

## Familiarity reduces aggression but does not modify acoustic communication in pairs of Nile tilapia (*Oreochromis niloticus*) and black-chinned tilapia (*Sarotherodon melanotheron*)

Akian Dieudonné Djétouan <sup>1,2,3</sup>, Yao Kouakou <sup>2</sup>, Parmentier Eric <sup>4</sup>, Clota Frederic <sup>5</sup>, Baroiller Jean-françois <sup>6,7</sup>, Bégout Marie-Laure <sup>3,\*</sup>

<sup>1</sup> Département Eaux, Forêts et Environnement, Institut National Polytechnique Félix HOUPHOUËT-BOIGNY Yamoussoukro ,Côte d'Ivoire

<sup>2</sup> Laboratoire de Biologie et Cytologie Animales, Unité de Formation et de Recherche Science de la Nature, Université NANGUI ABROGOUA Abidjan, Côte d'Ivoire

<sup>3</sup> MARBEC, Université Montpellier, CNRS, Ifremer, IRD, Palavas-les-Flots, France

<sup>4</sup> Laboratoire de Morphologie Fonctionnelle et Evolutive, AFFISH, Institut de chimie- B6C, Université de Liège ,Belgium

<sup>5</sup> Université Paris-Saclay, INRAE, AgroParisTech, GABI, Jouy-en-Josas, France

<sup>6</sup> Unité Mixte de Recherche 116, Institut des Sciences de l'Evolution de Montpellier, Centre de Coopération Internationale en Recherche Agronomique pour le Développement, Campus International de Baillarguet Montpellier ,France

<sup>7</sup> Institut des Sciences de l'Evolution de Montpellier, Centre National de la Recherche Scientifique, Institut de Recherche pour le Développement, Ecole Pratique des Hautes Etudes, Université de Montpellier ,France

\* Corresponding author : Marie-Laure Bégout, email address : [marie.laure.begout@ifremer.fr](mailto:marie.laure.begout@ifremer.fr)

### Abstract :

Reproduction involves multiple complex behaviours and the effects of familiarity on such social interactions are seldom described in fish. This is particularly true for sound production and communication within aggressive or non-aggressive context. Here we explore the effects of a common garden rearing without parental care of two closely related cichlid species (Nile tilapia *Oreochromis niloticus* and black-chinned tilapia *Sarotherodon melanotheron*) on their sound production features and social interactions. After 9 months in common garden rearing, from embryonic stage to first maturity, sound production and associated behaviours were recorded on specimens of the two species in intraspecific and interspecific pairings. We found that fish were able to produce the same kind of sounds as those recorded in similar context for their parents. Drums sounds were associated to chasing, lateral attack and courtship in *O. niloticus* and only in fleeing or avoidance in *S. melanotheron*. Specific grunts were produced in chasing, after biting and in nest building by *O. niloticus* and specific rolling sounds were associated to courtship in *S. melanotheron*. Sounds production and behaviours were not correlated to sex steroid levels but the number of sounds recorded in aggressive context was correlated to dominance in *O. niloticus*. We conclude that one generation of common garden rearing does not modify sound features which remain

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specific and innate in the two cichlids. Despite the familiarity, *O. niloticus* remained dominant on *S. melanotheron* but the aggressiveness between the two species decreased.

**Keywords** : acoustic signals, common garden, innate behaviour, tilapias

## 1 INTRODUCTION

According to Munakata and Kobayashi (2010), the reproductive behaviour of fish is innate and can take place without any experience or learning. In different fish species, this behaviour is associated to sound production that can be involved in mating choice, territory defence or spawning synchronization (Amorim *et al.*, 2003; 2004; Longrie *et al.*, 2008; 2013). Recent studies have however shown that sound production was innate in at least *Codoma ornata* (Johnston & Buchanan, 2007) and in *Oreochromis niloticus* (Longrie *et al.*, 2008).

The ability to distinguish conspecifics from heterospecific is crucial to avoid hybridizations between species (Turner *et al.*, 2001; Kéver *et al.*, 2018). However, the identification abilities are not universal and can show different levels of hierarchy. Species and even populations are capable of different levels of recognition, which may relate to their proximate ecology (Ward *et al.*, 2009). The distinction between conspecific and heterospecific individuals has been experimentally observed in different fish species in which social experience is not necessary for heterospecific recognition: the rock bass *Ambloplites rupestris* (Brown & Colgan, 1986), the Chinese bream *Parabramis pekinensis*, and the qingbo *Spinibarbus sinensis* (Tang *et al.*, 2017).

At a different level, the guppy *Poecilia reticulata* and sticklebacks *Gasterosteus aculeatus* are not only able to distinguish their conspecific, they also have the ability to identify individuals from the same siblings and choose generally to shoal with familiar individuals (Griffiths & Magurran, 1999; Frommen & Bakker, 2004; Ward *et al.*, 2009). In the framework of familiarity, animals learn to recognize the signature of familiar individuals during development and later discriminate these familiar individuals from unfamiliar ones. It does not imply to be kin or full sib (Mateo, 2004; 2017).

The ability of recognizing familiar individuals from unfamiliar, conspecifics from heterospecifics or kin from non-kin would be different among fish species and based on

different cues. Several studies on social behaviour in fish have shown that visual, olfactory or acoustic signals are involved in the recognition process of kin, sexual partners or predators (Brown & Brown, 1992; Brown & Brown, 1996; Sharp *et al.*, 2005; Plenderleith *et al.*, 2005; Mehlis *et al.*, 2008; Brown *et al.*, 2011; Xia *et al.*, 2018). For example, adult cichlid *Pelvicachromis taeniatus* use visual and chemical cues to discriminate unfamiliar kin from unfamiliar non-kin in mate choice context (Thünken *et al.*, 2007). Further, familiarity is known to reduce aggression among individuals in some species and can be also involved in the choice of sexual partner. For example in the guppy (*Poecilia reticulata*) in which unfamiliar males and females are preferred over familiar individuals (Ward & Hart, 2003; Frostman & Sherman, 2004; Mariette *et al.*, 2010).

Many cichlid species are widely known to provide parental care for their offspring until they are able to defend themselves and escape from a predator (Specker & Kishida, 2000; Desjardins *et al.*, 2007; Balshine & Sloman, 2011; Teresa & Freitas, 2011; Balshine, 2012). In *Neolamprologus pulcher*, the acquisition of such protective behaviour could take place and refine during parental care for which juveniles raised with their parents exhibited more aggressive and submissive behaviours to each other than those raised with siblings only (Arnold & Taborsky, 2010).

After parental care period, most juveniles fish are separated from their parents, live and evolve in shoal in natural streams (Lamboj, 2006). They cohabit or live in aggregation in the same habitat with other species hence such offspring do not have the opportunity to learn further behavioural repertoire (e.g. sounds production) from their parents. As a result their behaviour could be different from that of individuals which remained close to their parents. Fish behavioural patterns could be the result of learning experience or be innate or a combination of both (Kieffer & Colgan, 1992; Zheng *et al.*, 2005).

We hypothesize that two sympatric species living together could influence one another, affecting their specific behavioural pattern. *Oreochromis niloticus* (On) and *Sarotherodon melanotheron* (Sm) are both well-delineated biological species. *S. melanotheron* is monogamous species with a paternal or biparental mouthbrooding whereas *O. niloticus* males are polygamous and mouthbrooding is strictly maternal (Trevawas, 1983; Keenleyside, 1991). Interestingly, prezygotic barriers can be permeable since inter-specific hybridization between these both species has been observed in brackish environments (Amon *et al.*, 2013a). These two species are able to produce specific sounds in different behavioural context like chasing, fleeing, courtship and nest building (Akian *et al.*, 2020).

In this study, we aim to use both of these species to determine the influence of social environment on sound production features and associated behavioural repertoire in interspecific pairing context. Using specimens of both species that have shared a common developmental cycle from embryonic stage to sexual maturity without receiving parental care, the aim of this study is dual. We want to investigate whether the familiarity can impact acoustic behaviour and features during social interactions in reproductive pairing contexts.

## **2 MATERIALS AND METHODS**

### **2.1 Breeding in common garden**

Broodstock of both species (male and female) were raised at the station of the International Center for Agronomic Research for Development (CIRAD) of Montpellier (France) and transferred to the station of the National Institute for Ocean Science (Ifremer) of L'Houmeau (France). They were kept in intraspecific group in four polycarbonate tanks of 400 L. Water temperatures were maintained at 27 - 28 °C, dissolved oxygen concentration was higher than 4 mg.L<sup>-1</sup>, and pH between 7 and 8. Ammonia, nitrate and nitrite concentrations were 0.3 ± 0.2, 6.9 ± 5.2 mg.L<sup>-1</sup> and 0.5 ± 0.2 mg.L<sup>-1</sup> respectively. Fish were fed with a commercial food

for tilapia (EFICO YM 868, 3 mm, BIOMAR®, France) twice a day under a photoperiod of 12 hours of light.

First generation juveniles were obtained after collecting the incubating embryos during broodstock control in the mouth of male and female in *S. melanotheron* and females in *O. niloticus*. These embryos were incubated in two floating baskets installed in two tanks, one basket for each species. At 17 days post hatching (dph) and 13 dph for *O. niloticus* and *S. melanotheron* respectively, fry were counted and then dispatched (N= 150 of each species) into two interspecific groups in two 400 L tanks (Figure 1).

They were reared during 6 months (up to 197 dph for On and 193 dph for Sm) to reach their first sexual maturity, then weighed and sexed by observation of the genital papillae with a methylene blue solution. The following weight and length were obtained (mean  $\pm$  SD): 71.5  $\pm$  29.4 g and 15.4  $\pm$  2.0 cm for *O. niloticus* ♂; 53.0  $\pm$  19.7 g and 14.2  $\pm$  1.6 cm for *O. niloticus* ♀; 33.3  $\pm$  7.1 g and 12.8  $\pm$  0.9 cm for *S. melanotheron* ♂; 29.6  $\pm$  7.0 g and 12.0  $\pm$  0.9 cm for *S. melanotheron* ♀. These mature fish were subsequently distributed according to two interspecific pairing modes in expecting to get hybrids in four 400 L tanks (2 tanks per pairing mode: 1 = *O. niloticus* ♂  $\times$  *S. melanotheron* ♀; 2 = *S. melanotheron* ♂  $\times$  *O. niloticus* ♀) with a sex ratio male : female of 1 : 2 recommended for captive breeding of Tilapia (Salama, 1996; Nour *et al.*, 2008).

A total of 60 fish per tank were observed over 90 days and thereafter a subsample (n = 20 per sex and species) was used for acoustic and behavioural tests. ~~described in the following section~~

## 2.2 Experimental fish and pairing mode

After this 90 days period, the characteristics of the subsampled fish used for acoustic and behavioural test were as follows: males and female of *O. niloticus* were at 287 dph (mean  $\pm$  SD: 88.0  $\pm$  38.7 g and 16.7  $\pm$  2.4 cm for On ♂; 81.2  $\pm$  36.5 g and 16.3  $\pm$  2.1 cm for On ♀) and

*S. melanotheron* were 283 dph (mean  $\pm$  SD: 57.8  $\pm$  8.7 g and 15.4  $\pm$  0.7 cm for Sm  $\sigma$ ; 52.9  $\pm$  6.2 g and 14.9  $\pm$  0.4 cm for Sm  $\phi$ ). Considering the faster growth of *O. niloticus* than that of *S. melanotheron*, the larger individuals male and female were paired in *O. niloticus* intraspecific pairing and the smaller ones used to form interspecific pairs so that fish were of equal size within a pair.

Four aquariums (60  $\times$  25  $\times$  35 cm high) of 50 L each containing thermoregulated filtered water, were placed on a 4 cm thick polystyrene plate on a table in a soundproof room in the basement of Ifremer station in order to avoid background noise from the vibration transmitted from the ground to the aquariums. On the day before the behavioural recordings and before being transferred to the aquariums, fish were weighed, measured after benzocaine anaesthesia (0.1 mL<sup>-1</sup>, from a 10 % stock solution of Ethyl-p-aminobenzoate, Benzocaine, E1501, Sigma, St Louis, MO, USA, prepared by dissolving 100 g of Benzocaine in 1 L of 100 % ethanol) and dorsally marked with beads of different colours to ease sex recognition (Bégout *et al.*, 2012). The pairing was done according to 4 modes in the experimental aquariums (1 = *O. niloticus*  $\sigma$   $\times$  *O. niloticus*  $\phi$ ; 2 = *O. niloticus*  $\sigma$   $\times$  *S. melanotheron*  $\phi$ ; 3 = *S. melanotheron*  $\sigma$   $\times$  *O. niloticus*  $\phi$ ; 4 = *S. melanotheron*  $\sigma$   $\times$  *S. melanotheron*  $\phi$ ). To prevent pre-experimental physical and visual communication between individuals, the aquarium was divided into two equal-size compartments with a removable opaque PVC divider. For each pairing mode, ten couples were tested and fish were not used for more than one trial. Water in the aquariums was renewed after each pairing.

### 2.3 Sounds recording with associated behaviours

An uncalibrated hydrophone (High Tech, Inc -96-MIN, Saffron Walden, UK, frequency range: 2 Hz to 30 kHz, voltage sensitivity -164.9 dB re 1V /  $\mu$ Pa 1.06 mA) was placed in the centre of the aquarium the day after the isolation of fish. Then the movable divider that

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separated male and female was removed to allow the fish to be in contact. Simultaneously, for each session, the behaviours and sounds of the fish were recorded using a camera (SONY, HDR-PJ530) positioned 1.5 m away from the front of the aquarium. The hydrophone was connected to the camera and both connected to a computer. ~~We were thus able to record the sounds and associated behaviours.~~ Filter and aeration systems were removed from aquariums during recordings to reduce background noise and avoid disturbing the fish. For each pair, the recordings lasted 15 min and were performed 3 times a day from 10 am, 12 am and 2 pm, recording session were thus limited to the most active phases of the pairing experiment (Akian *et al.*, 2020). Food was not given to the fish during the recording sessions and another 400 L tank similar to their home tank was used to keep the fish after each pairing tests.

#### **2.4 Plasma steroids analyses**

At the end of the third recording session on each pair, fish were removed from aquariums. A blood sample (0.4–0.5 mL) was taken after anaesthesia in a bath of benzocaine (0.1 mL.L<sup>-1</sup>) on each individual and centrifuged (13000 rpm, 6 min, 4 °C). Then plasma was kept in Eppendorf tubes and stored in the freezer (-20 °C). For the dosage of sex steroids plasma was diluted 1/2000 for males and 1/500 for females for the determination of 11-ketotestosterone (11-kt) concentrations and 1/200 for the estradiol (E2) level in females (N = 20 by sex and species). A BioTek Synergy HT spectrofluorometer CAYMAN kit (Cayman Chemical Ann Arbor, MI, USA) and IBL kit (IBL Hamburg, Germany) were used to perform enzyme immuno assays for 11-kt and E2 respectively.

#### **2.5 Sounds and behaviours analyses**

Acoustic signals were extracted from video files using the software Audacity 2.0.4 (<https://audacityteam.org/>). Then sounds were digitized at 44.1 kHz (16-bit resolution) and analysed with Avisoft software -SASLAB Pro 4.33 (Glienicke, Brandenburg, Germany).

Sound's characteristics (number of pulses, duration (T), period of pulses or grunts (p), grunt duration (t) and number of grunts; Figure 2) were measured on the oscillograms. Power spectra (band pass lower cut-off frequency = 0.05 and upper cut-off frequency = 2.5 kHz, FFT size 1024 points, time overlap 95 % and Hanning window) were used to determine frequencies. The tilapia ethogram (Longrie *et al.*, 2013) and video observation help us to determine the behaviours associated with different sounds. Aggressive behaviours were biting, chasing, fleeing, avoidance, tail blow, lateral attack and non-aggressive behaviours were courtship, quiver, circle, nest building and no particular behaviour. The Ethovision XT and Observer Module (Noldus, The Netherlands) enabled encoding of the videos to determine the occurrences of the different behaviours. The sum of occurrences of aggressive behaviours (biting, chasing, tail blow, lateral attack) minus the sum of fleeing occurrences observed for each individual gave the calculation of the dominance index according to Renn *et al.* (2012).

## 2.6 Data analysis

The homogeneity of variances and normality of the data set of fish sizes, sound characteristics, E2 and 11-kt concentrations was verified with the Levene test. Data were compared between species according to the 4 pairing modes with the Tukey test. Non parametric Kruskal Wallis and Mann-Whitney multiple comparison tests were used when data did not fulfil normal distribution and homoscedasticity requirements. The Spearman correlation rank test ( $r_s$ ) was performed to determine the relationship between E2 and 11-kt concentrations and the number of sounds emitted, and the fish sizes and sound characteristics. Given the repeated observation of couples between 10 am and 2 pm, a repeated ANOVA was used to analyse the occurrences of the different behaviours. When a difference was observed, Tukey's post hoc test was used. Dominance index were tested using ANOVA-2 (species and sex as fixed factors) using the Newman-Keuls post hoc test to assess significant differences.

All statistical tests were performed on Statistica 10.0 (Statsoft, Tulsa, OK, USA) with the significance threshold  $P < 0.05$  for all tests.

## **2.7 Ethical statement**

All procedures performed in this study involving animals were in accordance with the ethical standards of the institution and followed European Directive 2010/63 UE. This project was performed under agreement EEA# 17-190-1 with project approval under APAFIS #263.

## **3 RESULTS**

### **3.1 Types of sound and production context by *O. niloticus* and *S. melanotheron***

Fish tested after common garden rearing emitted sounds in aggressive (biting, chasing, fleeing, avoidance, tail blow, lateral attack) or non-aggressive contexts (courtship, quiver, circle, nest building, and no particular behaviour) both in intraspecific and interspecific pairings.

Two types of sounds were recorded in *O. niloticus* male and female. These sounds are similar to drum sounds and grunt trains (Figure 2), both associated to aggressive behaviours (biting, chasing, fleeing, avoidance, tail blow, lateral attack) and non-aggressive behaviours (courtship, quiver, circle, nest building, and no particular behaviour). In *S. melanotheron* two types of sounds were also noted and are similar to drum sounds, generally observed during aggressive behaviour (avoidance, fleeing, lateral attack, biting) and the second are similar to rolling sounds produced during courtship behaviours (Figure 3).

### **3.2 Characteristics of drum sounds produced by *O. niloticus* and *S. melanotheron* during aggressive behaviours**

In male and female *O. niloticus*, drum sounds associated with aggressive behaviour are composed of 1 to 5 pulses (min-max) both in intraspecific and interspecific pairings. In *S.*

*melanotheron* the sounds measured during aggressive behaviour consist of 1 to 6 pulses. No significant difference was observed between males and females in the number of pulses of the sounds produced by the two species, whatever the pairing modes (Tukey post-hoc test,  $P > 0.05$ ).

In intraspecific pairings, drum sounds of *O. niloticus* males that are associated to aggressive behaviours lasted  $229 \pm 130$  ms with a period of  $104 \pm 56$  ms and a fundamental frequency of  $99 \pm 40$  Hz. In interspecific pairings with *S. melanotheron* female, these sounds lasted  $171 \pm 84$  ms, with a period of  $89 \pm 45$  ms and a fundamental frequency of  $93 \pm 36$  Hz. In intraspecific pairings, *O. niloticus* female drum sound associated to aggressive behaviours lasted  $244 \pm 122$  ms, with a period of  $116 \pm 51$  ms and a frequency of  $97 \pm 40$  Hz. Drum sound in *O. niloticus* female in interspecific context lasted  $153 \pm 116$  ms, with a period of  $95 \pm 56$  ms and  $113 \pm 44$  Hz for the frequency.

In intraspecific pairings, *S. melanotheron* male drum sounds associated to aggressive behaviours lasted  $304.8 \pm 130.2$  ms, with a period of  $106.6 \pm 50.4$  ms and a fundamental frequency of  $97.7 \pm 39.0$  Hz. These sounds in female lasted  $241.23 \pm 129.4$  ms, with a period of  $116.7 \pm 63.6$  ms and a fundamental frequency of  $115.2 \pm 41.1$  Hz. In interspecific pairings, *S. melanotheron* sound duration is  $242.2 \pm 137.4$  ms, with a period of  $105.4 \pm 52.1$  ms and fundamental frequency of  $110.5 \pm 37.4$  Hz for male, and  $230.5 \pm 127.7$  ms for duration,  $101.8 \pm 55.2$  ms for the period and  $101.4 \pm 35.5$  Hz for the frequency in female.

A detailed comparison of acoustic features (duration, period and frequency) produced in aggressive context can be found in Figure 4. By comparing species and pairings, we note that on one hand, there was no significant difference in the duration of sounds produced in the intraspecific pairings of the two species and in male *O. niloticus* paired to *S. melanotheron* female (Tukey post-hoc test,  $P > 0.05$ , Figure 4a). On the other hand, *O. niloticus* female

showed a significantly lower duration of sounds produced in interspecific pairings (Tukey post-hoc test,  $P < 0.05$ ). No significant difference in pulse period was noted in the intraspecific or interspecific pairing modes (Tukey post-hoc test,  $P > 0.05$ , Figure 4b). There was also no significant difference in frequency of the sounds between the sexes according to the pairing modes (Tukey post-hoc test,  $P > 0.05$ , Figure 4c). Moreover, sound frequency in *S. melanothereon* female was significantly lower (Tukey post-hoc test,  $P < 0.05$ ) when paired to *O. niloticus* male than in intraspecific context.

### **3.3 Characteristics of drum sounds in *O. niloticus* and rolling sound in *S. melanothereon* produced in non-aggressive behaviours**

Sounds emitted in a non-aggressive situation are composed of 1 to 6 (min-max) pulses in *O. niloticus* males and females without any significant difference (Tukey post-hoc test,  $P > 0.05$ ) according to the pairing modes and the sexes. In *S. melanothereon*, the number of pulses ranged from 1 to 26 with significantly (Tukey post-hoc test,  $P < 0.05$ ) higher values observed in intraspecific pairings.

Drum sound associated to non-aggressive behaviour in *O. niloticus* male lasted  $212.6 \pm 151.7$  ms with period of  $111.2 \pm 70.6$  ms and fundamental frequency of  $134.2 \pm 54.5$  Hz in intraspecific context. In *O. niloticus* female, values are  $201.1 \pm 123.2$  ms for the duration with period of  $114.8 \pm 57.5$  ms and fundamental frequency of  $128.5 \pm 44.3$  Hz.

In interspecific pairing, drum sound in *O. niloticus* male lasted  $224.0 \pm 171.8$  ms with period of  $108.8 \pm 62.9$  ms and fundamental frequency of  $120 \pm 28.1$  Hz. Drum sound in *O. niloticus* female lasted  $184.5 \pm 154.2$  ms with period of  $89.1 \pm 53.0$  ms and fundamental frequency of  $158.1 \pm 66.8$  Hz in interspecific pairing.

Rolling sound in *S. melanotheron* male lasted  $361.2 \pm 275.9$  ms with period of  $101.7 \pm 43.1$  ms and fundamental frequency of  $144.8 \pm 41.5$  Hz in intraspecific pairing. *S. melanotheron* female rolling sound duration is  $478.9 \pm 268.5$  ms with period of  $98.3 \pm 34.5$  ms and fundamental frequency of  $163.5 \pm 60.4$  Hz. These rolling sounds lasted  $392.4 \pm 222.8$  ms with period of  $94.9 \pm 42.7$  ms and frequency of  $149.1 \pm 59.1$  Hz in male and  $366.1 \pm 247.8$  ms with period of  $99.1 \pm 47.2$  ms and frequency of  $135.1 \pm 51.9$  Hz in female in interspecific context.

Drum and rolling sounds characteristics (duration, period and frequencies) are presented in Figure 5a and 5b. The duration of the sounds does not vary significantly in *O. niloticus* intraspecific context (Figure 5a), unlike in *S. melanotheron* pairing in which sound duration in females is significantly longer than in males (Tukey post-hoc test,  $P < 0.05$ ). In interspecific pairing, rolling sound durations are significantly higher ( $P < 0.05$ ) in *S. melanotheron*. No significant differences were noted in the period of sound in both species according to the pairing modes (Tukey post-hoc test,  $P > 0$ ; Figure 5b). Sounds frequencies did not vary significantly in intraspecific and interspecific pairings with *S. melanotheron* male and *O. niloticus* female (Figure 5c). Non-aggressive drum sound frequency in *O. niloticus* male was nonetheless significantly lower ( $P < 0.05$ ) than rolling sound frequency in *S. melanotheron* female in interspecific pairings.

### **3.5 Characteristics of grunt trains produced by *O. niloticus* during aggressive and non aggressive behaviours**

Grunts were only noted in *O. niloticus* in both intra and interspecific pairings and were related to aggressive (during chasing and before and after biting) or non-aggressive behaviours (nest building).

Grunts produced by *O. niloticus* in aggressive context were observed only in the intraspecific pairings. The number of grunts varies from 3 to 11 with no significant difference between

males and females. There were also not significant differences between sex in the others characteristics of grunts (duration, period and frequency, Tukey post-hoc test,  $P > 0.05$ , Table 1).

In non-aggressive context, the number of grunts in a sound ranged from 2 to 35 with no significant differences (Tukey post-hoc test,  $P > 0.05$ ) between sexes in intra and interspecific pairing. No significant differences ( $P > 0.05$ ) were observed in the duration of grunt trains between males and females, regardless of the pairing modes (Table 2). There were also no significant differences (Tukey post-hoc test,  $P > 0.05$ ) in the periods of grunts. In males and females, the frequency of grunts was however significantly lower (Tukey post-hoc test,  $P < 0.05$ ) in intraspecific context compared to interspecific context.

### **3.6 Number of sounds produced according to the context**

In both species, the number of sounds produced in aggressive and non-aggressive contexts did not vary significantly (Tukey HSD test,  $P > 0.05$ ) according to the recording times, regardless of the pairing mode. In *S. melanotheron* the number of drum sounds produced in aggressive context was significantly higher in interspecific pairings (Figure 6a). There was no significant difference (Tukey HSD test,  $P > 0.05$ ) in the number of sounds produced in non-aggressive context in *O. niloticus* male and female whatever the pairing mode, unlike *S. melanotheron* in which males and females showed a significantly higher value (Tukey HSD test,  $P < 0$ ).

### **3.7 Fish size and correlation with the characteristics of sounds**

Due to our experimental choice regarding fish sizes in the different pairing modes, we ended up recording sound production in fish of different sizes. In *O. niloticus*, the weight and length of male ( $117.2 \pm 29.6$  g /  $18.5 \pm 1.4$  cm) and female ( $112.2 \pm 22.5$  g /  $18.1 \pm 1.0$  cm) were significantly higher ( $P < 0.05$ ) in intraspecific pairings compared to values in interspecific pairings (male :  $58.8 \pm 19.9$  g /  $14.9 \pm 1.7$  cm ; female  $50.3 \pm 13.1$  g /  $14.4 \pm 0.9$  cm). In *S.*

*melanotheron* no significant difference (Tukey HSD test,  $P > 0.05$ ) existed in the fish sizes in the two pairing modes. Overall, no significant effects of fish size on sound production were observed within each pairing mode (Tukey HSD test,  $P > 0.05$ ). Further, no significant correlation ( $P > 0.05$ ) existed between the size of the fish (weight and length) and the characteristics of the sounds emitted (duration, period and frequencies).

### **3.8 Sexual steroids concentrations and correlation with weight and number of sounds emitted**

In males of both species 11-kt concentrations in the plasma (On =  $46.3 \pm 32.7$  ng.mL<sup>-1</sup>, Sm =  $42.5 \pm 30.3$  ng.mL<sup>-1</sup>) were significantly higher than those of females (On =  $2.1 \pm 1.8$  ng.mL<sup>-1</sup>, Sm =  $1.9 \pm 1.8$  ng.mL<sup>-1</sup>) (Kruskal Wallis test:  $H_{(7, 80)} = 59.3$ ,  $P < 0.05$ ). No significant differences (Kruskal Wallis test:  $H_{(7, 80)} = 59.3$ ,  $P > 0.05$ ) were noted for 11-kt concentrations values between the males of the two species. The same trend was observed in females for 11-kt and E2 concentrations (On =  $61.5 \pm 54.7$  ng.mL<sup>-1</sup>, Sm =  $41.5 \pm 35.9$  ng.mL<sup>-1</sup>; Mann-Whitney U test,  $P = 0.19$  for 11-kt and  $P = 0.27$  for E2). Correlations between weights and sex steroid concentrations (11-kt and E2) were not significant ( $P > 0.05$ ) (Table 3). In both species, Spearman's correlations between the number of sounds produced (in aggressive and non-aggressive contexts) and sex steroid concentrations (11-kt and E2) were not significant ( $P > 0.05$ ) in intra or interspecific pairings (Table 3).

### **3.9 Aggressiveness between fish and dominance index**

After the common garden, in *O. niloticus* and *S. melanotheron*, the aggressive acts in intraspecific and interspecific pairings consisted in chase, lateral attacks, biting, tail blow and fleeing. Mouth fighting was only observed in intraspecific pairings of both species. Aggressive behaviours were observed in all the 10 pairs tested in the different pairing modes, except 4 out of the 10 pairs tested in *S. melanotheron* intraspecific pairing in which no

aggressiveness was observed. On one hand, males *O. niloticus* were the most dominant against *O. niloticus* females and *S. melanotheron* females with dominance index significantly higher ( $P < 0.05$ ) than that measured for *O. niloticus* females (Figure 7a-b). On the other hand, *O. niloticus* males showed more aggressive acts against *O. niloticus* females than *S. melanotheron* females. In *S. melanotheron*, males were dominated by *O. niloticus* females in interspecific pairings (Figure 7c) and dominant against their conspecific females with a significant difference in dominance index ( $P < 0.05$ ) in intraspecific context (Figure 7d).

### **3.10 Correlation between dominance index, the number of sounds and the sexual steroid concentrations**

In *O. niloticus* males the dominance index was positively correlated ( $P < 0.05$ ) with the number of emitted sounds associated to aggressive behaviours in intra and interspecific contexts (Table 4). In *S. melanotheron*, the index was negatively correlated ( $P < 0.05$ ) to the number of sounds emitted in aggressive behaviours in interspecific pairings and positively correlated in non-aggressive context ( $P < 0.05$ ). Such negative correlation was also observed between dominance index and 11-kt concentration in *S. melanotheron* males ( $P < 0.05$ ). The correlations between the index and the number of sounds in non-aggressive behaviour, and the sex steroids were not significant ( $P > 0.05$ ) in *O. niloticus* (both sexes) and in *S. melanotheron* females.

## **4 DISCUSSION**

After 9 months of common garden rearing without parental care from embryonic stage to first maturity, our results showed that in both species, sound produced by offspring have similar features to that of their parents meaning common rearing does not alter fish vocalisation. In both species, sounds were associated with aggressive and non-aggressive behavioural contexts as observed in their parents (Akian *et al.*, 2020). Since the juveniles and young adults had no

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opportunity to learn from their parent's behaviour and acoustic signals, this result provides evidence that the ability to produce sounds is innate in *S. melanotheron* and provides confirmation regarding *O. niloticus* (Longrie *et al.*, 2008). Moreover, the comparison of sound features (number of pulses or grunts, duration and frequencies) from the tested fish in the present study with those of their parents obtained by Akian *et al.* (2020) do show a high degree of similarities, showing the rearing context did not influence vocal features. Two types of sounds were recorded in each of the two species. Drum sounds were produced in both species, specific grunt trains were produced by *O. niloticus* and specific rolling sounds were produced by *S. melanotheron*. These signals were associated with complex aggressive and non-aggressive behaviours as already observed in different cichlid species (Amorin *et al.*, 2003, 2004; Akian *et al.*, 2020).

Despite the time spent under the same rearing conditions, no spawning was observed in interspecific batches. As acoustic features are different between both species, it supports acoustic signals may be potentially involved in the mechanisms of reproductive isolation (Lobel, 1998; Ptacek, 2000; Amorin *et al.*, 2008). Since hybridizations between *O. niloticus* and *S. melanotheron* were obtained in previous studies (Amon *et al.*, 2013a; 2013b) and not in ours, we assume the lack of hybridization during our experiments is related to environmental factors (e.g. salinity, turbidity in lagoon) that changed the perception of the vocal signature of the two species or reduce their capacity of acoustic and visual communication. Indeed, altered environmental conditions can affect the sensory pathways (production, transmission, reception and response) of communication signals and therefore ecological interactions can be impaired (Kelley *et al.*, 2018; Rivest *et al.*, 2019). No differences were observed in the characteristics of sounds (duration, period, and frequency) produced by males and females during the aggressive contexts in intraspecific pairings in the two species. In the interspecific pairings, *O. niloticus* female showed shorter sound durations against *S. melanotheron* male

than during pairing with *O. niloticus* male; while *S. melanotheron* female had a lower sound frequency when paired to *O. niloticus* male than when female were in intraspecific context. This illustrates a decrease in the aggressiveness of *O. niloticus* female toward *S. melanotheron* male that could be attributed to the familiarity between the fish after a common garden. Indeed, in the study conducted in the parental generation (Akian *et al.*, 2020), we observed that sound duration of *O. niloticus* female in aggressive context was similar to that of *S. melanotheron* male in interspecific context and sound frequency in *S. melanotheron* female was not different regardless of the pairing mode.

No significant differences were observed between 11-kt levels in males and females of both species despite the larger size of male and female *O. niloticus* in intraspecific pairings. In male *O. niloticus* (weighing 88 g), 11-kt concentration ( $46.3 \pm 32.7 \text{ ng.mL}^{-1}$ ) was higher than  $31.6 \pm 8.1 \text{ ng.mL}^{-1}$  measured in males weighing 74 g by Toguyéni *et al.* (2009) in this species. In *S. melanotheron*, 11-kt min-max values (4.3 to  $111.7 \text{ ng.mL}^{-1}$ ) were higher than that measured on males of the same species with similar size during and just after the oral incubation (0.9 to  $22.0 \text{ ng.mL}^{-1}$ ) by Specker and Kishida (2000). This suggests that all the fish tested in the present study were at the same level of sexual maturity and that the lack of incubation would explain the high levels of 11-kt in *S. melanotheron* males. Indeed, despite the sexual maturity of the specimens of both species, no reproduction was observed in interspecific batches before sound recordings. In females, the 11-kt values were between 0.2 and  $7.8 \text{ ng.mL}^{-1}$  for *O. niloticus* and between 0.3 and  $8.6 \text{ ng.mL}^{-1}$  for *S. melanotheron* and were similar to those obtained by Toguyéni *et al.* (2009) in females of these two species. Despite the absence of spawning in interspecific batches, E2 values for *S. melanotheron* females were similar to that obtained by Specker and Kishida (2000) in mouthbrooding females or just after spawning (6.1 and  $13.2 \text{ ng.mL}^{-1}$  respectively).

Even with individual fish of similar sizes in each pairing mode, high standard deviations were still observed within 11-kt levels in males and E2 levels in female for each species. As suggested in their parents (Akian *et al.*, 2020), these interindividual differences could be related to the level of cortisol which would modify the concentrations of 11-kt in relation to the aggressiveness due to fish overcrowding in the breeding tank before the sound recordings (Consten *et al.*, 2001; Vasconcelos *et al.*, 2012). In addition, territorial males would have a higher 11-kt level as observed in the Cichlid *Astatotilapia burtoni* (Parikh *et al.*, 2006). Although there were differences in sex steroid levels, no significant correlation was observed with the size of the two species in each pairing mode. Similarly, the correlations were not significant between the concentrations of sex steroid and the number of sounds produced albeit, in the two species, the number of sounds were significantly associated with aggressive or non-aggressive behaviours in the four pairing modes. This result is similar to the observations made on the parents of the two species tested in the same contexts (Akian *et al.*, 2020). In addition, the present study on offspring suggests that a high level of androgen does not necessarily support aggressiveness since *S. melanotheron* males with high 11-kt levels were still dominated by *O. niloticus* females. We did not however test male x male or female x female interactions and direct hierarchy establishment as Taves *et al.* (2009) did, and where they unravel sex specific androgen roles in intra-sex contests in *Neolamprologus pulcher*. It is very likely that mature male x female interactions trigger more complex endocrine pathways than intra-sex pairings and additional steroids should be analysed such as testosterone as well as sampling at additional time (*e.g.* before and after the pairing). Such broaden information could allow a better understanding of fish endocrine correlates of behavioural responses observed in reproductive context. Indeed social contexts such as social hierarchy and interspecific pairings may explain the aggressive behaviours observed in some tested pairs. The dominance of *O. niloticus* on *S. melanotheron* is corroborated by dominance index that

were positively correlated with the number of sounds associated to aggressive behaviours in *O. niloticus* male and negatively correlated in *S. melanotheron* male and female in interspecific pairings.

Further, our results showed that the aggressiveness and the dominance of *O. niloticus* on *S. melanotheron* was overall conserved. Indeed, we have observed the same types of dominance of *O. niloticus* on *S. melanotheron* in the different interspecific pairing modes (Akian *et al.*, 2017). However, with common garden rearing, aggression seems to be reduced in some pairings likely because of the familiarity between the paired fish. Our observations seem to corroborate the findings of several authors that familiarity reduces the aggressiveness between *Leuciscus cephalus*, *Phoxinus phoxinus* and *Neolamprologus pulcher* (Ward *et al.*, 2003; Frostman & Sherman, 2004; Jordan *et al.*, 2009). In *S. melanotheron* intraspecific pairings, reduction of aggressiveness between the fish could be explained by the fact that females or males that have not yet reproduced would be less aggressive between themselves and would more easily accept an opposite sex congener. The females in this case would be less demanding than females with a reproductive experience that choose larger males (Legendre & Trébaol, 1996). Aggressiveness between fish in some intraspecific pairing of *O. niloticus* and *S. melanotheron* suggests differences in personality between individuals of the same species (Martins *et al.*, 2011). It remains to assess if other signals besides visual and acoustic cues, e.g. chemical cues, could be involved in the choice or acceptance of the sexual partner (Plenderleith *et al.*, 2005).

In conclusion, this experiment confirms sounds are innate in *O. niloticus* (Longrie *et al.*, 2008) and shows for the first time it is also the case in *S. melanotheron*. Specimens of both species do thus not require prior learning from their parents to produce sounds associated with various aggressive and non-aggressive complex behaviours. Furthermore, rearing the two

species together in the same tank from the embryonic stage to adult stage (first maturity only) does not influence the sound features which remain characteristic of each species. Finally, despite the familiarity between the fish tested, there was still dominance of *O. niloticus* on *S. melanotheron* but the aggression level between the two species seems to be decreasing in intensity.

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## AUTHOR CONTRIBUTIONS

D. D. A., Y. K., F. C., J-F. B., and M-L. B. designed the research, D. D. A., Y. K., and M-L. B. performed the research, D. D. A., Y. K., E. P., and M-L. B. analysed the data, D. D. A., Y. K., E. P., F. C., J-F. B., and M-L. B., wrote the manuscript, M-L. B. supervised the study.

**ORCID MLB 0000-0003-1416-3479**

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## Figures captions

**FIGURE 1.** Schematic diagram of the rearing in common garden for 9 months until the acoustic tests of the juveniles of *Oreochromis niloticus* and *Sarotherodon melanotheron* (dph: days post hatching) were performed in pairs.

**FIGURE 2.** Oscillograms of the drumming sound (top) and a train of grunts (bottom) produced by a parent (left) and by F1 offspring (right) in *Oreochromis niloticus*. (T) represents the duration of the sound, (p) the period of two pulses, and (t) the duration of a grunt. Relative amplitude means that the hydrophone we used was not calibrated.

**FIGURE 3.** Oscillograms of a drumming sound (top) and a rolling sound (bottom) produced by a parent (left) and by F1 offspring (right) in *Sarotherodon melanotheron*. (T) represents the duration of the sound and (p) the period between two pulses. Relative amplitude means that the hydrophone we used was not calibrated.

**FIGURE 4.** Characteristics of drum sounds associated with aggressive behaviours in *Oreochromis niloticus* and *Sarotherodon melanotheron* in intraspecific ( $\text{On}\text{♂} \times \text{On}\text{♀}$ ,  $\text{Sm}\text{♂} \times \text{Sm}\text{♀}$ ) and interspecific ( $\text{On}\text{♂} \times \text{Sm}\text{♀}$ ,  $\text{Sm}\text{♂} \times \text{On}\text{♀}$ ) pairing contexts. The letters a and b on the histogram indicate significant differences (Tukey test,  $P < 0.05$ ).

**FIGURE 5.** Characteristics of drum sounds associated with non-aggressive behaviours in *Oreochromis niloticus* and rolling sound in *Sarotherodon melanotheron* in intraspecific ( $\text{On}\text{♂} \times \text{On}\text{♀}$ ,  $\text{Sm}\text{♂} \times \text{Sm}\text{♀}$ ) and interspecific ( $\text{On}\text{♂} \times \text{Sm}\text{♀}$ ,  $\text{Sm}\text{♂} \times \text{On}\text{♀}$ ) pairing contexts. The letters a, b and c on the histogram indicate significant differences (Tukey test,  $P < 0.05$ ).

**FIGURE 6.** Number of sounds produced in aggressive (A) and non-aggressive (B) situations in *Oreochromis niloticus* and *Sarotherodon melanotheron* according to pairing modes.

**FIGURE 7.** Dominance index among males and females in *Oreochromis niloticus* and *Sarotherodon melanotheron* after the "common garden" according to the pairing mode

(N=10). Letters indicate significant differences between male and female (ANOVA and Newman and Keuls test,  $P < 0.05$ ).

FIGURE 1

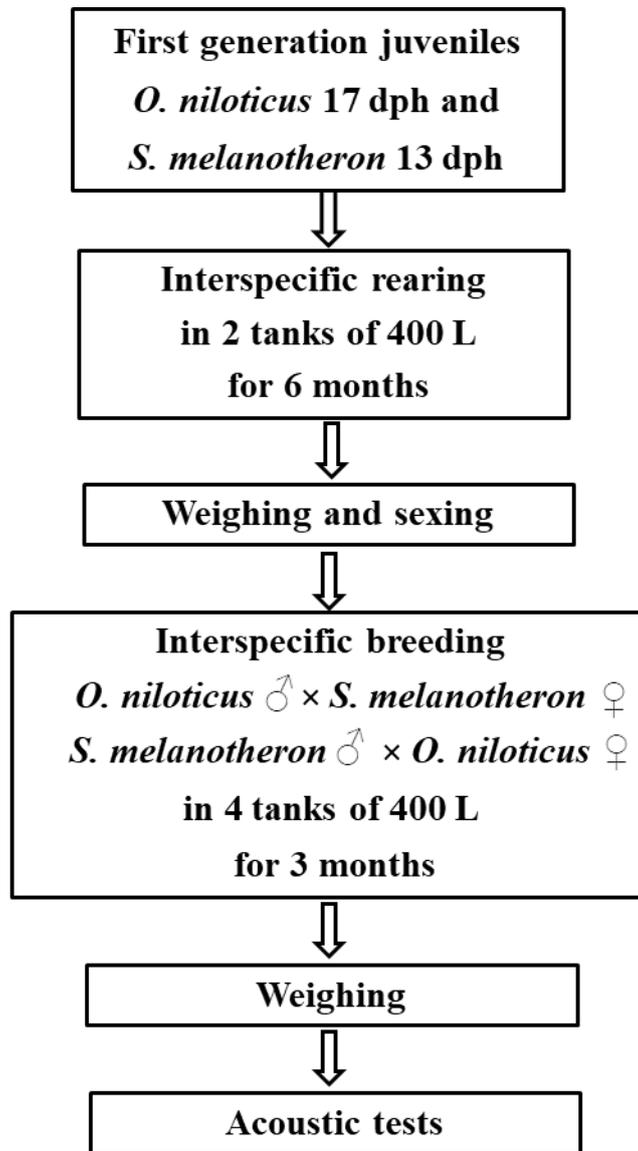


FIGURE 2

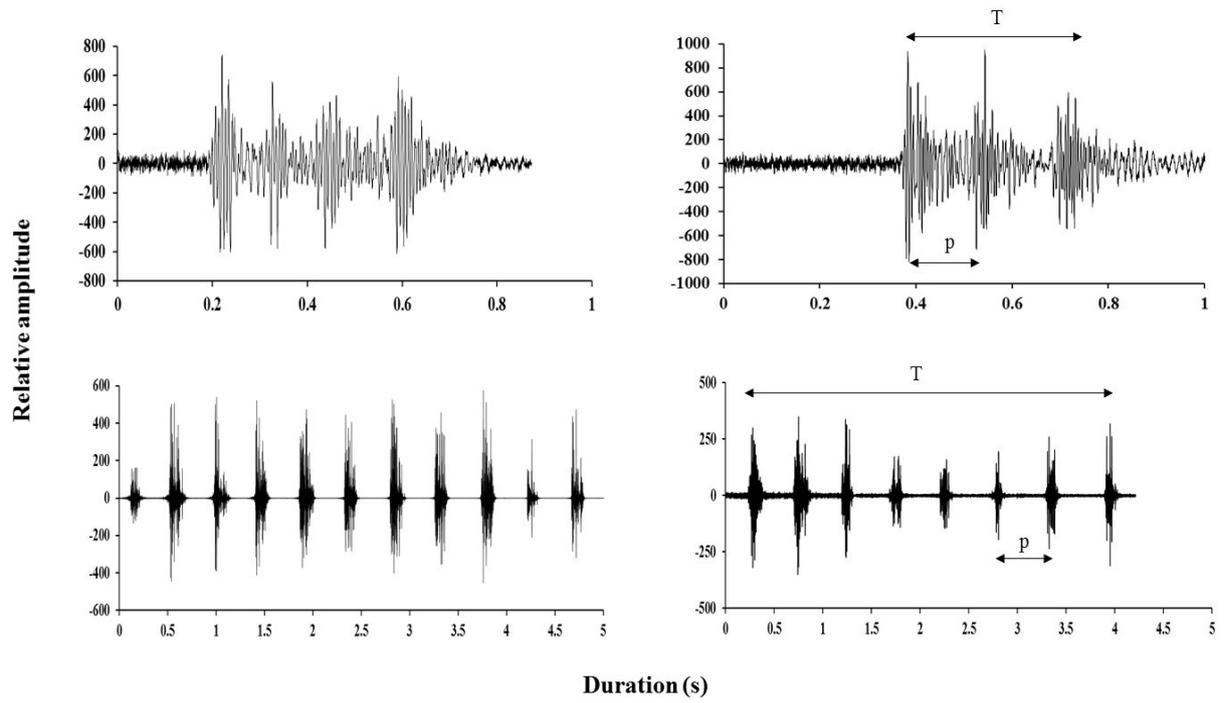


FIGURE 3

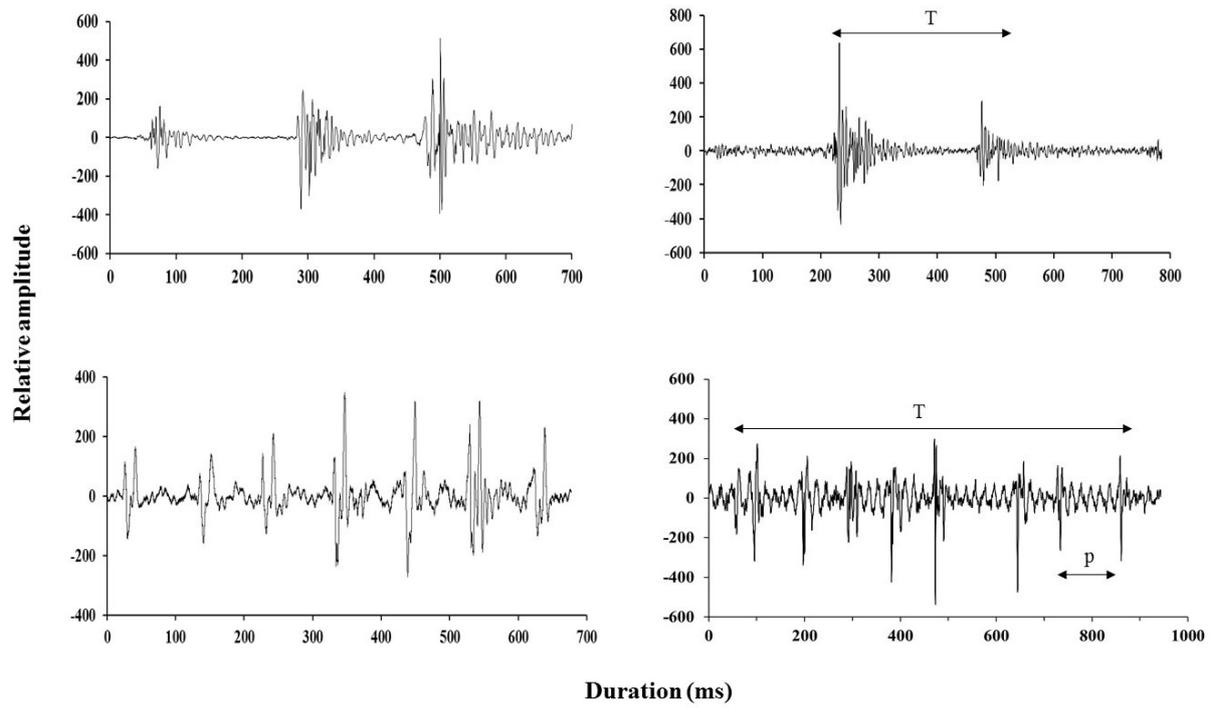


FIGURE 4.

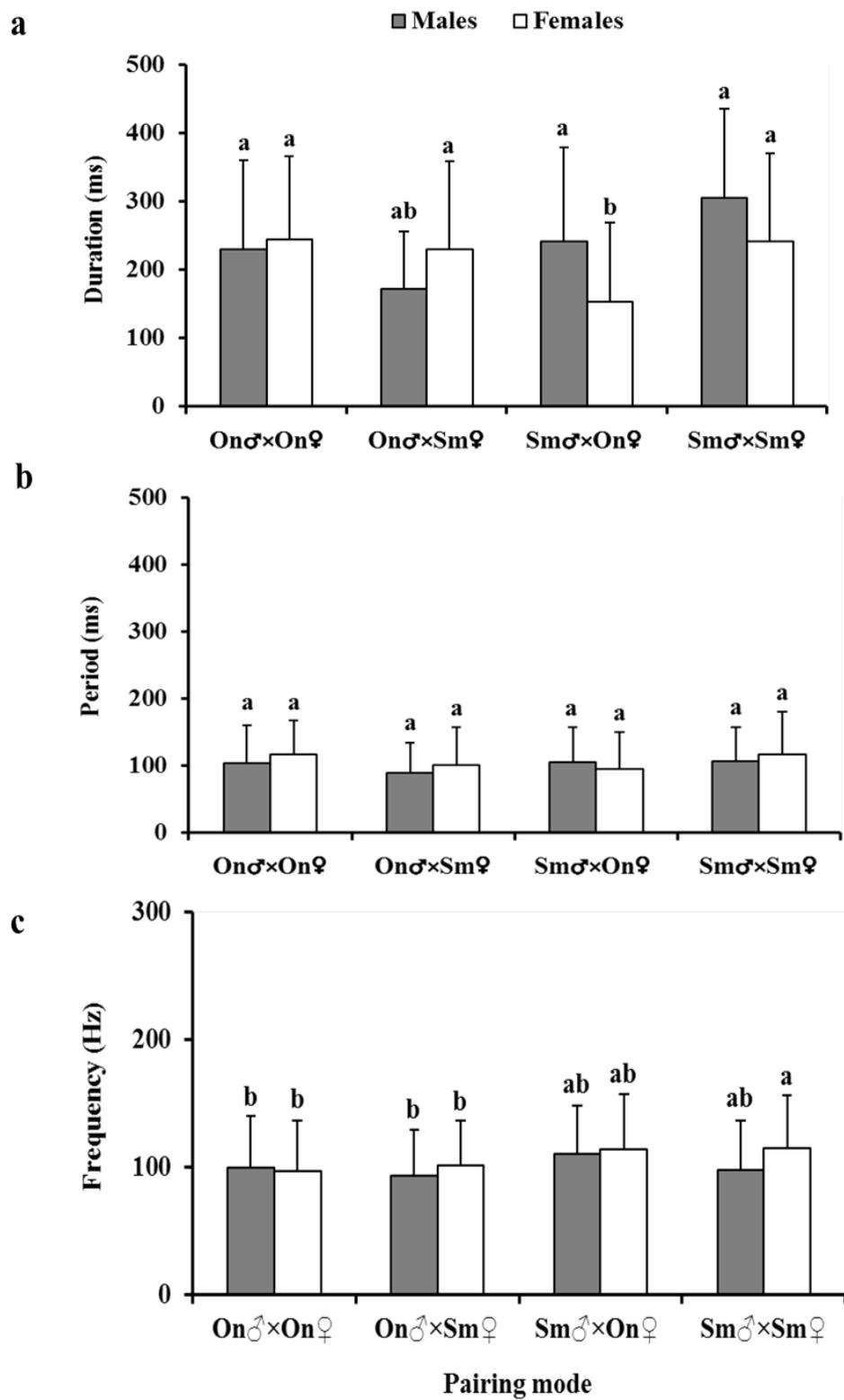


FIGURE 5.

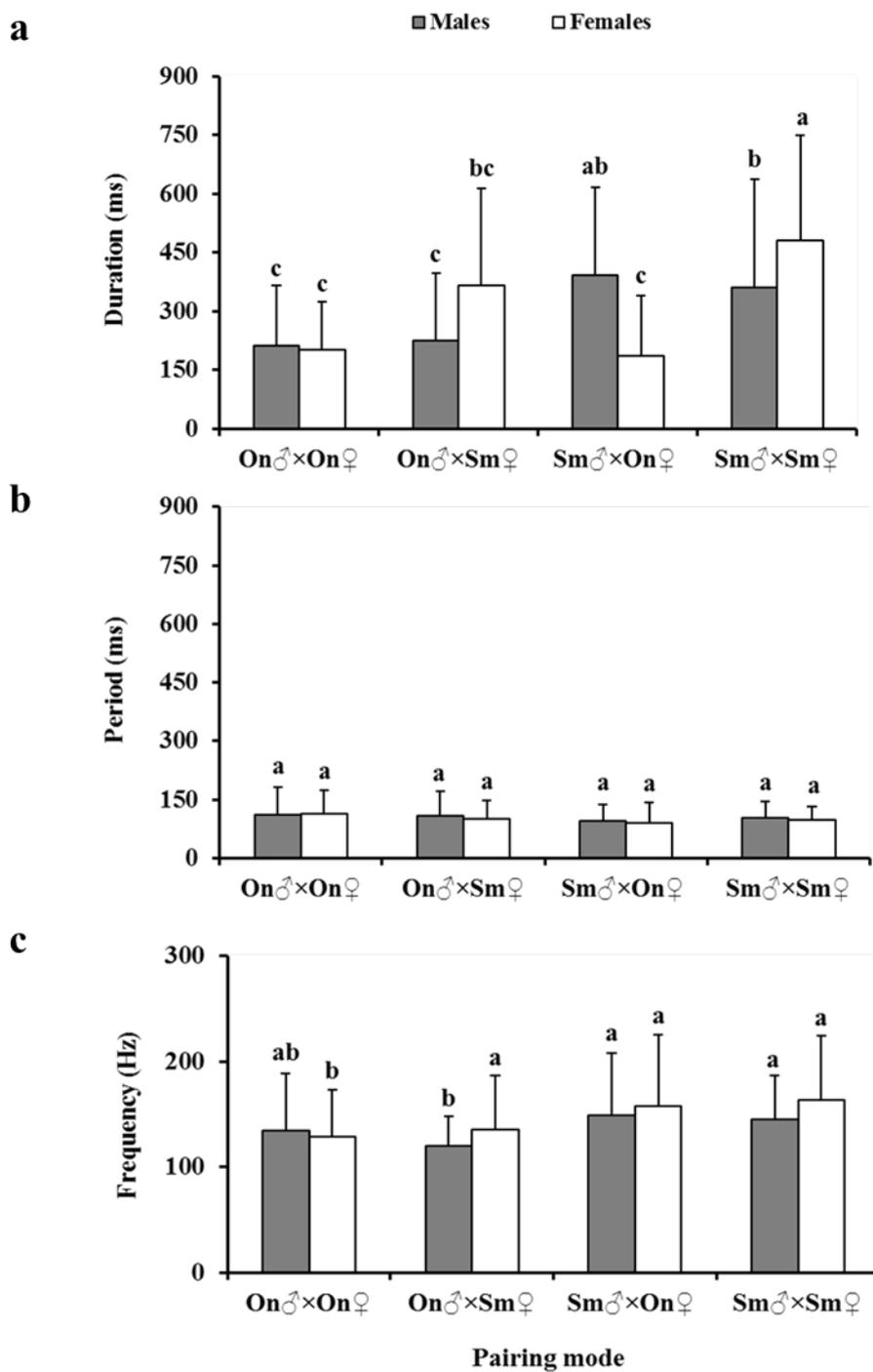


FIGURE 6.

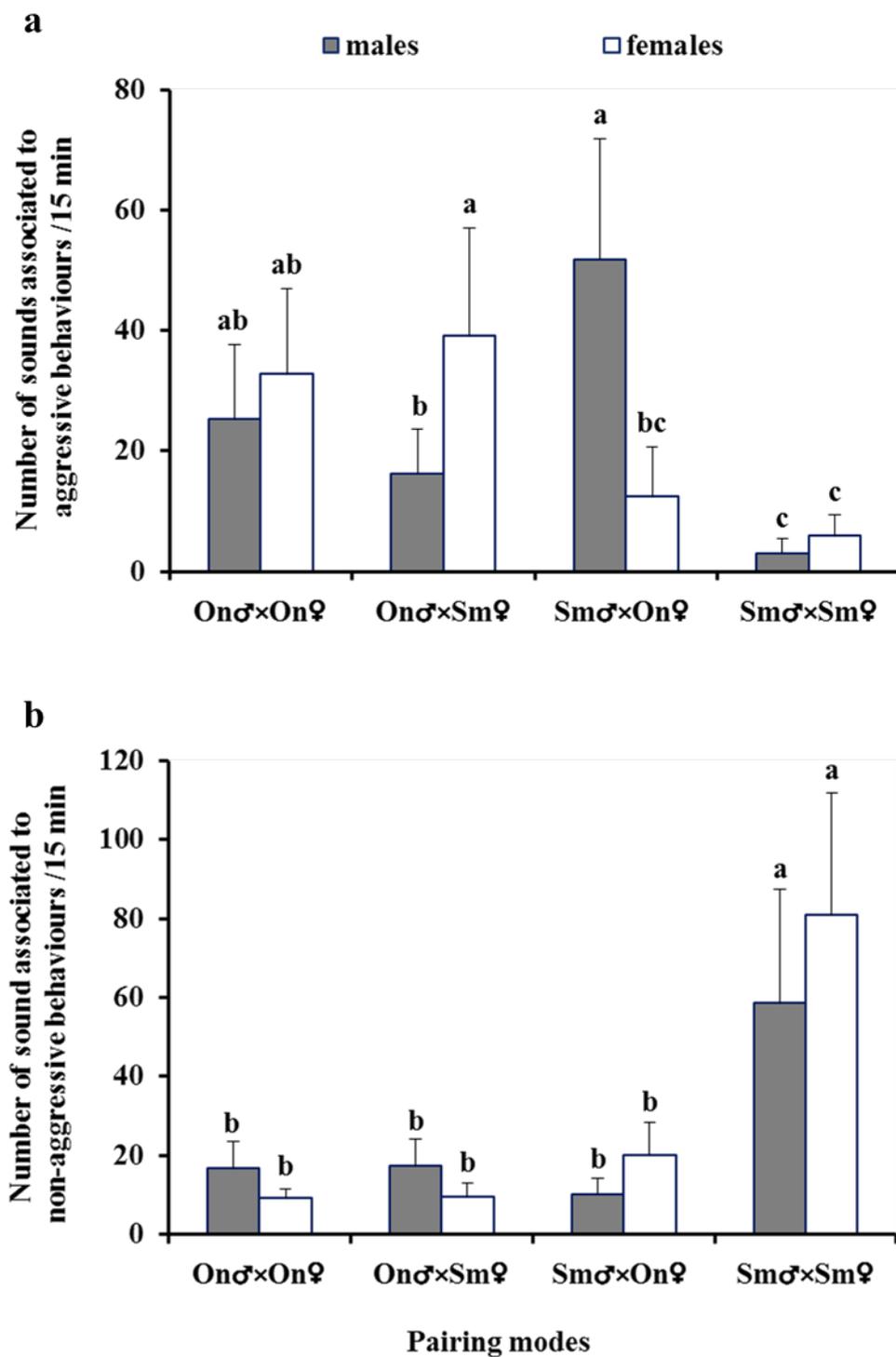
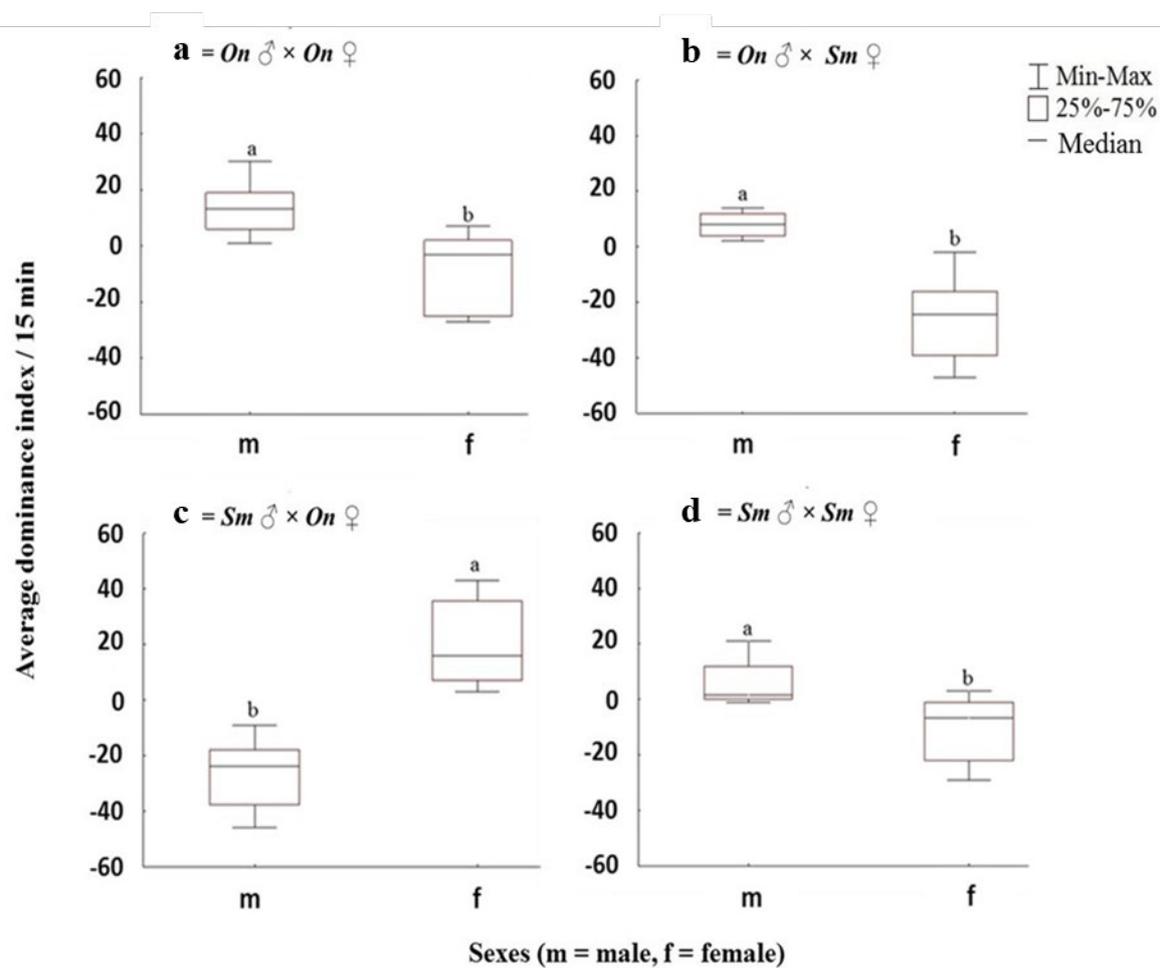


FIGURE 7



## Tables captions

**TABLE 1.** Characteristics of the grunt trains emitted by *Oreochromis niloticus* during aggressive behaviours in intraspecific pairing. The letters a in the columns indicate no significant differences according to Tukey's post-hoc test ( $P > 0.05$ ).

**TABLE 2.** Characteristics of the grunt trains emitted by *Oreochromis niloticus* during non-aggressive behaviours during intraspecific and interspecific pairing with *Sarotherodon melanotheron*. The letters a and b in the columns indicate significant differences according to Tukey's post-hoc test ( $P < 0.05$ ).

**TABLE 3:** Spearman's correlation ( $R_s$ ) between sex steroids (11-ketotestosterone (11-kt) and estradiol (E2)) concentrations with the weight and the number of sounds produced by *Oreochromis niloticus* (male On ♂ and female On ♀) and *Sarotherodon melanotheron* (male Sm ♂ and female Sm ♀) in intraspecific (On ♂ × On ♀; Sm ♂ × Sm ♀) and interspecific pairing (On ♂ × Sm ♀; Sm ♂ × On ♀).

**TABLE 4.** Spearman's correlations between the dominance index and the number of sounds and sex steroid (11-ketotestosterone (11-kt) and estradiol (E2)) concentrations in *Oreochromis niloticus* (male On ♂ and female On ♀) and *Sarotherodon melanotheron* (male Sm ♂ and female Sm ♀) in intraspecific (On ♂ × On ♀; Sm ♂ × Sm ♀) and interspecific (On ♂ × Sm ♀; Sm ♂ × On ♀) pairings. The asterisks (\*) indicate a significant correlation ( $P < 0.05$ ).

TABLE 1

Pairing modes		Sound characteristics					
		Number of sounds analyzed	Sound Duration (ms)	Number of grunts	Grunt duration (ms)	Period of grunts (ms)	Fundamental frequency (Hz)
<i>O. niloticus</i> ♂ × <i>O. niloticus</i> ♀	♂	27a	2578.6 ± 1377.7a	6.1 ± 2.9a	148.3 ± 15.4a	489.0 ± 81.7a	566.9 ± 93.3a
	♀	23a	2367.5 ± 1476.4a	5.3 ± 2.4a	155.8 ± 13.2a	522.7 ± 34.6a	633.7 ± 133.9a

TABLE 2

Pairing modes		Sound characteristics					
		Number of sounds analyzed	Sound Duration (ms)	Number of grunts	Grunt duration (ms)	Period of grunts (ms)	Fundamental frequency (Hz)
<i>O. niloticus</i> ♂ × <i>O. niloticus</i> ♀	♂	38	4669.4 ± 3441.8a	8.1 ± 4.9a	161.7 ± 44.4a	586.2 ± 91.5a	386.1 ± 139.3b
	♀	29	5227.9 ± 3408.6a	11.4 ± 7.2a	157.6 ± 33.5a	509.3 ± 96.5a	367.9 ± 112.3b
<i>O. niloticus</i> ♂ × <i>S. melanotheron</i> ♀	♂	27	4042.4 ± 2352.9a	10.2 ± 6.1a	164.8 ± 51.1a	613.2 ± 127.9a	460.2 ± 103.7a
<i>S. melanotheron</i> ♂ × <i>O. niloticus</i> ♀	♀	41	4859.1 ± 2953.6a	9.1 ± 5.8a	190.2 ± 36.3a	595.6 ± 109.4a	453.9 ± 132.4a

TABLE 3

Pairing modes	specie/sex	N	11-kt-weight		E2-weight		11-kt-Number of sounds associated to aggressive behaviours		11-kt- Number of sounds associated to non-aggressive behaviours		E2- Number of sounds associated to aggressive behaviours		E2- Number of sounds associated to non-aggressive behaviours	
			Rs	p-value	Rs	p-value	Rs	p-value	Rs	p-value	Rs	p-value	Rs	p-value
On ♂ × On ♀	On ♂	10	-0.22	0.53			0.02	0.95	0.15	0.67				
	On ♀	10	0.35	0.33	-0.61	0.06	0.45	0.18	0.01	0.97	0.45	0.19	-0.07	0.85
On ♂ × Sm ♀	On ♂	10	-0.50	0.14			0.15	0.69	-0.58	0.08				
	Sm ♀	10	-0.04	0.91	-0.09	0.80	0.56	0.09	-0.43	0.21	0.42	0.22	0.41	0.23
Sm ♂ × On ♀	Sm ♂	10	-0.01	0.99			0.55	0.09	0.17	0.64				
	On ♀	10	0.08	0.83	-0.60	0.07	0.36	0.30	-0.26	0.47	0.18	0.61	-0.22	0.53
Sm ♂ × Sm ♀	Sm ♂	10	0.28	0.43			-0.07	0.85	-0.28	0.42				
	Sm ♀	10	-0.07	0.85	0.32	0.36	-0.31	0.37	-0.55	0.09	0.39	0.25	-0.02	0.96

TABLE 4

Pairing modes specie/sex		Dominance Index							
		Number of sounds associated to aggressive behaviours		Number of sounds associated to non-aggressive behaviour		11-kt		E2	
		Rs	p-value	Rs	p-value	Rs	p-value	Rs	p-value
On ♂ × On ♀	On ♂	0.84	0.00*	-0.15	0.67	0.15	0.69		
	On ♀	-0.47	0.16	0.29	0.42	-0.19	0.59	-0.03	0.93
On ♂ × Sm ♀	On ♂	0.81	0.00*	0.54	0.11	0.13	0.71		
	Sm ♀	-0.99	0.00*	-0.13	0.72	-0.56	0.09	-0.47	0.17
Sm ♂ × On ♀	Sm ♂	-0.88	0.00*	0.64	0.04*	-0.65	0.04*		
	On ♀	0.56	0.09	0.38	0.27	0.45	0.17	0.42	0.23
Sm ♂ × Sm ♀	Sm ♂	0.55	0.10	-0.24	0.51	0.25	0.48		
	Sm ♀	-0.51	0.13	-0.08	0.82	-0.04	0.92	-0.51	0.13