1   The initial response of females towards congeneric males matches the propensity to hybridise

2   in *Ophthalmotilapia*.

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26   Abstract:

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28   Cichlid radiations often harbour closely related species with overlapping niches and

29   distribution ranges. Such species sometimes hybridise in nature, which raises the question

30   how can they coexist. This also holds for the Tanganyika mouthbrooders *Ophthalmotilapia*

31   *ventralis* and *O. nasuta*. Earlier studies found indications of asymmetrical hybridisation with

32   females of *O. ventralis* accepting males of *O. nasuta*, but not the other way around. We

33   hypothesised that this was due to differences in the capacity for species recognition. Given the

34   higher propensity of *O. ventralis* females towards hybridisation, we expect a reduced ability

35   for species recognition in *O. ventralis* females, compared to *O. nasuta* females. We staged

36   two experiments, one focusing on 22 female *O. nasuta* and one on 21 female *O. ventralis*.

37   These fish were placed in one half of a tank and briefly exposed to a conspecific or a

38   heterospecific male, a conspecific female, or nothing (control). Female response was

39   evaluated by scoring six tracking parameters and by noting the occurrence of ten discrete

40   behaviours before and during the encounter. Females always responded to the presence of

41   another fish by approaching it. Remarkably, for both *O. nasuta* and *O. ventralis*, we did not

42   find a different response between encounters with conspecific males and females. However,

43   in agreement with our hypothesis, *O. nasuta* females behaved differently towards conspecific

44   or heterospecific males, whereas *O. ventralis* females did not. When presented with a

45   heterospecific male, *O. nasuta* females performed a lower number of 'ram' behaviours.

46   Additionally, they never displayed the 'flee' behaviour, a component of the species' mating

47   repertoire that was seen in all but one of the presentations with a conspecific male. Our

48   findings show that differences in species recognition at first encounter predict to a large

49   degree the outcome of the mating process, even in the absence of mating behaviour.

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2

51   Introduction

52

53   Speciation is traditionally seen as a gradual build-up of reproductive isolation between

54   diverging populations (Mayr 1982; Coyne and Orr 2004). The classical view that this is a slow

55   process that occurs between allopatric populations has recently been challenged by genomic

56   findings (Marques et al. 2019) that showed how hybridisation can drive rapid speciation

57   (Seehausen 2004; Jiggins et al. 2008). However, as unrestrained gene flow inevitably

58   homogenizes the genomes of diverging lineages (Roux et al. 2016), the question remains what

59   mechanisms keep incipient species separated. In scenarios of sympatric, closely related species,

60   the ability to correctly distinguish between conspecific and heterospecific mates is probably

61   crucial to maintain the integrity of the species (Sullivan 2009).

62

63   Mating is the end point of a complex decision-making process in which several potential mates

64   are evaluated (Luttbeg et al. 2001). An encounter with a potential mate can be seen as the first

65   step in this process. The outcome of this initial contact can have profound implications on

66   fitness, either through the refusal of suitable mates, or through the acceptance of suboptimal

67   partners. Therefore, we expect intra- and interspecific differences in individual responses when

68   confronted with a choice of partners. Additionally, the preference for a given mate can depend

69   on environmental, social and intrinsic parameters, explaining variation in preference both

70   between and within species, and between and within individuals (Pfennig 2008; Sommer-

71   Trembo et al. 2017). Assortative mate selection was traditionally seen as a sequential process

72   in which individuals first assess whether 'the other' is a conspecific and then assess its quality

73   as a mate (Mayr 1982). However, empirical data and theory suggest that assessment of species

74   and quality are not independent steps (Sullivan 2009; Mendelson and Shaw 2012) as the

75   specific status of an individual could be judged using the same cues as its quality. Additionally,

3

76 adaptive hybridisation has been observed in several taxa (overview in Mendelson and Shaw

77 2012), indicating that preferred mates are not necessarily always conspecific.

78

79 Cichlids of the large East African Lakes form endemic, species-rich radiations (Salzburger

80 2018). Several suggested "key adaptations" of cichlids, such as their pharyngeal jaws, help to

81 explain their evolution into numerous trophic niches (Kocher 2004). However, a large

82 proportion of these closely related species coexist without apparent eco-morphological

83 differences (Van Oppen et al. 1998). Since most cichlid assemblages are relatively young,

84 several taxa may be classified as incipient species and they retain the potential to hybridise.

85 The oldest East African lake, Lake Tanganyika, however, contains a mature cichlid radiation

86 (Salzburger 2018) in which most species are well-delineated (Ronco et al. 2020). However,

87 even between well-delineated biological species, boundaries can be permeable and molecular

88 studies identified several instances of inter-specific hybridisation (Rüber et al. 2001;

89 Koblmüller et al. 2007; Nevado et al. 2011). Such examples allowed us to select a case to study

90 the importance of prezygotic, behavioural isolation after, or at the last stages, of the speciation

91 process.

92

93 *Ophthalmotilapia* Pellegrin, 1904 species are maternal mouth brooders that occur on the rocky

94 and intermediate (rocky patches separated by sand) shores of Lake Tanganyika. The genus

95 contains four currently accepted valid species: *O. ventralis* (Boulenger 1898), *O. boops*

96 (Boulenger 1901), *O. heterodonta* (Poll and Matthes 1962) and *O. nasuta* (Poll and Matthes

97 1962) (Hanssens et al. 1999). They are sexually dimorphic maternal mouthbrooders with

98 territorial males that protect a spawning site, and females that aggregate in feeding schools

99 when they are not breeding. *Ophthalmotilapia* males possess egg-shaped lappets at the distal

100 ends of their elongated pelvic fins that are unique among Great Lake cichlids (Poll 1986). These

4

101   lappets function as egg dummies during the species' mating behaviour in a similar way as the

102   egg spots on the anal fins of the so-called 'modern' haplochromines (sensu Salzburger et al.

103   2007; Theis et al. 2012). During the mating process, the female deposits the eggs and almost

104   immediately takes them into her mouth. By snapping at the egg dummies, which are situated

105   close to the genital opening of the male, the intake of sperm is thought to be facilitated,

106   increasing the fertilisation rate of the eggs within the female's mouth (Salzburger et al. 2007).

107

108   The four species of *Ophthalmotilapia* have different but partially overlapping, distribution

109   ranges. *Ophthalmotilapia nasuta* is the sole species in the genus with a patchy but lake-wide

110   distribution. The sister species *O. heterodonta* and *O. ventralis* have non-overlapping ranges

111   with the former occurring in the northern half and the latter in the southern third of the Lake.

112   The fourth species, *O. boops* only occurs along a rather limited stretch of Lake Tanganyika's

113   south-eastern shoreline. There, it prefers sites where large stones are available (Konings 2019).

114   This is the only part of the lake where up to three species of *Ophthalmotilapia* occur in sympatry

115   (Hanssens et al. 1999).

116

117   Although specimens of *Ophthalmotilapia* can be easily assigned to one of the valid species

118   (with the possible exception of *O. heterodonta* and *O. ventralis*, see Hanssens et al. 1999), a

119   phylogeographic study discovered gene flow among these species. Nevado et al. (2011)

120   observed that specimens of *O. nasuta* often carried mitochondrial DNA of the other species,

121   whereas the opposite was much less often the case. They suggested that this pattern either has

122   a postzygotic, (*e.g.* by cyto-nuclear incompatibilities that affects mutual crossbreedings

123   differently) or a prezygotic (*e.g.* by an asymmetry in reproductive behaviour that results in a

124   different resistance towards hybridisation) cause. The latter scenario implies that females of all

125   species would occasionally mate with *O. nasuta* males, while *O. nasuta* females would be much

126 less inclined to mate with heterospecific males. It also implies that the female hybrid offspring

127 would backcross into *O. nasuta*. This scenario agrees with the recent description of a successful

128 mating between a female *O. ventralis* and a male *O. nasuta* (Kéver et al. 2018). Reproductive

129 isolation in closely related species of East African cichlids is mostly maintained through

130 prezygotic isolation (Turner et al. 2001). Hence, models that describe speciation in cichlids

131 emphasize the importance of female mate choice in the initial stages of the speciation process

132 (Danley and Kocher 2001).

133

134 The Lake Tanganyika cichlids assemblage contains species with profoundly different mating

135 strategies (Ronco et al. 2020). *Ophthalmotilapia* stands out by its extreme sexual dimorphism

136 and female-biased reproductive investment. Although the correlation between reproductive

137 investment, sexual selection and choosiness is well-established, it still remains debated whether

138 choosiness is an evolutionary outcome (sensu Trivers 1972), or rather a determinant of

139 differences between the sexes in parental investment (Thomas and Szekely 2005). Using Lake

140 Tanganyika cichlids, Gonzales-Voyer et al. (2008) showed support for the latter hypothesis.

141 Regardless of the evolutionary mechanism, females should be considered the choosy sex in

142 *Ophthalmotilapia* (sensu Wirtz, 1999). Therefore, if a prezygotic mechanism explains the

143 asymmetric pattern observed in nature (Nevado et al. 2011), it would be caused by differences

144 between females of the different species in accepting matings with heterospecific males. As

145 increased capacity for species recognition leads to increased preference in the choosier sex

146 (Kozak & Boughman, 2009), we predict to see an interspecific difference in female response

147 to conspecific and heterospecific males. As males of the different *Ophthalmotilapia* species

148 have very similar courtship behaviours, in which the few species-specific elements are

149 insufficient to prevent hybridisation (Kéver et al. 2018), females would mainly rely on other

150 cues like colour patterns, body size and pheromones. Although the reproductive behaviour of

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151   *Ophthalmotilapia* species is well documented (Haesler et al. 2011; Immler and Taborsky 2009;

152   Kéver et al. 2018), little is known on how *Ophthalmotilapia* species recognize conspecifics and

153   select potential mates.

154

155   In this study, we will investigate species recognition, which was, in spite of its shortcomings

156   (Mendelson and Shaw 2012), defined as ''a measurable difference in behavioural response

157   toward conspecifics as compared to heterospecifics''. We will study this by comparing the

158   ability of female *O. nasuta* and *O. ventralis* to distinguish con- from heterospecific males. Often,

159   dichotomous mate choice trails, in which a female can choose between two males, are used for

160   such studies. Such a setup includes an aspect of male competition, whereas we are interested in

161   the response of females in the absence of such behaviour. Additionally, with dichotomous trails

162   one cannot discriminate mate preference from species recognition. Although we expect species

163   recognition to be a good predictor of female mate preference, we did not aim to quantify

164   preference directly. Instead, we choose to investigate differences in species recognition by

165   presenting a set of *O. ventralis* and *O. nasuta* females to males of both species separately and

166   by measuring a range of behavioural variables.

167

168   This study described and compared the initial behavioural response of *O. ventralis* and *O.*

169   *nasuta* females towards males of both species in an aquarium setting. In view of the pattern of

170   asymmetric hybridisation discovered among these species (Nevado et al. 2011), we expected a

171   difference in the capacity for species recognition between females of *O. ventralis* and *O. nasuta*.

172   Specifically, we hypothesized that *O. nasuta* females would be able to differentiate between

173   conspecific and heterospecific males at the initial stages of an encounter. For *O. ventralis*

174   females, however, we expected that this capacity would be less pronounced or absent.

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7

176   Materials and Methods

177   *Experimental setting*

178

179   We performed two independent experiments using females and males of *O. ventralis* and *O.*

180   *nasuta*. The first focused on the behaviour of focal *O. nasuta* females (ON experiment), the

181   second on that of focal *O. ventralis* females (OV experiment). All individuals were wild caught

182   off the coast of Ulwile Island or nearby Kala, on the mainland (Tanzania). Specimens were

183   acquired as juveniles and hence had no prior mating experience. They were kept in

184   monospecific aquaria per species until they reached maturity. Hence, all females used had

185   similar ages. Males were older and most had prior mating experience (Kéver et al. 2018). We

186   verified the origin of a random selection of these fishes by sequencing the mitochondrial control

187   region, and by comparing these sequences with the data collected by Nevado et al. (2011). The

188   experiments were performed at the aquarium facilities of the University of Liège. Prior to the

189   onset of the experimental trials, the sex of the specimens was checked by visually inspecting

190   their genital papillae. Female specimens were kept jointly but isolated from males and

191   heterospecific specimens in a separate tank for at least two weeks. This tank was devoid of

192   hiding places, in order to prevent the development of territoriality. During that period, males

193   were kept in monospecific tanks in which they were visually isolated from each other using

194   opaque partitions. We kept all specimens in the same condition for at least two weeks with

195   photoperiod: 12:12 h L:D, water temperature: 26±1°C, carbonate hardness: >8 dKH. Fishes

196   were fed daily ad libitum with 'Tropical Spirulina forte' mini-granules.

197

198   We used three identical experimental aquaria (88cm*50cm*40cm with water level ca. 40cm),

199   which we divided into two equal parts by a perforated transparent partition (separation wall),

200   through which fishes could not pass, and by an opaque wall (visual barrier) that could be

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201   removed (Fig. 1A). A flower pot was placed on each side of the separation to allow the fish to

202   take refuge. We kept the fishes in these aquaria for at least twelve hours before they were used

203   in the experimental trials. During the trial, the visual barrier was removed.
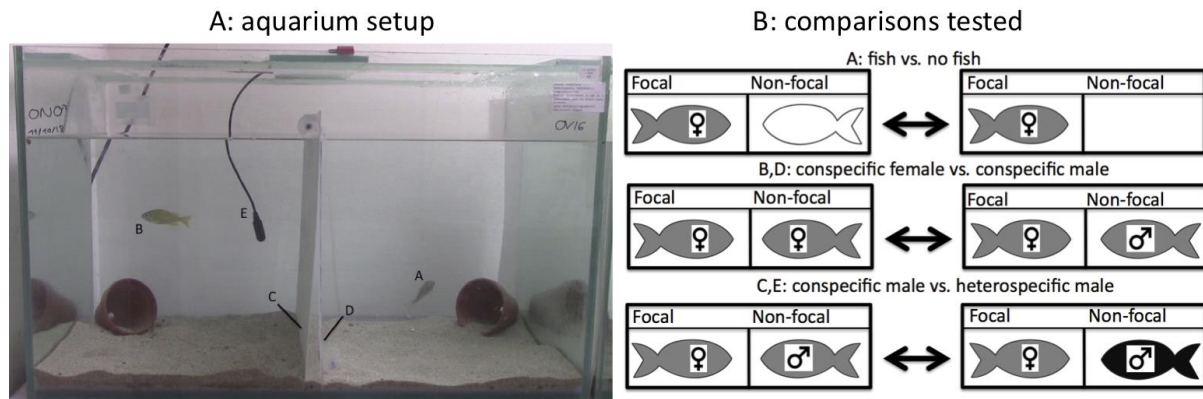

Figure 1. Experimental setup. A: aquarium setup: A focal female of either *O. nasuta* or *O. ventralis* (here *O. nasuta*) (A) was placed in one half of the experimental tank whereas no fish, a conspecific female or a hetero- or conspecific male was placed in the other half (here an *O. ventralis* male) (B). The tank was divided in two by a transparent wall (D) and a visual barrier (C), a microphone (E) was placed on the side of the non-focal specimen and an empty flowering pot was placed in both halves of the tank, allowing fishes to take refuge. Video and audio recordings were made 15 minutes prior and 45 minutes after the visual barriers were removed. B: Contrasts tested using permanova: A: focal females presented with another fish *vs*. with no fish, B: focal females presented with a conspecific female *vs*. a conspecific male, C: focal females presented with a conspecific *vs*. a heterospecific male, D: conspecific females and males presented to a focal female and E: conspecific and heterospecific presented to a focal female. Black and grey fishes represent different species, the white fish represents all possible non-focal specimens used.

We recorded the behaviour of focal specimens (*O. nasuta* females or *O. ventralis* females) in

four different experimental conditions. They were either exposed to (i) no other specimen (Co),

(ii) a conspecific female (CF), (iii) a conspecific male (CM) or (iv) a heterospecific male (HM)

(Supplement 1). For each experiment (ON and OV) and for each experimental condition (i to

iv), we conducted a minimum of five replicates. We filmed (using a CANON Legria HF R606)

the entire aquarium (i.e. focal and non-focal fishes) during one hour: from 15 minutes before to

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223   45 minutes after the visual barrier was removed. Experimenters were only briefly present in the

224   room to remove the visual barrier. As *O. ventralis* males are known to produce weak-pulsed

225   sounds during the inviting behaviour (Kéver et al. 2018), we used a HTI Min-96 hydrophone

226   (−164.4 dB re. 1 V μPa−1; bandwidth 2 Hz and 30 kHz, MS, USA), connected to a Tascam

227   DR-05 recording (TEAC, Wiesbaden, Germany) at a 44.1 kHz sampling rate to record sounds

228   during the whole experiment. The hydrophone was positioned near the separation wall, at half

229   the height of the water column, on the side of the non-focal specimen. At the start of each trial,

230   we switched off the aeration of the tank so that sounds could be recorded. However, these

231   recordings were not analysed, as we detected no communication sounds. After each trial, the

232   focal female was euthanized. Both the focal and the non-focal specimens were weighed. Focal

233   specimens were measured, dissected and the stage of gonad development was scored following

234   Panfili et al. (2006).

235

236   We performed a total of 28 ON and 21 OV experimental trials, with a maximum of three trials

237   per day (Supplement 1). However, after the dissections (see below), we observed that six focal

238   *O. nasuta* females from the first set of trials possessed male or ambiguous gonads (Supplement

239   2). These specimens were referred to as floater males and the recordings for these trials were

240   not analysed. As we suspected that these specimens had changed sex, we photographed the

241   genital papillae of the focal females that were to be used subsequently, two weeks before the

242   onset of the experimental trials. A comparison between papillae of the same individuals after

243   two weeks revealed that some could be identified as males. This either confirmed that a sex

244   change did indeed take place in several specimens, or showed that the examination of genital

245   papillae was insufficient for sex determination in subadult *Ophthalmotilapia*. These specimens

246   were not studied. After each trial, the aquarium was cleaned and the water fully renewed.

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10

248   *Collection of tracking and qualitative behavioural data*

249

250   Video files were converted into JPG images using Adapter v2.1.6 (available at

251   https://www.macroplant.com), capturing one frame per second and saving it as an 8-bit, gray-

252   scale JPG file. Images taken within ten seconds before or after an experimenter was performing

253   an action (i.e. removing the wall) were discarded from analyses. We chose to analyse the same

254   number of frames for all trials within the ON and the OV experiment, respectively. For the ON

255   experiment, this resulted in a minimum of 721 and 2186 frames collected before and after the

256   removal of the separation wall, respectively. For the OV experiment, 871 and 2685 frames were

257   available for analyses. Both focal and non-focal specimens were tracked using the ImageJ v1.49

258   (Schneider et al. 2012) software package.

259

260   Given the presence of both light- and dark- coloured backgrounds in the aquarium setting, the

261   set of frames was studied twice. Specimens that were present before a light coloured

262   background were tracked by inverting black and white values whereas specimens present before

263   a dark-coloured background were tracked using non-inverted images. For computational

264   reasons, analyses were performed on subsets of the data containing a maximum of 1,000 frames.

265   For each set of images, a subset of 30 frames was used to create a background using the plug-

266   in '*Image stack merger plus*'. Backgrounds were removed using the image calculator and the

267   resulting frames were transformed into black-and-white images using the threshold function

268   with '*MaxEntropy*' as the methodology. Images were adjusted using the '*erode*' and '*dilate*'

269   functions to remove noise and to obtain a better representation of the fishes. The resulting image

270   series was then used for tracking using the plug-in '*MTrack2*', in which tracks were summarized

271   as the x- and y- coordinates of the centroids of the tracked object. The quality of the automated

272   tracking was checked by visually inspecting each of the frames. When the software failed to

273   track a specimen that was clearly present in the final images, coordinates were added manually.

274   Finally, tracks obtained from both datasets, inverted and non-inverted, were combined. When

275   a specimen was recognized by both methods, *e.g.* when the fish was partially before a light-

276   and partially before a dark-coloured background, the average of the coordinates was used.

277   When tracking data was missing, the average value of the coordinates of the previous and the

278   next positions were used. This is justified, as missing data either corresponded to fish that

279   remained stationary for many frames, and could hence not be distinguished from the

280   background, or to fish that hid behind the terracotta flowerpots (Fig. 1). Frames collected before

281   and after the removal of the visual barrier were analysed separately. Coordinates were shifted

282   using the lower- and anterior-most point of the separation wall as the origin, and rotated by

283   setting the anterior water level as a reference for the horizontal plane. Finally, all coordinates

284   were transformed from pixels to centimetres using the dimensions of the aquaria. Tracks were

285   visualized by plotting all individual positions as well as the shift in average position of a

286   specimen before and after the removal of the barrier (Supplement 3).

287

288   For each specimen, six tracking parameters were calculated from the coordinates (Table 1).

289   Each parameter was calculated three times: once using coordinates obtained for 721/871

290   (OV/ON experiment respectively) seconds before (before) the removal of the visual barrier,

291   once using coordinates obtained during 721/871 seconds (after1) after the visual barrier was

292   removed and finally using coordinates obtained during 2186/2685 seconds (after2) after the

293   visual barrier was removed, hence including the after1. Additionally, ten specific behaviours

294   were defined based on Baerends and Baerends-Van Roon (1950). These were encoded and

295   recorded as point events in Boris v. 2.72 open source software (Friard and Gamba 2016) (Table

296   1). This data was collected during the same three periods: before, after1 and after2. We choose

297   these time periods, as we aimed to study the immediate response following presentation to

298   another fish. Teleosts respond to changing social situations at two scales, by an immediate

299   change in behaviour, and by a modification of neural circuits (Oliveira 2012). The latter is

300   driven by the regulation of immediate early genes (IEG). Following Oliveira (2012), we

301   assumed that the transcription of IEG occurs roughly 15 min (after1), and their translation 45

302   min (after2) after the stimulus. Behaviours displayed within ten seconds before or after an

303   experimenter was performing an action (removing the wall) were discarded from the analyses.

304

305   **Table 1. Tracking parameters and point events recorded for the focal and non-focal individuals during the**

306   **experimental trials.** For point events, interpretation of the behaviour was added.

Tracking parameters	Description	
Dist.wall	Distance to the visual barrier (% of length of compartment).	
Dist.fish	Distance to the fish on the other side of the transparent wall (cm).	
Sp	Average speed (cm/s).	
SpX	Horizontal speed (cm/s).	
SpY	Vertical speed (cm/s).	
Height	Mean height (% of height of water column).	
Point events	**Description**	**Interpretation**
Chase	The fish suddenly swims very fast towards the other fish and rams (or almost rams) the separation wall.	Contextual: agonistic and male courtship behaviour (Kéver et al. 2018)
Flee	The fish suddenly swims away from the other fish.	Contextual: agonistic and female courtship behaviour (Kéver et al. 2018)
Lateral	Lateral display: The fish positions itself perpendicular to the other fish, keeping its head slightly downwards, erects its fins and bends its body.	Signal movement (Baerends and Baerends-Van Roon 1950), agonistic behaviour (Kéver et al. 2017)
Frontal	Frontal display: The fish faces the other fish head up and erects its fins.	Signal movement (Baerends and Baerends-Van Roon 1950), agonistic behaviour (Kéver et al. 2018)
Bite	Biting the wall: The fish bites the separation wall (possibly trying to bite the other fish).	Signal movement (Baerends and Baerends-Van Roon 1950), agonistic behaviour (Kéver et al. 2018)
Ram	Ram into the wall: The fish tries to enter the other part of the aquarium and rams (not very fast) the separation wall.	
Sand	Sand picking: The fish takes sand in its mouth.	Courtship behaviour when linked to construction of bower (Kéver et al. 2018), signal movement when nipping off a substrate (Baerends and Baerends-Van Roon 1950).
Spasm	A quick, strong, and unilateral contraction of the trunk musculature that results in a displacement of the head and the caudal fin in the same direction.	Contextual: comfort behaviour or signal movement. Observed in courtship behaviour or inter-territorial fights depending on the genus (Baerends and Baerends-Van Roon 1950).

13

Tail	Tail-wagging: Exaggerated movements of the caudal fin (+ caudal part of the dorsal fin). At its zenith, the movement of the caudal fin is completely counterbalanced by backpedalling.	Signal movement (Baerends and Baerends-Van Roon 1950), courtship behaviour (Kéver et al. 2018).
Flicker	Pelvics flickering: The fish quickly and alternatively moves its right and left pelvic fins.	Comfort behaviour (Baerends and Baerends-Van Roon 1950)

307

308 *Statistical analyses*

309

310 Prior to testing differences in species recognition, we visually explored the combined datasets

311 of tracking parameters and point events. We did this by performing principal component

312 analysis (PCA) and canonical variate analyses (CVA) in Past 3.14 (Hammer et al. 2001). The

313 former allowed for an unbiased visualization of the variation in the data and was performed on

314 the correlation matrices. The latter was conducted to maximize the differentiation between the

315 different groups. Separate analyses were performed for each period of time recorded (before,

316 after1, after2) and for each experiment (ON or OV). Point events that were not recorded during

317 one of these periods were disregarded and missing values (i.e. for Dist.fish in the control

318 condition Co) were treated using mean value imputation. Prior to the analyses, each of the

319 tracking parameters and point events was normalised. This was done for each experiment (OV

320 and ON), and for each of the time periods ('before', 'after1', 'after2' and 'shift') separately.

321

322 We used permanova to compare the behaviour of both focal and non-focal specimens in five

323 different comparisons (Fig. 1B). With this non-parametric test, we could include all recorded

324 behaviours. This allowed us to measure species recognition via differences in behaviour (sensu

325 Mendelson and Shaw 2012) without defining *a priori* in what variable specimens would differ.

326 In order to reduce the number of comparisons, we restricted ourselves to only biologically

327 relevant contrasts. For focal specimens, we compared the behaviour between (A) females that

328 were presented with another fish *vs*. with no fish, (B) focal females that were presented with a

329 conspecific female *vs*. a conspecific male, and (C) females that were presented with a

14

330   conspecific *vs*. a heterospecific male. Two additional comparisons were tested for the non-focal

331   individuals. We tested (D) whether conspecific females and males respond differently to a focal

332   female and (E) whether conspecific and heterospecific males respond differently to a focal

333   female. Even though our main goal was to test whether females of *O. nasuta* and *O. ventralis*

334   differed in behavioural response towards conspecific and heterospecific males (C), we tested

335   the four other comparisons as well, following the recommendations of Moran (2003) and

336   Nakagawa (2004).

337

338   Tests were performed using non-parametric permanova, using the *pairwise.adonis* function, of

339   the R package *vegan* on the combined data of tracking parameters and point events. This

340   approach was chosen as the conditions for multivariate normality were violated. When

341   permanova revealed significant differences, we verified whether this could be due to dispersion

342   effects (Anderson 2006). For this, in view of the size of the dataset, non-parametric Mann-

343   Whitney U tests were performed on the within group dispersions from the mean, calculated

344   using the *betadisper* function implemented in the R package *vegan* (Oksanen et al. 2017). A

345   difference in dispersion across groups does, however, not contradict a difference in their

346   average values. Hence, the data was visually explored, using PCA, to assess whether dispersion

347   could be present together with a difference in average values. For each comparison revealed

348   significant by permanova, Mann Whitney U non-parametrical tests were performed on each of

349   the variables separately in order to detect which of these caused the difference between the

350   treatments. We choose this non-parametric approach as the assumptions of normality were often

351   not met. When significant, the effect sizes of these variables were estimated using Hedge's g,

352   which was calculated using the estimationstats.com web application (Ho et al. 2019).

353

354   In order to test whether the observed differences in behaviour depended on the visual presence

355   of another specimen, and whether these differences were already visible at the first stages of

356   the encounter, permanova tests were performed on data collected before the removal of the

357   visual separation (before) as well as on data collected over a short (after1) and a long (after2)

358   period of time after this separation was removed. Finally, an additional test was conducted

359   which removed individual variation between the different treatments. For this, behavioural

360   shifts were calculated for each tracking parameter and point event by subtracting the values of

361   the 'before' period before from those of the 'after1' period (shift). All tests were performed

362   separately for the ON and the OV experiment. As behaviour can be influenced by gonad

363   development and weight of the focal and non-focal specimens, Mann Whitney U tests were

364   performed to check whether these differed between the treatments. Such tests were also

365   performed on the amount of frames in which fishes could not be tracked. All statistical analyses

366   were performed using Past 3.14 (Hammer et al. 2001) and R (R core team 2017).

367

368   Results

369

370   We separately analysed two experiments, one focusing on *O. nasuta* and one on *O. ventralis*

371   females (ON and OV experiment). In the ON experiment, two males of *O. ventralis* performed

372   advanced courtship behaviours. After the encounter, these males started to swim in circles, in

373   fast and erratic movements. This was accompanied by tail wagging, generally displayed close

374   to the partition wall. These males often bit the hydrophone and picked up and moved around

375   sand (49 and 45 times within 45 min *vs.* 0 for the other males). One of these two males (*O.*

376   *ventralis* male presented to ON38) also tried to chase the female (79 times) and presented the

377   egg dummies of its pelvic fins (5 times). This behaviour stopped immediately when the

378   experimenter removed the female fish. During the encounter, these males turned dark grey, to

379   almost black, which was swiftly reversed after the experimental trial. As we designed our

380   experiment to study behavioural response in the absence of courtship behaviour, we removed

381   these outliers from all analyses. All ten point events were observed in at least one of the fishes

382   in the ON experiment, whereas 'tail' (*i.e.* tail wagging) was never observed in the OV

383   experiment (Supplement 4).

384

385   *Visualization of the behavioural data*

386

387   We visually explored the data using PCA and CVA to compare the behaviour of all specimens

388   used in each of the two experiments. In the PCAs conducted on the behavioural data collected

389   before the removal of the barrier, values of all females as well as of conspecific males

390   overlapped (Fig. 2A,B), suggesting a highly similar behaviour. However, heterospecific males

391   were (somewhat) separated from all other specimens by their higher values for PC1 (ON

392   experiment) or PC2 (OV experiment). The loadings of the main PCs (Supplement 5.1) suggest

393   that this difference was due to a more active swimming behaviour (Sp, SpX, SpY) higher up in

394   the water column (height) for *O. ventralis* males (ON experiment) and a higher number of point

395   events (ram, sand, bite) performed at the floor of the aquarium (height) for the *O. nasuta* males

396   (OV experiment), prior to their presentation to a heterospecific female. The fact that *O. nasuta*

397   males build true bowers could explain the higher number of point events, especially 'sand'.

398   Additionally, *O. nasuta* might be less at ease close to the water surface as it generally occurs in

399   deeper waters than *O. ventralis* (Konings 2019). The CVAs also reflected the behavioural

400   differences of heterospecific males (Fig. 3A,B), as they had higher values for the first CVs. The

401   behaviours that contributed strongly to the separating PCs, also contributed to the main CVs

402   (Supplement 5.2).

403

17

404   The PCAs performed on the data collected 15 minutes after the removal of the barrier foremost

405   showed how the focal females that were used as controls behaved differently than the specimens

406   that were presented with another specimen (Fig. 2C,D). For both experiments, this can be

407   explained by control females spending less time closer to the wall (Dist.wall), and performing

408   less agonistic behaviour (ram, lateral, flee). We did not observe any additional separation in the

409   PCA of the OV experiment (Fig. 2D). In the ON experiment, values for (heterospecific) *O.*

410   *ventralis* males stood out by their high values for PC1, whereas those of (conspecific) *O. nasuta*

411   males had mostly low values for this axis (Fig. 2C). The tracking parameters Sp, SpX, SpY all

412   had high, positive contributions to PC1 (Supplement 5), reflecting that *O. ventralis* males kept

413   swimming actively when the barrier was removed in the ON experiment.

414

415   We carried out CVAs on the same datasets (Fig. 3C,D). For both experiments, the control

416   females stood out by their high values for CV1. This could again be explained by their higher

417   values for Dist.Wall. In the ON experiment, (heterospecific) *O. ventralis* males stood out by

418   their low values for CV1, which would be attributed to their more active swimming behaviour

419   (Sp, SpX, SpY). Conspecific *O. nasuta* males stood out by their low values for CV2, which

420   could be due to the higher occurrence of 'sand' and 'bite' behaviour. Values for *O. nasuta*

421   females that were presented with another fish had more intermediate values for CV1 and CV2.

422   However, values of *O. nasuta* females that were presented to a conspecific male clustered

423   between values of those males and of those of the other females. Similarly, females that were

424   presented to a heterospecific male had values that were intermediate between those of these

425   males and those of the other females (Fig. 3C). This suggests that the behaviour of focal females

426   shares characteristics with the behaviour of the non-focal fishes presented to them. In the CVA

427   of the OV experiment, (heterospecific) *O. nasuta* males stood out by their low values for CV1

428   and high values for CV2. This was most influenced by the higher occurrence of point

18

429   behaviours (ram, spasm, sand, flicker). Values of *O. ventralis* males overlapped with those of

430   female specimens that were presented with another fish (Fig. 3D). These patterns remained

431   present when performing similar analyses on the data collected 45 minutes after the removal of

432   the visual barrier (Supplement 6). Plotting the shift in average position before and after the

433   removal of the barrier revealed how almost all specimens moved towards the wall when

434   presented with another specimen. Additionally, this showed that *O. nasuta* specimens, on

435   average, spent more time closer to the bottom whereas *O. ventralis* specimens were more often

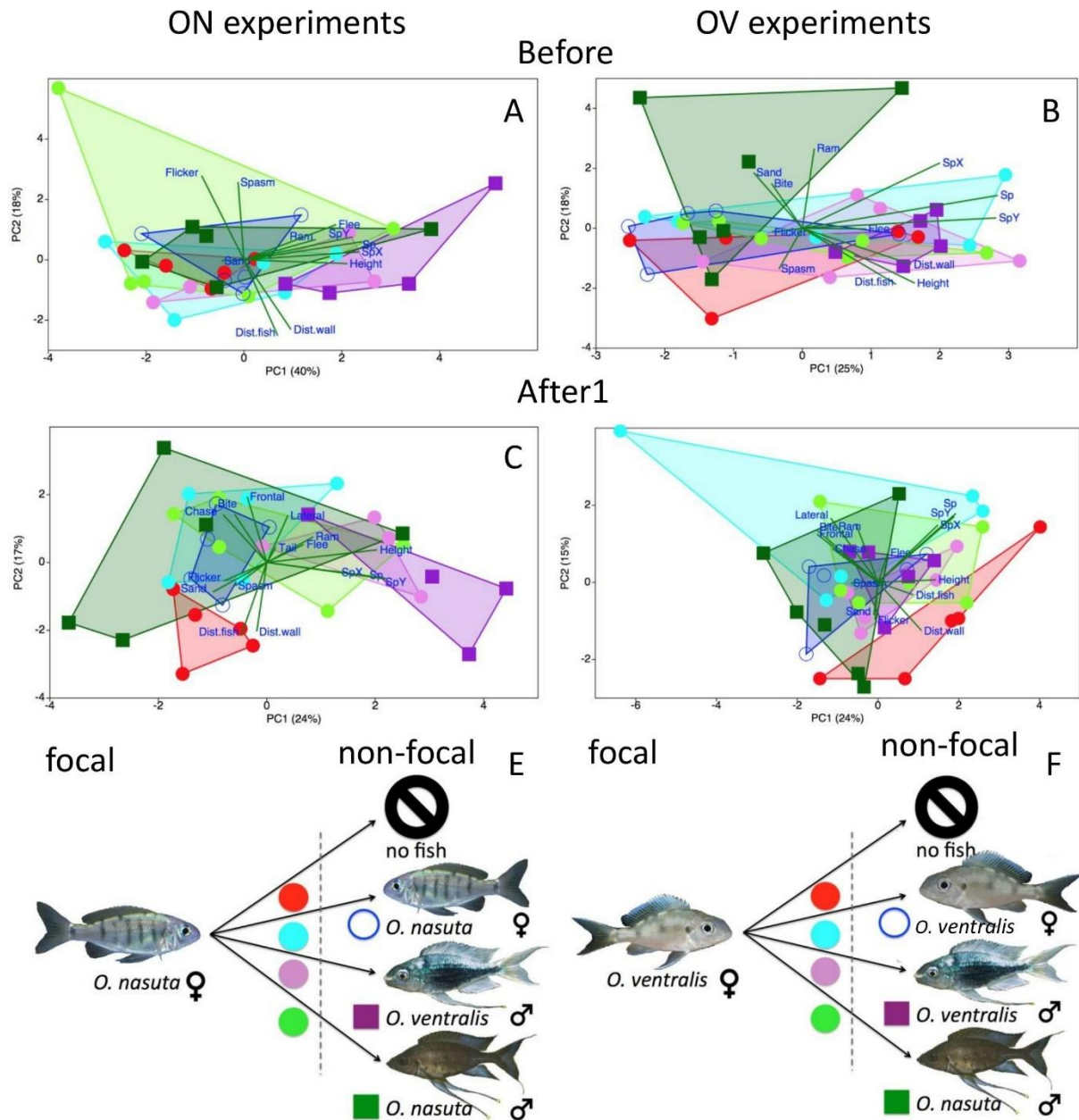436   found higher up in the water column (Supplement 7).

Figure 2. Principal component analyses performed on the behavioural data collected 15 min before (A, B) and 15 min after (C, D) the visual barrier was removed in the ON (*O. nasuta*, left) and the OV (*O. ventralis*, right) experiments. Symbols on the scatter plots for the ON and OV experiment as in E and F, respectively, with full circles denoting focal females presented with no fish (red), a conspecific female (blue) an *O. ventralis* male (purple), and an *O. nasuta* male (green), empty circles denote non-focal conspecific females and full squares *O. ventralis* (purple) and *O. nasuta* (green) males. Explained variances are added to the axes.
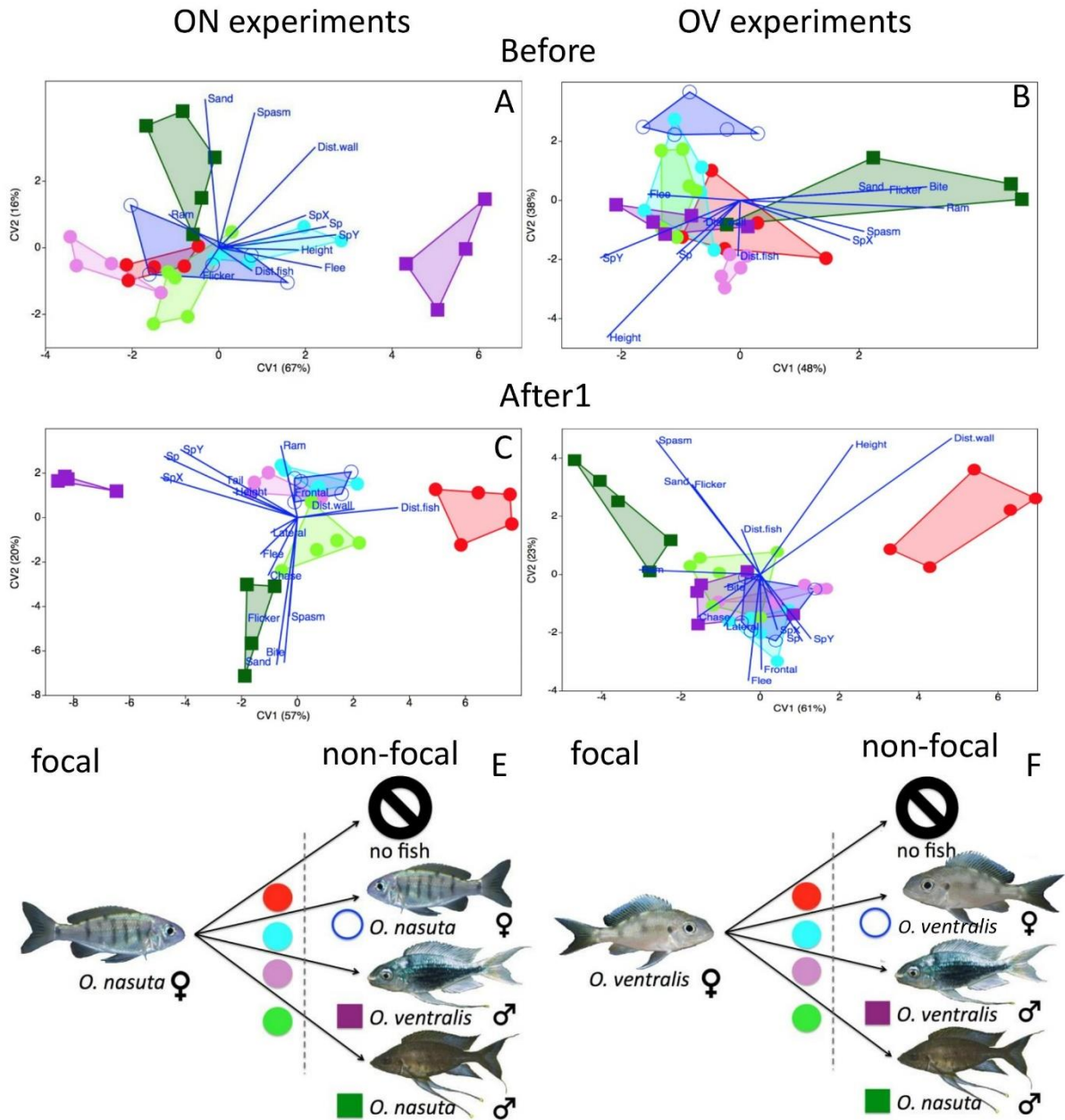
20

Figure 3. Canonical variate analyses on the behavioural data collected 15 min before and 15 min after the visual barrier was removed in the ON (*O. nasuta*, left) and the OV (*O. ventralis*, right) experiment. Symbols on the scatter plots for the ON and OV experiments as in E and F, respectively, with full circles denoting focal females presented with no fish (red), a conspecific female (blue) an *O. ventralis* male (purple), and an *O. nasuta* male (green), empty circles denote non-focal conspecific females and full squares *O. ventralis* (purple) and *O. nasuta* (green) males. Explained variances are added to the axes.

21

454   *Behaviour of focal females*

455

456   We tested whether the behavioural responses of females of *O. nasuta* and *O. ventralis* differed

457   in three different comparisons (Fig. 1B), i.e. when they were presented to (A) another fish *vs.*

458   no fish, (B) a conspecific female *vs.* a conspecific male, and (C) a conspecific *vs.* a

459   heterospecific male. Within each of the comparisons in the ON experiment, we detected no

460   significant difference in the gonad development of the focal females, in their weights, in the

461   weights of the non-focal fishes, and in the percentage of missing frames. The same applies for

462   the OV experiment, although here, non-focal females had lower body weights than conspecific

463   males. Prior to the removal of the visual barrier, no significant difference in behaviour was

464   recorded for focal females from the different treatments in both experiments, and for all three

465   comparisons (Table 2).

466

467   For the three comparisons, and for both experiments (ON and OV), we performed permanova

468   on the behavioural data recorded during the 15 minutes after the removal of the visual barrier

469   (Table 2). For both the OV and the ON experiment, this revealed a significant difference in the

470   behaviour of focal females that were not presented to another fish (controls) and focal females

471   that were presented with another fish (comparison A). For the ON experiment, we could not

472   exclude that these differences stem from dispersion effects. However, these dispersion effects

473   can be explained by the (much) lower variation in values for control females compared to those

474   of other focal females. As visualisation of the data showed a clear separation of both groups,

475   we can assume that a difference in dispersion is present together with a difference in average

476   values (Fig. 2C). Mann-Whitney U tests revealed that controls differed from other focal females

477   by their higher values for the variable Dist.wall (ON: 46.3 +-18.2 *vs.* 20.1 +-8.8, p=0.005,

478   g=1.32; OV: 55.2 +-13.3 *vs*. 20.8 +-9.7, p=0.002, g=1.13) and their lower values for 'ram' (ON:

479   8.6+-12.1 *vs*. 74.5+-54.0, p=0.005, g=-2.14; OV: 8.0 +-11.9 *vs*. 48.8 +-38.4, p=0.014, g=-3.12).

480

481   **Table 2. PERMANOVA performed on the behavioural parameters of the ON (*O. nasuta*) and OV (*O.**

482   ***ventralis*) experiment.** Tests were performed on the data collected during 15 minutes before (B), 15 minutes after

483   (A1) and 45 minutes after (A2) the removal of the opaque wall as well as on behavioural shifts (S) calculated by

484   subtracting data recorded during 15 minutes before from that of 15 min after the removal of the wall (A1-B). We

485   tested five comparisons (Fig. 1B): by comparing the behaviour of focal females that were presented with (A)

486   another fish *vs*. with nothing, (B) with a conspecific female *vs*. a conspecific male, and (C) with a conspecific *vs*.

487   a heterospecific male. We further compared (D) the behaviour of non-focal conspecific males vs. females, and (E)

488   conspecific *vs*. heterospecific males. Behaviours that were neither recorded before or after the removal of the wall,

489   'tail' and 'sand' for the ON, and 'flicker' and 'sand' for the OV experiment, were excluded. For the first

490   comparison, Dist.fish was excluded, as it could not be calculated. Values in bold are significant at the 0.05 level.

491   For these comparisons, ° and † denote that the assumption of equal dispersion was violated at the 0.05 and 0.01

492   levels.

	C	A	B	C	D	E
ON	B	F:0.31; p: 0.789	F:0.95; p: 0.423	F:0.91; p: 0.474	F:1.17; p: 0.339	F:1.15; p: 0.24
	A1	**F:7.28; p: 0.012 †**	F:0.42; p: 0.768	**F:11.33; p: 0.016 °**	F:0.45; p: 0.651	F:0.55; p: 0.73
	A2	**F:4.71; p: 0.027 †**	F:0.49; p: 0.553	**F:8.13; p: 0.040**	F:0.66; p: 0.545	F:0.85; p: 0.47
	S	**F:8.40; p: 0.006 †**	F:1.03; p: 0.416	**F:8.75; p: 0.018 †**	F:1.18; p: 0.332	F:0.25; p: 0.93
OV	B	F:0.23; p: 0.762	F:1.62; p: 0.203	F:0.85; p: 0.387	F:3.19; p: 0.061	**F:5.13; p: 0.013**
	A1	**F:8.00; p: 0.005**	F:0.91; p: 0.386	F:0.40; p: 0.546	F:0.28; p: 0.633	F:0.48; p: 0.524
	A2	**F:4.28; p: 0.026**	F:0.78; p: 0.438	F:0.48; p: 0.610	F:0.09; p: 0.837	F:0.49; p: 0.525
	S	**F:7.61; p: 0.004**	F:0.89; p: 0.418	F:0.87; p: 0.407	F:0.42; p: 0.679	F:0.73; p: 0.447

493

494   Unexpectedly, in both experiments, we did not observe a difference in behaviour between focal

495   females that were presented with a conspecific female or a conspecific male (comparison B).

496   However, when comparing the behaviour of focal females presented with conspecific and

497   heterospecific males (comparison C), a difference became evident between the ON and the OV

498   experiment. In support of our hypothesis, females of *O. nasuta* responded differently towards

499   conspecific and heterospecific males, whereas females of *O. ventralis* did not (Table 2). Mann-

500   Whitney U tests revealed that this was due to the lower number of observed 'ram' behaviours

501   (43.8+-17.9 *vs.* 138+-52.9; p=0.04, g=2.25) in *O. nasuta* females that were presented to *O.*

23

502   *nasuta* males compared to those presented to *O. ventralis* males. Additionally, *O. nasuta*

503   females never performed a 'flee' behaviour when presented to an *O. ventralis* male, whereas

504   this was observed in all but one of the *O. nasuta* females presented to a *O. nasuta* male (0 *vs.*

505   2.6+-2.3; p=0.04, g=-1.33). We obtained highly similar results when analysing the data

506   collected 45 minutes after the visual barrier was removed, or when analysing the shift data

507   (Table 2).

508

509   *Behaviour of non-focal specimens*

510

511   We tested whether the behavioural response of conspecific females and males differed when

512   presented to a focal female (D) and whether the behavioural response of conspecific and

513   heterospecific males differed when presented to a focal female (E). Unexpectedly, permanova

514   revealed a difference in the behaviour of *O. nasuta* and *O. ventralis* males in the OV experiment,

515   prior to the removal of the barrier. Mann-Whitney U tests revealed that this was due to the

516   higher average vertical swimming speed of *O. ventralis* males compared to *O. nasuta* males

517   (SpY 1.7+-0.5 *vs.* 0.7+-0.4, p= 0.014, g=2.05). None of the other comparisons was shown to be

518   significantly different, neither before, nor after the removal of the barrier (Table 2).

519

520   Discussion

521   Summary

522

523   We tested the initial response of *O. nasuta* and *O. ventralis* females towards conspecific and

524   heterospecific males. In support of our hypothesis, *O. nasuta* females differentiated between

525   conspecific and heterospecific males, whereas *O. ventralis* females did not. Visualisation of the

526   data revealed that *O. nasuta* females mirrored the behaviour of the males to which they were

24

527 presented. We also presented females of both species with a conspecific female or with nothing

528 (control). Although females always responded to the presence of another fish, their behaviour

529 did not differ when presented with conspecific males and females. Comparisons of non-focal

530 specimens did not reveal any differences in behaviour after presentation to a focal female.

531 However, before the removal of the wall, males of *O. ventralis* and *O. nasuta* behaved

532 differently in the OV experiment.

533

534 *Responses of* Ophthalmotilapia *females*

535

536 Nevado et al. (2011) discovered signatures of unidirectional hybridisation in *Ophthalmotilapia*,

537 which could either be explained by cyto-nuclear incompatibilities, or by asymmetric mate

538 choice. The latter explanation implies that *O. nasuta* females would discriminate more strongly

539 against heterospecific males, than females of *O. ventralis*. This is supported by our experiments.

540

541 As we did not present focal females with heterospecific females, we cannot say that the

542 observed species recognition in *O. nasuta* females was due to a different response towards

543 males, or towards all specimens of the other species. However, as females in *Ophthalmotilapia*

544 are non-territorial and therefore often encounter heterospecific congeners, we expect that the

545 female response is specific to heterospecific males. Unexpectedly, females of both species

546 behaved similarly towards conspecific males and females. Hence, we cannot say whether we

547 observed sexually motivated behaviour, or just the routine behaviour of a (isolated) female that

548 encounters a conspecific individual. In the wild, non-breeding females of both species

549 aggregate in large feeding groups (Konings 2019). Hence, being isolated for 12 hours, as was

550 the case prior to the start of the experiment, represents an unnatural situation for

551 *Ophthalmotilapia* females. It would, therefore, not be unlikely if *Ophthalmotilapia* females are

25

552   behaviourally hardwired to reunite immediately with conspecifics, regardless of whether these

553   are female or male.

554

555   Females of *O. nasuta* only performed the 'flee' behaviour towards conspecific males and

556   displayed the 'ram' behaviour less frequently. Although we found no evidence that this

557   behaviour is sexually motivated, male chasing and female fleeing (i.e. 'flee') form the first steps

558   in the mating process of *Ophthalmotilapia* (Kéver et al. 2018). The ram behaviour, on the other

559   hand, was seen in all experimental trials in which a focal female was presented to another fish.

560   Indeed, the main difference in behaviour between focal females of both species that were, or

561   were not, presented with another fish was the amount of time spent close to the wall, and in the

562   display of the 'ram' behaviour.

563

564   We discovered that several of the presumed females that we planned to use in the experiments

565   had been, or changed into, males. This was also observed for one of the non-focal females in

566   the ON experiment, which was kept isolated from males after the experiment (Supplement 2).

567   Although there are several reports of sex changes occurring in cichlids (Peters 1975; Naish and

568   Ribbink 1990), evidence hereof remained, until now, limited. As no reproductions were

569   recorded when these specimens still had a female morphology, we cannot rule out that they

570   were males that delayed the development of the conspicuous male colouration and the elongated

571   pelvic fins. Hence, we did not provide solid evidence of sex change in *Ophthalmotilapia*.

572

573   *Interpretation*

574

575   Although we uncovered a significant difference in the behaviour of *O. nasuta* females that were

576   presented with a conspecific and a heterospecific male, permanova did not reveal a significant

26

577   interspecific difference in the behaviour of the males. This could indicate that *O. nasuta* females

578   interpreted behaviour differently when displayed by *O. nasuta* or by *O. ventralis* males. Such

579   species- or sex-dependent interpretation of behaviour is known for several cichlid species, in

580   which territorial males present themselves identically towards both visiting females and

581   intruding males (Baerends and Baerends-Van Roon 1950). Visual exploration of the data,

582   however (Fig. 3C, Supplement 6), revealed a potential difference in male behaviour, which was

583   mirrored by female response. As female *O. ventralis* did not appear to differentiate between

584   conspecific and heterospecific males, one could ask why hybridisation is not even more

585   prevalent. However, we only examined the very first stage in a potential mating process, so

586   other differences that are present in the mating behaviour between both species could be

587   responsible for this. For example, *O. ventralis* males display a specific late mating behaviour,

588   called 'invite', which *O. nasuta* males never display (Kéver et al. 2018). Additionally, hybrids

589   might have a lower fitness. In order to reach mitochrondrial introgression, female hybrids would

590   also need to mate with *O. nasuta* males. This is not unlikely given that, in cichlids, female mate

591   choice is influenced, via imprinting, by the maternal phenotype (Verzijden and ten Cate 2007;

592   Verzijden et al. 2008).

593

594   *The role of males*

595

596   Asymmetric propensities towards hybridisation are expected in the intermediate stages of

597   reproductive isolation (Arnold et al. 1996). This has been observed in a variety of animal taxa

598   including lungless salamanders (Verrell 1990), spadefoot toads (Pfennig 2007), swordtails

599   (Crapon de Caprona and Ryan 1990), pupfishes (Strecker and Kodric-Brown 1999), and several

600   cichlids (Egger et al. 2008; Nevado et al. 2011). Although most examples are related to female

601   mate choice, these patterns can also be caused by asymmetries in male choosiness (Svensson

27

602 et al. 2007). Although male mate choice is common in fishes (Wong and Jennions 2003; Werner

603 and Lotem 2003), we choose to focus on the role of females (see introduction, Seehausen et al.

604 2008; Sefc et al. 2017). However, the mode of fertilization in *Ophthalmotilapia* could also have

605 an influence on male choosiness. Haesler et al. (2011) studied the reproductive behaviour of *O.*

606 *ventralis*, but it can be assumed that the behaviour of its congeners is highly similar. In *O.*

607 *ventralis*, a ripe female will visit the territories of several males, either to spawn, or just to

608 collect additional ejaculates. Subsequently, sperm competition will take place within her mouth,

609 resulting in clutches with multiple sires (Haesler et al. 2011). Given that this dilutes the effect

610 of a 'wrong' choice, a female can afford to be less choosy. Differences in both male and female

611 courtship effort towards genetically distant or similar mates have been documented in another

612 mouth brooding cichlid: *Tropheus* Boulenger, 1898 (Zoppoth et al. 2013). However, *Tropheus*

613 species are sexually monomorphic and both sexes are territorial. Additionally, *Tropheus* males

614 invest significantly more in raising the clutch, by providing the female access to their feeding

615 territories. As males of *Ophthalmotilapia* do not share their resources, we can expect these

616 males to be less choosy than those of *Tropheus*. Additionally, a substantial role of male mate

617 choice is not supported by our data, as we did not observe a difference in behaviour between

618 non-focal males of *O. nasuta* and *O. ventralis* when presented with females of the two species.

619 It should be noted, however, that two *O. ventralis* males that displayed mating behaviour

620 towards *O. nasuta* females were excluded from the analyses.

621

622 Whereas our experiments only revealed the capacity for species recognition in females of *O.*

623 *nasuta*, we cannot conclude that males cannot distinguish between females of the two species.

624 Whereas the males of *O. nasuta* and *O. ventralis* behaved differently when a visual barrier was

625 present, no significant difference was found after its removal. This could imply that males of

626 the two species behave in a similar way when presented with a conspecific or a heterospecific

28

627   female. However, an alternative explanation would be that males recognise conspecific and

628   heterospecifics, and use this knowledge to court females using a repertoire appropriate to the

629   species. Although this was found in sister species pairs of freshwater sticklebacks (*Gasterosteus*

630   spp. L. 1758) (Kozak et al. 2009), our experimental design did not allow us to test this in

631   *Ophthalmotilapia.*

632

633   We cannot exclude that morphological, physiological and behavioural features that distinguish

634   *O. nasuta* males from males of congeners could have caused the asymmetric pattern of

635   introgression. Foremost, *O. nasuta* males become larger and possess longer pelvic fins. This

636   feature could render them more attractive as *O. ventralis* females have a preference towards

637   males with strongly elongated pelvic fins (Haesler et al. 2011). As a change in the feature

638   associated with attractiveness can alter species recognition in the mating process (Phelps et al.

639   2006), the extra-long pelvic fins of *O. nasuta* males could serve as a super-natural stimulus

640   (sensu Tinbergen 1948). Additionally, even though Haesler et al. (2011) found no correlation

641   between female choice and male body length in *O. ventralis*, they did observe that larger males

642   outcompeted their rivals in sperm competition within the females' mouth. Additionally, sperm

643   of *O. nasuta* remains viable for a significantly longer amount of time than that of *O. ventralis*

644   (Morita et al. 2014). Lastly, *O. nasuta* males construct true bowers (elaborate, crater-shaped

645   sand mounts), whereas the nests of males of the other species of *Ophthalmotilapia* only consist

646   of a small area of cleaned rock, or of a small pit in the sand (Konings 2019).

647

648   *The importance of visual cues*

649

650   Although animals can use multiple kinds of cues to assess the quality of a potential mate, their

651   final assessment depends on the overall information available. This is exemplified by female

652 mate choice in the allopatric swordtail species *Xiphophorus nigrensis* Rosen 1960 and *X.*

653 *pygmaeus* Hubbs and Gordon 1943. Here, mating preferences differed depending on whether

654 visual, olfactory or a combination of both cues were available (Crapon de Caprona and Ryan

655 1990). Different responses to visual and olfactory cues were also shown for females of

656 sympatric *Cyprinodon* Lacipède, 1803 pupfish species from Lake Chichancanab (Mexico).

657 Here, different degrees of asymmetric discrimination of males were observed depending on

658 whether females had access to visual or olfactory information (Strecker and Kodric-Brown

659 1999). In species-rich systems and in species that form leks, such as *Ophthalmotilapia* spp.,

660 females must be able to rapidly assess the quality of a potential mate (Barlow 2002).

661 *Ophthalmotilapia* females could use the morphological characteristics, conspicuous bowers

662 and/or stereotypical displays of males to distinguish them from sympatric congeners. However,

663 even though multiple cues can be involved, mate choice decisions in radiations are often based

664 on just a small amount of (combinations of) these traits (Hohenlohe and Arnold 2010).

665

666 The separation wall used in our experiments contained holes that allowed for the exchange of

667 water between both compartments. Hence, besides visual clues, the fishes most likely also

668 received olfactory and acoustic information. Although visual cues were suggested to be the

669 primary factor in species-isolating, female mate choice in other cichlids (Jordan et al. 2003;

670 Kidd et al. 2006), we cannot rule out the importance of other types of information. Studies have

671 shown that olfactory (Blais et al. 2009; Plenderleith et al. 2005), acoustic (Nelissen 1978;

672 Amorim et al. 2004; Kéver et al. 2018) and behavioural (Barlow 2002) information can also

673 influence the mating process. Although Seehausen and van Alphen (1998) showed a certain

674 hierarchy of information, where other cues are taken into account when visual information is

675 absent or masked, other experiments showed that female cichlids are more likely to select the

676 right male when both olfactory and visual cues are available (Plenderleith et al. 2005; Blais et

677 al. 2009). When visual information suffices for mate recognition, the behaviour throughout the

678 mating process, i.e. potentially leading toward spawning, does not need to diverge between

679 closely-related species (Barlow 2002). This may explain why spawning behaviour of

680 *Ophthalmotilapia* is remarkably similar across the genus (Kéver et al. 2018) and why

681 differently-coloured, sympatric mbuna cichlids from Lake Malawi have identical courtship

682 behaviours (McElroy and Kornfeld 1990).

683

684 *Ecological reasons for asymmetric hybridisation*

685

686 Although they can be found in sympatry, *O. ventralis* is more associated with the rocky shores

687 of Lake Tanganyika, whereas *O. nasuta* has a wider ecological range. At rocky shores, *O.*

688 *ventralis* can be one of the most abundant cichlid species (Sturmbauer et al. 2008). Hence, for

689 an *O. ventralis* female, a random encounter with another *Ophthalmotilapia* male is much more

690 likely to result in a conspecific than a heterospecific encounter. In contrast, for an *O. nasuta*

691 female venturing into the preferred *O. ventralis* habitat, a conspecific encounter would be less

692 often the case. Therefore, the ability to discriminate between conspecific and heterospecifics

693 would be less important for females of *O. ventralis* than for those of *O. nasuta*. A similar

694 interpretation was given to explain asymmetries in female discrimination of sympatric

695 *Cyprinodon* species, where the species with the highest abundance had the lowest choosiness

696 (Strecker and Kodric-Brown 1999). Although a species' propensity for discrimination could be

697 a consequence of its distribution range, the opposite could also hold. Species that are better in

698 recognizing conspecifics are more likely to maintain the integrity of their gene pool. Hence,

699 they could be better in colonising habitats that have already been occupied by related species.

700 Finally, we showed that substantial behavioural differences can be observed between closely-

701 related species. This should be a warning to be cautious when assuming similarities in the

702 behaviour of certain (model) organisms and related taxa.

703

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705

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710

711 *Ethical note*

712

713 All specimens were obtained from a certified commercial supplier (Cichlidenstadl, Alerheim,

714 Germany). Specimens were housed in the aquarium facilities of the University of Liège.

715 Experimental procedures were performed in accordance with Belgian law, and approved by the

716 University of Liège Institutional Animal Care and Use Committee (protocol #1759) in

717 accordance with the regulations of the ethical committee of the University of Liège. All

718 manipulations were performed by a FELASA-certified experimenter. The data and script are

719 deposited on Zenodo under: 10.5281/zenodo.5534485.

720

721 *Conflict of interest disclosure*

722

723 The authors of this article declare that they have no financial conflict of interest with the content

724 of this article. MVS is one of the PCI Zool recommenders.

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726   References

727

728   Amorim MCP, Knight ME, Stratoudakis Y, Turner GF. 2004. Differences in sounds made by

729   courting males of three closely related Lake Malawi cichlids. J Fish Biol. 65(5):1358-1371.

730   Anderson MJ. 2006. Distance-based tests for homogeneity of multivariate dispersions.

731   Biometrics. 62(1):245–253.

732   Arnold SJ, Verrell PA, Tilley SG. 1996. 1996 The evolution of asymmetry in sexual isolation:

733   A model and a test case. Evolution 50:1024-1033

734   Barlow GW. 2002. How behavioural studies contribute to the species problem: a piscine

735   perspective. Fish Fish. 3(3):197-212.

736   Baerends GP, Baerends-Van Roon JM. 1950. An introduction to the study of the ethology of

737   the cichlid fishes, Leiden, The Netherlands, E. J. Brill.

738   Blais J, Plenderleith M, Rico C, Taylor MI, Seehausen O, van Oosterhout C, Turner GF. 2009.

739   Assortative mating among Lake Malawi cichlid fish populations is not simply predictable from

740   male nuptial colour. BMC Evol Biol. 9(53):53.

741   Coyne JA, Orr HA. 2004. Speciation. Sunderland, MA: Sinauer Associates.

742   Crapon de Caprona MD, Ryan MJ. 1990. Conspecific mate recognition in swordtails,

743   *Xiphophorus nigrensis* and *X. pygmaeus* (Poeciliidae): olfactory and visual cues. Anim Behav.

744   39(2):290-296.

745   Danley PD, Kocher TD. 2001. Speciation in rapidly diverging systems: lessons from Lake

746   Malawi. Mol Ecol. 10:1075-1086.

747   Egger B, Obermüller B, Eigner E, Sturmbauer C, Sefc KM. 2008. Assortative mating

748   preferences between colour morphs of the endemic Lake Tanganyika cichlid genus *Tropheus*.

749   Hydrobiologia. 615:37-48.

750   Friard O, Gamba M. 2016. BORIS: A Free, Versatile Open-Source Event-Logging Software

751   for Video / Audio Coding and Live Observations. Methods Ecol Evol. 7(11):1–6.

752   Gonzalez-Voyer A, Fitzpatrick JL, Kolm N. 2008. Sexual selection determines parental care

753   patterns in cichlid fishes. Evolution 62(8): 2015-2026.

754   Haesler MP, Lindeyer CM, Otti O, Bonfils D, Heg D, Taborsky M. 2011. Female

755   mouthbrooders in control of pre- and postmating sexual selection. Behav Ecol. 22(5):1033–

756   1041.

757   Hammer Ø, Harper DA, Ryan PD. 2001. PAST: Paleontological statistics software package for

758   education and data analysis. Palaeontologia Electronica. 4:9pp.

759   Hanssens M, Snoeks J, Verheyen E. 1999. A Morphometric Revision of the Genus

760   *Ophthalmotilapia* (Teleostei, Cichlidae) from Lake Tanganyika (East Africa). Zool J Linn Soc-

761   Lon. 125(4):487–512.

762   Ho J, Tumkaya T, Aryal S, Choi H, Claridge-Chang A. 2019. Moving beyond P values: data

763   analysis with estimation graphics. Nat Met. 16(7):565-566.

764   Hohenlohe PA, Arnold SJ. 2010. Dimensionality of mate choice, sexual isolation, and

765   speciation. PNAS. 107(38):16583-16588.

766   Immler S, Taborsky M. 2009. Sequential polyandry affords post-mating sexual selection in the

767   mouths of cichlid females. Behav Ecol Sociobiol. 63(8):1219–1230.

768   Jiggins CD, Salazar C, Linares M, Mavarez J. 2008. Hybrid trait speciation and *Heliconius*

769   butterflies. Philos T R Soc B. 363(1506):3047-3054.

770   Jordan R, Kellogg K, Juanes F, Stauffer J. 2003. Evaluation of female mate choice cues in a

771   group of Lake Malawi mbuna (Cichlidae). Copeia. 2003(1):181-186.

772   Kéver L, Parmentier E, Derycke S, Verheyen E, Snoeks J, Van Steenberge M, Poncin P. 2018

773   Limited possibilities for prezygotic barriers in the reproductive behaviour of sympatric

774   *Ophthalmotilapia* species (Teleostei, Cichlidae). Zoology. 126:71-81.

34

775 Kidd MR, Danley PD, Kocher TD. 2006. A direct assay of female choice in cichlids: all the

776 eggs in one basket. J Fish Biol. 68(2):373–384.

777 Kocher TD. 2004. Adaptive evolution and explosive speciation: the cichlid fish model. Nat Rev

778 Genet. 5(4):288-298.

779 Koblmüller S, Duftner N, Sefc KM, Aibara M, Stipacek M, Blanc M, Egger B, Sturmbauer C.

780 2007. Reticulate phylogeny of gastropod-shell-breeding cichlids from Lake Tanganyika - the

781 result of repeated introgressive hybridisation. BMC Evol Biol. 7:7.

782 Konings A. 2019. Tanganyika cichlids in their natural habitat. 4th edition. El Paso, TX: Cichlid

783 press.

784 Kozak GM, Boughman JW. 2009. Learned conspecific mate preference in a species pair of

785 sticklebacks. Behav Ecol. 20: 1282–1288.

786 Kozak GM, Reisland M, Boughmann JW. 2009. Sex differences in mate recognition and

787 conspecific preference in species with mutual mate choice. Evolution. 63: 353-365.

788 Luttbeg B, Towner MC, Wandesforde-Smith A, Mangel M, Foster SA. 2001. State-dependent

789 mate-assessment and mate-selection behavior in female threespine sticklebacks (*Gasterosteus*

790 *aculeatus*, Gasterosteiformes: Gasterosteidae). Ethology. 107(6):545-558.

791 Mayr E. 1982. The growth of biological thought: Diversity, evolution and inheritance.

792 Cambridge, MA: Harvard University Belknap Press.

793 Marques DA, Meier JI, Seehausen O. 2019. A combinatorial view on speciation and adaptive

794 radiation. Trends Ecol Evol. 34(6):531-544.

795 Mendelson TC, Shaw KL. 2012. The (mis)concept of species recognition. Trends Ecol Evol.

796 27(8):421-427.

797 McElroy DM, Kornfeld I. 1990. Sexual selection, reproductive behaviour, and speciation in the

798 mbuna species fock of Lake Nyasa (Pisces: Cichlidae). Environ Biol Fish. 28:273-284.

799   Moran MD. 2003. Arguments for rejecting the sequential Bonferroni in ecological studies.

800   Oicos. 100(2):403-405.

801   Morita M, Awata S, Yorifuji M, Ota K, Kohda M, Ochi H. 2014. Bower-building behaviour is

802   associated with increased sperm longevity in Tanganyikan cichlids. J Evolution Biol.

803   27(12):2629–2643.

804   Naish KA, Ribbink AJ. 1990. A preliminary investigation of sex change in *Pseudotropheus*

805   *lombardoi* (Pisces: Cichlidae). Environ Biol Fish. 28:285–294.

806   Nakagawa S. 2004. A farewell to Bonferroni: the problem if low statistical power and

807   publication bias. Behav. Ecol. 15:1044-1045.

808   Nelissen MHJ. 1978. Sound production by some Tanganyikan cichlid fishes and a hypothesis

809   for the evolution of their communication mechanisms. Behaviour. 64(1-2):137–147.

810   Nevado B, Fazalova V, Backeljau T, Hanssens M, Verheyen E. 2011. Repeated unidirectional

811   introgression of nuclear and mitochondrial DNA between four congeneric Tanganyikan

812   cichlids. Mol Biol Evol. 28(8):2253-2267.

813   Oksanen JF, Blanchet G, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara

814   RB, Simpson GL, Solymos PM, Stevens HH, Szoecs E, Wagner H. 2017. vegan: Community

815   Ecology Package. R package version 2.4-5.

816   Panfili J, Thior D, Ecoutin J-M, Ndiaye P, Albaret J-J. 2006. Influence of salinity on the size at

817   maturity for fish species reproducing in contrasting West African estuaries. J Fish Biol.

818   69(1):95-113.

819   Peters HM. 1975. Hermaphroditism in cichlid fishes. pp. 228–235.In: R. Reinboth (ed.)

820   Intersexuality in the Animal Kingdom. Springer-Verlag, Berlin.

821   Pfennig KS. 2007. Facultative mate choice drives adaptive hybridization. Science, 318(5852):

822   965.

823   Phelps SM, Rand AS, Ryan MJ. 2006. A cognitive framework for mate choice and species

824   recognition. Am Nat. 167(1):28–42.

825   Plenderleith M, van Oosterhout C, Robinson RL, Turner GF. 2005. Female preference for

826   conspecific males based on olfactory cues in a Lake Malawi cichlid fish. Biol Lett. 1(4):411-

827   414.

828   Poll M. 1986. Classification des cichlidae du Lac Tanganyika: Tribus, genres et espèces. Bull

829   Acad R Belg. 8:1-163.

830   R core team. 2017. R: A Language and environment for statistical computing. R foundation for

831   statistical computing, Vienna, Austria.

832   Ronco F., Büscher H. H., Indermauer A., Salzburger W. 2020. The taxonomic diversity of the

833   cichlid fish fauna of ancient Lake Tanganyika, East Africa. J. Great Lakes Res. 46: 1067-1078.

834   Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image

835   analysis. Nat Met. 9(7):671-675.

836   Roux C, Fraïsse C, Romiguier J, Anciaux Y, Galtier N, Bierne N. 2016. Shedding light on the

837   grey zone of speciation along a continuum of genomic divergence. PLOS Biol.

838   14(12):e2000234.

839   Rüber L, Meyer A, Sturmbauer C, Verheyen E. 2001. Population structure in two sympatric

840   species of the Lake Tanganyika cichlid tribe Eretmodini: Evidence for introgression. Mol Ecol.

841   10(5):1207–1225.

842   Salzburger W. 2018. Understanding explosive diversification through cichlid fish genomics.

843   Nat Rev Genet. 19:705-717.

844   Salzburger W, Braasch I, Meyer A. 2007. Adaptive sequence divergence in a colour gene

845   involved in the formation of the characteristic egg-dummies of male *Haplochromis* cichlid

846   fishes. BMC Biol. 5(51):13pp.

847   Seehausen O. 2004. Hybridisation and adaptive radiation. Trends Ecol Evol. 19(4):198-207.

848 Seehausen O, van Alphen JJM. 1998. The effect of male coloration on female mate choice in

849 closely related Lake Victoria cichlids (*Haplochromis nyererei* complex). Behav Ecol Sociobiol.

850 42:1–8.

851 Seehausen O, Terai Y, Magalhaes IS, Carleton KL, Mrosso HDJ, Miyagi R, van der Sluijs I,

852 Schneider MV, Maan ME, Tachida H, Imai H, Okada N. 2008. Speciation through sensory

853 drive in cichlid fish. Nature. 455:620-626.

854 Sefc KM, Mattersdorfer K, Ziegelbecker A, Neuhüttler N, Steiner O, Goessler W, Koblmüller

855 S. 2017. Shifting barriers and phenotypic diversification by hybridisation. Ecol Lett. 20(5):651-

856 662.

857 Sommer-Trembo C, Plath M, Gismann J, Helfrich C, Bierbach D. 2017. Context-dependent

858 female mate choice maintains variation in male sexual activity. R Soc Open Sci. 4: 170303.

859 Strecker U, Kodric-Brown A. 1999. Mate recognition systems in a species flock of Mexican

860 pupfish. J Evolution Biol. 12(5):927- 935.

861 Sturmbauer C, Fuchs C, Harb G, Damm E, Duftner N, Maderbacher M, Koch M, Koblmüller

862 S. 2008. Abundance, distribution, and territory areas of rock-dwelling Lake Tanganyika cichlid

863 fish species. Hydrobiologia. 615(1):57-68.

864 Sullivan BK. 2009. Mate recognition, species boundaries and the fallacy of "species

865 recognition". Open Zool. 2:86-90.

866 Svensson EI, Karlsson K, Friberg M, Eroukhmanoff F. 2007. Gender differences in species

867 recognition and the evolution of asymmetric sexual isolation. Curr Biol. 17(22):1943–1947.

868 Tinbergen N. 1948. Social releasers and the experimental method required for their study.

869 Wilson Bull. 60(1):6-51.

870 Turner GF, Seehausen O, Knight ME, Allender CJ, Robinson RL. 2001. How many species of

871 cichlid fishes are there in African lakes. Mol Ecol. 10(3):793-806.

872   Theis A, Salzburger W, Egger B, 2012. The function of anal fin egg-spots in the cichlid fish

873   *Astatotilapia burtoni*. PLoS ONE 7(1):e29878.

874   Thomas GH, Székely T. 2005. Evolutionary pathways in shorebird breeding systems: sexual

875   conflict, parental care, and chick development. Evolution 59(10): 2222–2230.

876   Trivers RL. 1972. Parental investment and sexual selection. in Sexual selection and the descent

877   of man, EBG. Campbell, ed. (136-179). Aldine, Chicago.

878   Van Oppen MJH, Turner GF, Rico C, Robinson RL, Deutsch JC, Genner MJ, Hewitt GM. 1998.

879   Assortative mating among rock-dwelling cichlid fishes supports high estimates of species

880   richness from Lake Malawi. Mol Ecol. 7(8):991-1001.

881   Verrell PA. 1990. Frequency of interspecific mating in salamanders of the plethodontid genus

882   *Desmognathus*: different experimental designs may yield different results. J Zool. 221(3):441-

883   451.

884   Verzijden MN, Korthof REM, ten Cate C. 2008. Females learn from mothers and males learn

885   from others. The effect of mother and siblings on the development of female mate preferences

886   and male aggression biases in Lake Victoria cichlids, genus *Mbipia*. Behav Ecol Sociobiol. 62:

887   1359–1368.

888   Verzijden MN, ten Cate, C. 2007. Early learning influences species assortative mating

889   preferences in Lake Victoria cichlid fish. Biol lett 3(2): 134-136.

890   Werner NY, Lotem A. 2003. Choosy males in a haplochromine cichlid: first experimental

891   evidence for male mate choice in a lekking species. Anim Behav. 66(2):293-298.

892   Wirtz, P. 1999. Mother species-father species: unidirectional hybridization in animals with

893   female choice. Animal Behaviour. 58:1–12.

894   Wong BMB, Jennions MD. 2003. Costs influence male mate choice in a freshwater fish. P Roy

895   Soc B-Biol Sci. 270(Suppl 1):S36-S38.

896   Zoppoth P, Koblmüller S, Sefc KM. 2013. Male courtship preferences demonstrate

897   discrimination against allopatric colour morphs in a cichlid fish. J Evolution Biol. 26(3): 577-

898   586.

899

900   Additional material

901

902   Supplement 1. Summary of the experiments.

903

904   Supplement 2. Indications for sex change observed in *O. nasuta* females. Top row: gonads of

905   some of the specimens that were female in external phenotype but had male or ambiguous

906   gonads (ON33, 35, 26, 43). Vertical left: female gonads in developmental stage 3 (ON34), 4

907   (ON24) and 5 (ON37) respectively (Panfili et al. 2006). Bottom: two presumed non-focal *O.*

908   *nasuta* females used in the ON experiment, one of which could have undergone transition after

909   the experiments, and horizontal right, the ventral area of the same specimens, with A: anal pore

910   and UG: urogentital pore.

911

912   Supplement 3. Visualization of tracking data. The position of each fish is plotted for each

913   second in which specimens were tracked with the positions recorded before and after the

914   removal of the opaque wall (grey) coloured differently. Ellipses denote the area in which 90%

915   of tracks are situated, large dots denote the average positions before and after the removal of

916   the barrier and arrows shows the change in mean position. The separation wall is visualized as

917   a meshed partition. The average speed before (v0) and after (v1) the removal of the barrier is

918   plotted for each tracked specimen with data for focal specimens given in bold. Abbreviations

919   (ON: *O. nasuta*, OV, *O. ventralis*, F: female, M; male)

920

921   Supplement 4. Summary of the data.

922

923   Supplement 5. Loadings and variance of the main axes of the PCAs and CVAs conducted in

924   the study.

925

926   Supplement 6. Principal component analyses and Canonical variate analyses performed on the

927   behaviours recorded 45 min after the visual barrier was removed of the ON (*O. nasuta*, left)

928   and the OV (*O. ventralis,* right) experiment. Symbols on the scatter plots for the ON and OV

929   experiment as in E and F, respectively, with full circles denoting focal females presented with

930   no fish (red), a conspecific female (blue) an *O. ventralis* male (purple), and an *O. nasuta* male

931   (green), empty circles denote non-focal conspecific females and full squares *O. ventralis*

932   (purple) and *O. nasuta* (green) males. Explained variances are added to the axes.

933

934   Supplement 7. Shift in average position of the specimens analysed 15min before and 15min

935   after the removal of the separation wall for the ON (above) and OV (below) experiment. Focal

936   specimens are all visualised on the left, and non-focal specimens on the right. Dimensions in

937   cm, with the vertical bar representing the separation wall. Dashed arrows represent individual

938   fishes, bold arrows the average per treatment. Colours, for focal females (ON and OV)

939   presented with no fish (red), a conspecific female (turquoise) an *O. ventralis* male (pink), and

940   an *O. nasuta* male (light green), and non-focal specimens (right): conspecific females (blue),

941   *O. ventralis* males (purple) and *O. nasuta* males (green).