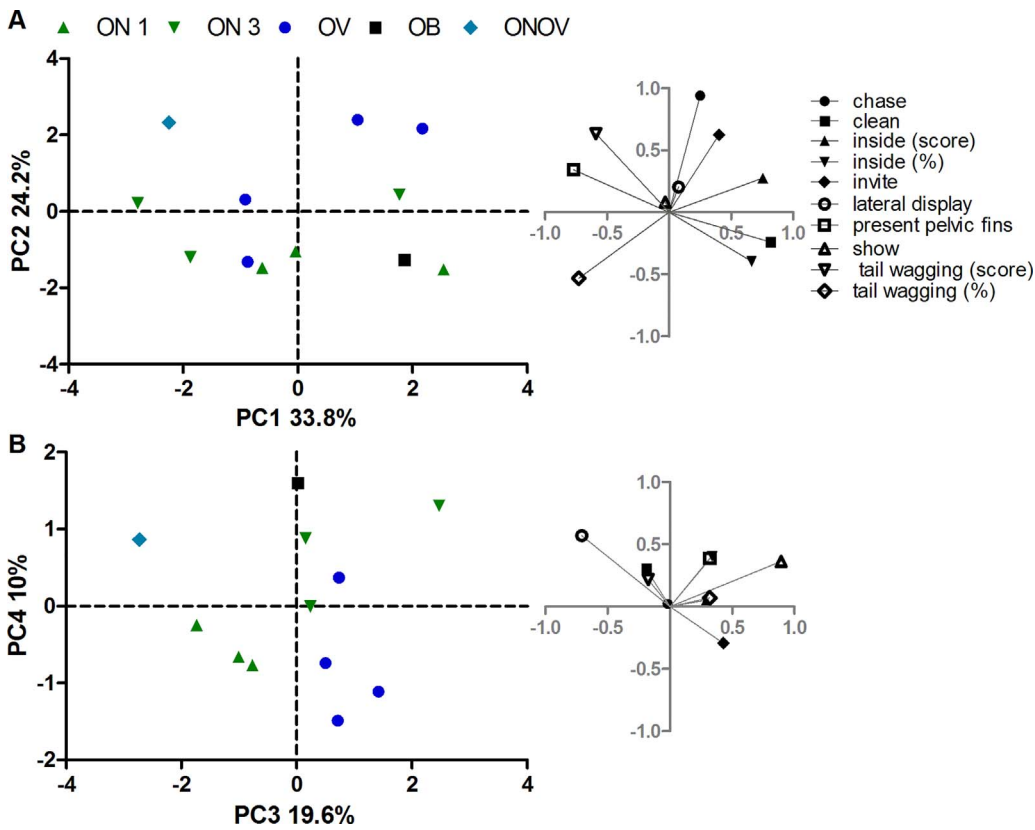
Fig. 4. Principal component analysis based on the behaviours recorded for *Ophthalmotilapia* mating males. (A) Coordinates of the individuals (left) and loadings of the variables (right) on PC1 vs. PC2. (B) Coordinates of the individuals (left) and loadings of the variables (right) on PC3 vs. PC4. OV: the male *O. ventralis* mated with a female *O. ventralis*. ON1: the male *O. nasuta* mated with a female *O. nasuta* and there was no other male in the tank. ON3: the male *O. nasuta* mated with a female *O. nasuta* and there were two other males in the tank. OB: the male *O. boops* mated with a female *O. boops*. ONOV: the male *O. nasuta* mated with a female *O. ventralis*. Score: number of events recorded. %: (total behaviour duration/total analysis duration) x 100.
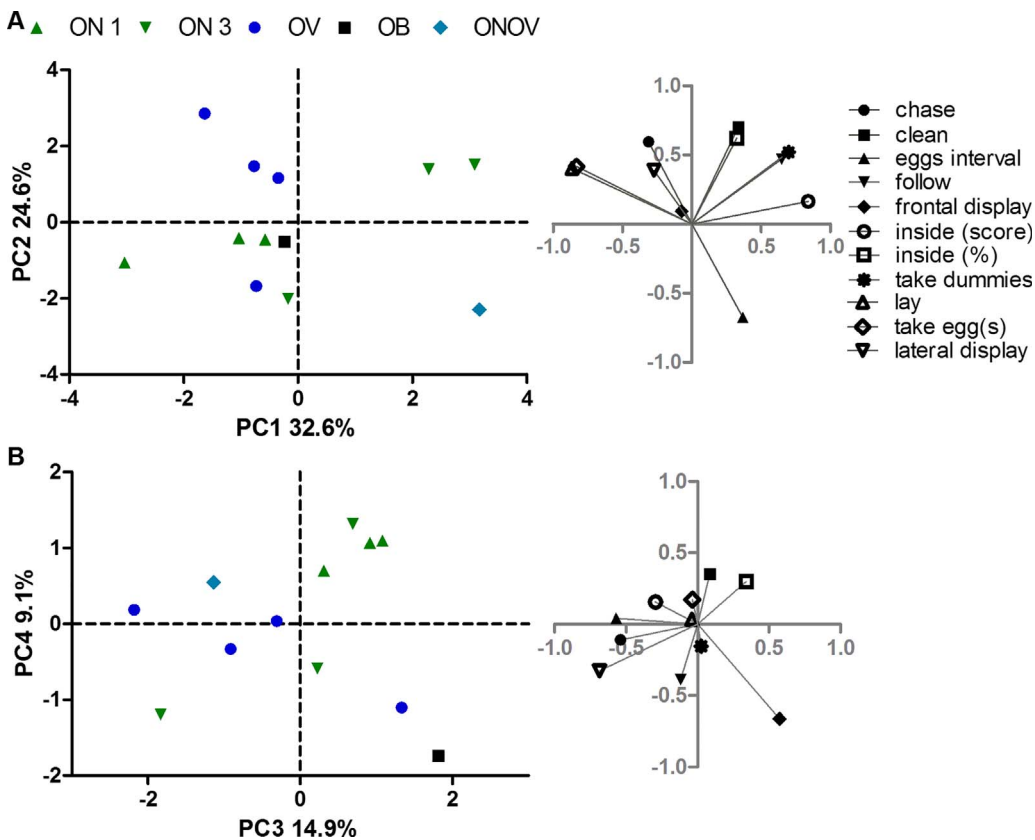

Fig. 5. Principal component analysis based on the behaviours recorded for *Ophthalmotilapia* mating females. (A) Coordinates of the individuals (left) and loadings of the variables (right) on PC1 vs. PC2. (B) Coordinates of the individuals (left) and loadings of the variables (right) on PC3 vs. PC4. OV: the male *O. ventralis* mated with a female *O. ventralis*. ON1: the male *O. nasuta* mated with a female *O. nasuta* and there was no other male in the tank. ON3: the male *O. nasuta* mated with a female *O. nasuta* and there were two other males in the tank. OB: the male *O. boops* mated with a female *O. boops*. ONOV: the male *O. nasuta* mated with a female *O. ventralis*. Egg interval: mean duration between the laying of two successive eggs. Score: number of events recorded. %: (total behaviour duration/total analysis duration) x 100.

of the principal component analysis were investigated. Regarding this analysis, male and female behaviours show much overlap among species (Figs. 4 and 5). However, there was less overlap on the PC3 vs PC4 graph of the male PCA; ON 1 and OV 1 males form two distinct groups on the third axis (Fig. 4C), and ON 1 and ON 3 males form two separate groups along PC3 and PC4 (Fig. 4C), these axes being related to the high loadings of the number of 'show' and 'lateral display' (Fig. 4D). In both analyses (male and female behaviour), the heterospecific interaction clearly separated from the conspecific ones on a combination of the first and second axis and, for males, also on the third axis (Figs. 4 and 5).

We saw no 'invite' behaviour in any of the seven reproduction events involving males of *O. nasuta* (Table 3), which also explains part of their position in the male PCAs (Fig. 4). This behaviour was recorded 20 times during the OB reproduction and 161 times during the OV reproductions (0, 40, 56, and 65 times). Although it was not performed during the heterospecific (ONOV) reproduction, the female *O. ventralis* did enter the bower and laid eggs with the *O. nasuta* male. This was, however, the reproduction with the lowest number of eggs laid (7 eggs) and the longest inter-egg intervals (Table 4). During the OV 1, OB 1, and ON 1 reproductions much more eggs were laid (22.5 ± 3.3, 17, and 21.3 ± 5.9 eggs per reproduction, respectively). The number of eggs laid during ON 3 reproductions was also low (13.3 ± 0.6 eggs per reproduction).

The statistical comparisons of behaviour focused on the four OV reproductions and the three ON 1 reproductions recorded with a single male in the tank. We could not include OB and ONOV reproductions to these analyses because we recorded only one reproduction for these setups. Except for the 'invite' behaviour (absent in *O. nasuta*), the courting and mating behaviours displayed by *O. ventralis* and *O. nasuta* males are quantitatively (T^2 Hotelling = 4227.7, F5,1 = 169.1, $P = 0.06$) and qualitatively similar. For the males, we only found a significant difference for the number of 'show' events. This was higher in *O. ventralis* (t = 15.2, dl = 5, $P = 0.00002$). Similarly, we found only one difference in the behaviour of the females: females of *O. ventralis* followed males (t = 4.8, dl = 5, $P = 0.0049$) more often than females of *O. nasuta*.

3.3. Sound production

Among the recorded behaviours, sounds with a potential communicative value were only recorded during the 'invite' behaviour (Fig. 3), which implies that we recorded no sound for *O. nasuta* (ON 1, ON 3, and ONOV). This acoustic signal was recorded only when the invite behaviour was intense (i.e. when the spasm movement was very quick) and performed very close to the hydrophone. We analysed 51 pulses recorded from three different *O. ventralis* males. These broadband frequency pulses (Fig. 6) had most energy between 100 and 750 Hz (mean ± SD, 310 ± 176 Hz) and had relatively short pulse duration (8.8 ± 4.1 ms). They were emitted singly or in pairs, except for one sound that looked like a pulse train. In this case, nine pulses were emitted at a pulse period of 157 ± 32 ms.

4. Discussion

Despite the variation in the number of steps between the spawning bouts of a reproduction event, the reproductive behaviours of the three *Ophthalmotilapia* species (*O. ventralis*, *O. nasuta*, and *O. boops*) were similar. The only qualitative difference was the absence of the 'invite' behaviour and the associated sound in *O. nasuta*. As in *O. ventralis*, male *O. boops* may produce sounds because they also display strong spasms during the 'invite' behaviours and as those spasms were associated with sound production in the former species (see Section 4.1 for more details). However, this needs to be confirmed as no hydrophone was recording during the OB reproduction. The comparison of *O. nasuta* and *O. ventralis* reproductive behaviour also revealed some quantitative differences: male *O. ventralis* performed significantly more 'shows' and
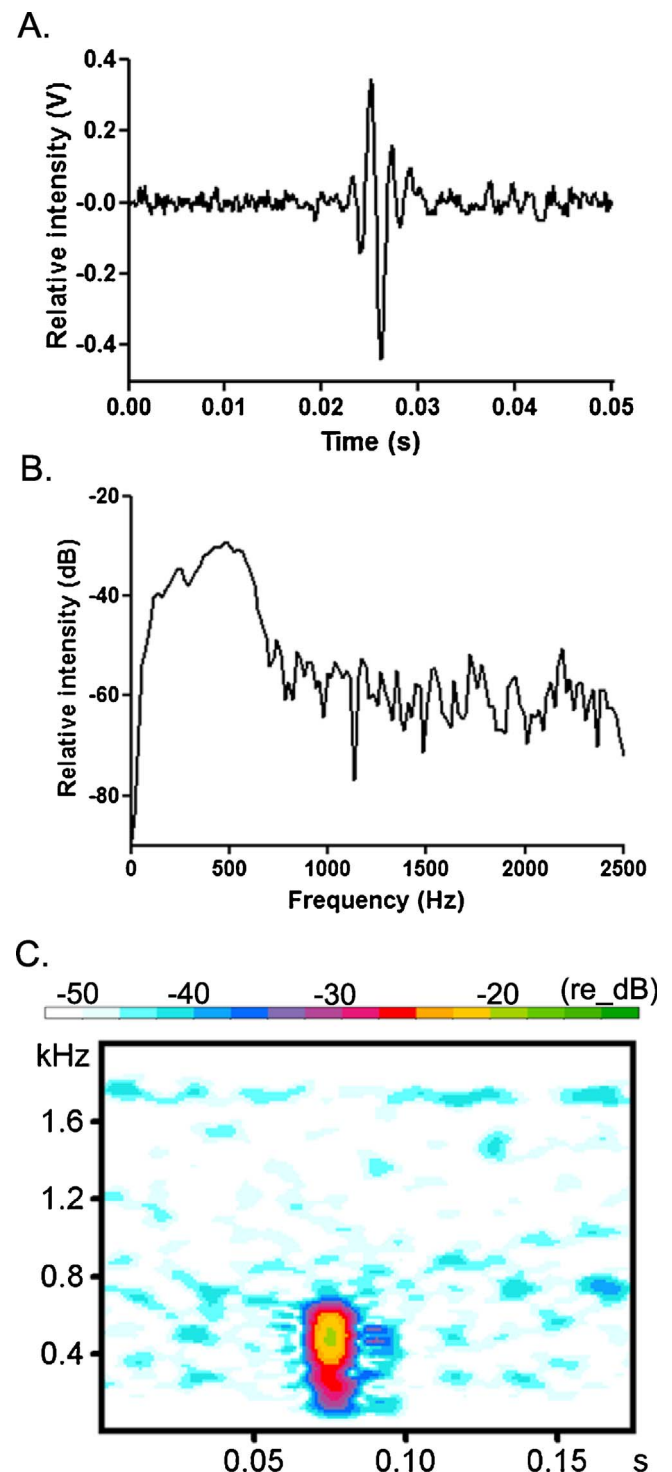
Fig. 6. Acoustic signal associated with the 'invite' behaviour of a male *Ophthalmotilapia ventralis*. (A) Waveform, (B) logarithmic power spectrum, and (C) spectrogram (FFT length: 256 points, frame size: 75%, flat top window, overlap: 98.4%) of an 'invite' pulse.

O. ventralis females followed the male significantly more often. Interestingly, Konings (2014) suggested that the large crater bowers of *O. nasuta* stand out from the surrounding rocks and must be obvious to the females. Male *O. ventralis* and *O. boops* may perform the 'invite' behaviour and show their bower more often to the female because the latter is less conspicuous and potentially less appealing to the female.

The maternal mouthbrooding that resulted from the heterospecific reproduction only lasted for two days, the female laid very few eggs (only 7), and the inter-egg interval was longer. This suggests that when

mating with a heterospecific male, the motivation of the *O. ventralis* female to produce a large clutch size may be lower than when mating with a conspecific male. Selz et al. (2014) reported similar trends for two Lake Victoria cichlids tested using a 2-way mate choice design. It has also been shown that female songbirds stimulated by conspecific songs lay more eggs than females triggered by heterospecific songs (Bentley et al., 2000). In other words, differences in male courtship signal can explain lower female inclination to lay eggs.

4.1. Terminological considerations related to 'quiver', 'invite' and 'tail wagging'

Although the 'quiver' display has been reported for many cichlid species (e.g. Baerends and Baerends-Van Roon, 1950b; Rowland, 1978; Lobel, 1998; Rice and Lobel, 2003; Amorim et al., 2004, 2008; Simoes et al., 2008; Bertucci et al., 2010; Verzijden et al., 2010; Maruska et al., 2012), we did not observe this behaviour during any of the twelve reproductions of the *Ophthalmotilapia* species. This is surprising, since Haesler et al. (2009), and Immler and Taborsky (2009) mentioned that *O. ventralis* males 'quivered' at the entrance of the nest. It is possible that *Ophthalmotilapia* does not display the 'quiver' behaviour in tanks, but this seems unlikely because this behaviour was often recorded in artificial conditions for other cichlid species (e.g. Amorim et al., 2008; Bertucci et al., 2010; Maruska et al., 2012). It seems more plausible that the terminology was used in a different meaning by Haesler et al. (2009) and Immler and Taborsky (2009) to describe the intense tail wagging observed when the male shows the location of the bower to the female. During quivering, the fish is generally positioned head down and with its head bent sideways over a small angle (Baerends and Baerends-Van Roon, 1950b). Often, this movement is repeated a few times and then carried out alternately to the left and to the right. The centre of the movement lies just behind the operculae and, if it is intense, it propagates backwards along the body and the tail is swept appreciably sideways. In contrast, during tail wagging, the action consists of vigorous lateral body undulations with the propulsive force counterbalanced by beating the pectoral fins forwards. Finally, the 'invite' behaviour that males use to guide a gravid female to their bower was recorded in *O. ventralis* and *O. boops* and consists of flexing the body and performing a spasm like those observed during quivering. However, whereas a 'quiver' is composed of successive spasms, the 'invite' is composed of a single spasm (or several isolated ones) (Baerends and Baerends-Van Roon, 1950b).

4.2. Sound associated with reproductive behaviour in Ophthalmotilapia

Multiple pulsed sounds have been recorded for many haplochromines from lakes Malawi and Victoria (listed in Longrie et al., 2013) and for some Tropheini (Nelissen, 1978), an endemic LT tribe nested within the Haplochromini (Day et al., 2008). Among the endemic species of the Great African Lakes, *O. ventralis* and *O. nasuta* (Ectodini), and *Neolamprologus pulcher* (Lamprologini) are the only non-haplochromine species in which sound production has been investigated. In *N. pulcher,* which is a highly social cichlid, no communicative sound has been recorded (Pisanski et al. 2014). In *Ophthalmotilapia*, sounds were only recorded when intense 'invite' events were performed near the hydrophone. Since these single-pulsed sounds were very weak, their communicative value is questionable. It would be interesting to collect data from the many other LT tribes to trace the evolutionary history of sound production in cichlids from the Great African lakes.

During the 'invite' behaviour of *O. ventralis,* every single pulse sound was associated with a spasm movement. The hydrodynamic component resulting from the spasm movement may be important: this behaviour was always performed close to the receiver. The role of the lateral line in non-contact aggressive interactions has been demonstrated in the cichlid *Astatotilapia burtoni* (Butler and Maruska, 2015). Like the 'invite' behaviour in *Ophthalmotilapia*, opponents are very close (< 1 body length) to the signal emitter in *A. burtoni*. In the Nile tilapia, *Oreochromis niloticus* (Cichlidae), fast and bilateral contractions of the anterior hypaxial musculature were shown to be responsible for sound emission (Longrie et al., 2009). A similar mechanism was reported in a more distantly related species, the pyramid butterflyfish (Chaetodontidae), *Hemitaurichthys polylepis* (Boyle and Tricas, 2010). The fast-lateral movements of the head observed for *O. ventralis* (also visible in the high-speed movies in the supplementary online Appendix) suggest that their sounds result from a fast and unilateral contraction of the anterior part of the trunk musculature. The pattern observed in *Ophthalmotilapia* sound production is like the one observed during the production of submissive sounds in the clownfish (Pomacentridae), *Amphiprion frenatus* (Dr. O. Colleye, personal communication). The latter sounds were always emitted in a submissive posture while exhibiting head shaking movements (Colleye and Parmentier, 2012). A common factor in the submissive sounds of clownfish and the inviting sounds of *Ophthalmotilapia* is that they are associated with behaviours that convey a message of non-aggression. Since they necessitate no specific features, sound production mechanisms involving fast contractions of the hypaxial musculature may have evolved independently in several Perciformes.

4.3. Egg deposition and sperm release in Ophthalmotilapia

Reproduction time in *O. ventralis* and *O. nasuta*, measured as the time between the deposition of the first and the last egg, lasted approximately half an hour (28.2 ± 22.9 min for *O. ventralis* and 23.1 ± 15.8 min for *O. nasuta*). This is comparable to the 35 min on average observed for *O. ventralis* reproduction in the wild (Immler and Taborsky, 2009). In each of the three species, the mating female left the bower of the male several times during spawning. In the field, female *O. ventralis* generally visit and collect the sperm from several males (Haesler, 2007; Immler and Taborsky, 2009; Haesler et al., 2011). For the three *O. nasuta* reproductions recorded in a multiple males' setup, all eggs from a single clutch were laid in a single bower, but the three females visited the territory of the two dominant males during reproduction and two of them even entered both bowers.

Although every male recorded in this study built a bower, our data corroborate that *O. nasuta* is a crater-building species (i.e. species that build a sand mount with a crater at its top) and *O. ventralis* a non-crater building species (Konings, 2014). Even though all the reproductions analysed in this study took place in bowers, it is not an indispensable condition for a female *O. ventralis* to mate since we observed a mouthbrooding female in a reserve tank devoid of any substratum. Crater-builders are believed to release sperm in the crater before the female enters the bower while non-crater building species (e.g. those that manage a sandy or rocky patch) with egg dummies or egg spots release sperm directly into the mouth of females (Morita et al., 2014). The same authors argued that a crater shape prevented sperm from being washed away and they found increased sperm longevity in crater-building species. Our results do not support the difference of sperm uptake in crater and non-crater species proposed by Morita et al. (2014). In all but one of the reproductions, the females indubitably swam towards male egg dummies with an opened mouth (*O. ventralis*: 8 ± 4.4 times; *O. nasuta*: 7.2 ± 9.5 times; *O. boops*: 7 times; *O. ventralis* female/*O. nasuta* male: 11 times). In addition, every male stopped at the entrance of the bower, tail wagging vigorously just before the female entered the nest. This behaviour was generally followed by a pelvic fin presentation inside the bower. Furthermore, Haesler (2007) found that the sperm of *O. ventralis* was viable for at least 900 s which is longer than all the sperm longevities reported by Morita et al. (ca. 550 s on average for *O. nasuta* and ca. 400 s on average for *O. ventralis*). Because *O. ventralis* females collect the sperm of several males but do not lay eggs in each bower (Haesler et al., 2011), the time a female spends collecting sperm from different males could be measured to test an alternative hypothesis explaining the interspecific differences in sperm

longevity of LT cichlids. Sexual selection acting during sperm shopping by the female was already suggested to be responsible for the sequential polyandry in *O. ventralis* (Immler and Taborsky, 2009).

4.4. Hybridisation between sympatric species of Ophthalmotilapia

The repeated introgression of mitochondrial DNA from *Ophthalmotilapia* species into *O. nasuta* (Nevado et al., 2011) suggests that male *O. nasuta* are (or were) able to mate with congeners. Here, we recorded a heterospecific mating between a male *O. nasuta* and a female *O. ventralis,* which also suggests there are no hermetic prezygotic barriers between males of *O. nasuta* and females of *O. ventralis*, at least when there is no conspecific partner available. However, this female laid only very few eggs and the time interval between the deposition of two successive eggs was very long. Moreover, although she repeatedly swam with an open mouth towards the egg dummies of the male, the mouthbrooding was aborted after a couple of days. This could be a choice made by the female because there were relatively few eggs but it may also have resulted from postmating incompatibilities (e.g. no fertilization, no hatching, or lower survival rate). However, after this study, and in a different setting, we did obtain viable offspring from reproductive events between male *O. nasuta* and female *O. ventralis*. It should be noted that, also here, no conspecific males were present and that only four hybrid specimens survived. Using internally calibrated clocks, Stelkens et al. (2009) estimated that a complete inviability of haplochromine hybrids was reached 4.4 my after a speciation event (8.5 and 18.4 my with relaxed clocks calibrated using cichlid fossil records and the fragmentation of Gondwanaland, respectively) while Koblmüller et al. (2004) considered that species within the *Ophthalmotilapia* clade evolved during a diversification event that may have occurred between 2.5 and 3 MYA. As they used different calibration points, comparisons between these two studies should be considered with caution. However, theses estimations suggest that a complete inviability of *Ophthalmotilapia* hybrids is unlikely though postmating incompatibilities could be relatively high. Since we could not identify hermetic behavioural barriers to the hybridisation of *Ophthalmotilapia* species living in sympatry, further investigations are needed to pinpoint the role of other pre- and/or postzygotic isolations.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.zool.2017.12.001.

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