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Bile acids as putative social signals in Mozambique tilapia (Oreochromis mossambicus)

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ABSTRACT

Chemical cues provide potential mates with information about reproductive status and resource-holding potential. In the Mozambique tilapia (*Oreochromis mossambicus*), males can distinguish female reproductive status through chemical cues, and accessibility of males to females depends on their position in the hierarchy, determined in part by chemical cues. Here, we hypothesized that tilapia faecal cues are attractive to conspecifics once released into the water. C18 solid-phase extracts of faeces from dominant males and pre-ovulatory females evoked stronger olfactory epithelium electrical responses (EOG) than, respectively, subordinate males and post-spawning females. Mass spectrometry of the reverse-phase C18 high-performance liquid chromatography fractions of these extracts with highest EOG, identified by amino acids and bile acids. Faeces from pre-ovulatory females contain significantly higher concentrations of cholic acid (CA) and taurocholic acid (TCH) than both post-spawning females and males. A pool of amino acids had no effect on aggression or attraction in males. However, males were attracted to the scent of pre-ovulatory female faeces, as well as CA and TCH, when applied separately. This attraction was accompanied by increased digging behaviour compared to the odour of post-spawning females. CA and TCH exert their action through separate receptor mechanisms. These findings are consistent with a role for faeces – and bile acids therein – in chemical communication in this species, acting as an attractant for males to reproductive females.

1. Introduction

Teleost fish rely on conspecific chemical cues or pheromones released to the water for vital social functions such as kin recognition, parent-offspring interaction, dominance and sexual behaviours [1,2]. The compounds involved are generally small molecules such as amino acids, nucleotides, steroids, bile acids and prostaglandins, which include hormones or hormonal byproducts [3–5]. The pheromones may be a single compound, such 3keto petromyzonol sulfate released by sea lamprey (*Petromyzon marinus*) males or prostaglandin $F_{2\alpha}$ released by ovulating goldfish (*Carassius auratus*) females, or a mixture of compounds such as the panoply of bile acids released by sea lamprey larvae or the dominance pheromone in Mozambique tilapia, *Oreochromis mossambicus* [3,6,7]. Goldfish, which employ a promiscuous or polygynandrous mating system involving intense sperm competition, are a good example of a species using reproductive pheromones, steroids and prostaglandins for chemical communication. Female goldfish at the end of vitellogenesis release a pheromone mixture dominated by androstenedione (AD) that promotes agonistic behaviour among males. With the luteinizing hormone (LH) surge prior to oocyte final maturation, the ratio of 4-pregnen-17,20β-diol-3-one (17,20β-P) to AD increases and males start chasing conspecifics and spermiogenesis is stimulated mediated by LH. At ovulation, females produce $PGF_{2\alpha}$ which acts in the brain to trigger female sex behaviour and is released together with its metabolite 15keto-$PGF_{2\alpha}$ as a postovulatory pheromone stimulating both male spawning behaviours and additional LH increase [8,9]. The routes of release of these molecules depend mainly on the site of production and polarity of the compounds, some being released from special glands in the skin, or through the gills, urine, semen and, possibly, faeces [10–19].

The first evidence of faeces as a source of chemical cues, comes from coho salmon, *Oncorhynchus kisutch*, being attracted by conspecific faeces [20]. Furthermore, ovulated and non-ovulated Mozambique tilapia females, have different olfactory sensitivity to faeces, [21] and chameleon cichlid, *Australoheros facetus,* had different sensitivity to intestinal fluid from dominant and subordinate males [15]. Intestinal fluid has been

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shown to contain, among other compounds, amino acids [22] and bile acids [23,24], both of which have been implicated in chemical communication [25].

The Mozambique tilapia, hereafter 'tilapia', is a maternal mouth-brooding African cichlid in which males congregate in "leks" where they establish a social hierarchy and dig and aggressively defend pits ("nests"). Ripe females then visit these leks and choose one or more males with which to spawn [26–28]. Territorial males release pulses of urine as a vehicle for pheromones when they encounter conspecifics [10,29]. The urine contains a pheromone composed of two epimeric steroids (20α- and 20β-pregnanetriol 3-glucuronate), which primes the female reproductive axis to produce the maturation-inducing steroid 17,20β-P [2]. The urine also contains a pheromone that signals dominance and lowers conspecific aggressiveness, although its chemical identity is still unknown [7,10,29–31]. Furthermore, it has been shown that other excretory products of tilapia, such as faeces, might be used as vehicle to carry putative pheromonal compounds that can be discerned by olfactory epithelium and convey information about the donor's physiological status [21]. As males seek to attract the best females to their nests, faeces could convey information about their feeding/nutritional and reproductive status. The nutritional status of females is important because they do not feed while mouth brooding [10].

Given the high olfactory potency of tilapia faeces [21] and the paucity of information available, the present study was conducted to investigate their possible involvement in chemical communication with the following three objectives: first, to isolate the putative pheromonal compound(s) present in the faeces from males and females; second, to assess the olfactory sensitivity of tilapia to the identified compound(s); and third, to determine their behavioural effect(s).

2. Materials and methods

2.1. Establishment of social hierarchies and collection of samples

Animal experiments were carried out under license 0421/000/000/2020 issued by the Directorate-General for Food and Veterinary of Portugal, following Directive 2010/63/EU on the protection of animals used for scientific purposes.

Sexually mature tilapia were maintained in mixed-sex groups (2 males and 4 females) in 300 L tanks with a glass front, sand substratum and under-gravel filters, at 27 °C and 12 h light:12 h dark photoperiod. Fish were fed once a day with a commercial cichlid diet (Sparos Lda., Olhão, Portugal). Individual fish were tagged using coloured plastic labels (T-Bar anchor FD94, Floy Tag, Seattle, WA, USA) attached to the muscle near the dorsal fin. Seven days after the establishment of the mixed-sex groups, males in each tank were observed daily to access their social rank. The frequency of submissive behaviours (escape, flight, and light coloration) and dominance behaviours (biting, chasing, circling, digging, courtship with females and dark coloration) were recorded. For each male, a daily dominance index was calculated as the ratio of the sum of dominance behaviours over the total number of dominant and subordinate behaviours [32]. Reproductive cycle length of individual females was determined by recording their spawning date over a three-month period. After each spawning, the fertilized eggs were removed from the mother's mouth to trigger a new ovulation cycle. Only regularly cycling females, with a mean cycle length of 17 days (mean ± standard deviation; 17.0 ± 5.1) were used. Females were considered 'pre-ovulatory' on the day prior to their predicted ovulation (i.e., just before spawning). Females that passed two days since their last spawning were considered as 'post-spawning' [21]. Fish were wrapped in a thin wet towel to cover the urogenital pore and avoid contamination of faeces samples with urine. Faeces were obtained by gently squeezing the abdomen anterior to the anus of dominant males (dominance index I_D: 1; standard length L_S: 14.2 ± 0.6 cm; weight: 56.9 ± 2.2 g; $n = 10$; mass of collected faeces = 0.25 ± 0.03 g per male), subordinate males (I_D: 0; L_S: 13.2 ± 0.7 cm; weight: 52.5 ± 2.3 g; $n = 10$; mass of collected faeces = 0.24 ± 0.04 g per male), pre-ovulatory females (L_S: 13.0 ± 0.5 cm; weight: 56.0 ± 2.8 g; $n = 7$; mass of collected faeces = 0.13 ± 0.02 g per female), and post-spawning females (L_S: 12.5 ± 0.6 cm; weight: 51.9 ± 3.4 g; $n = 7$; mass of collected faeces = 0.12 ± 0.02 g per female). Faeces were stored at −20 °C until use.

2.2. Sample fractionation

Faeces were thawed at room temperature and weighed. A 1:1 (w/v) ratio of distilled water was added, vortexed thoroughly and centrifuged at 10,000 rpm for 5 min. The supernatant was collected and ran through a C18 solid phase extraction (SPE) cartridge (Waters Sep-Pak™, Milford, U.S.A) following the manufacturers' instructions. The methanol eluate was separated using a HPLC system with UV detector (Model D-14,163, Smartline KNAUER, Berlin, Germany). The HPLC column was a silica based C18 column (Luna™ omega 3 μm polar. 150 × 2.1 mm, Phenomenex Inc, Portugal). The mobile phase was methanol (HPLC grade) and distilled water containing 0.001 % formic acid, at 0.6 ml.min^{-1} flow rate and 24 °C over 30 min. The gradient profile was isocratic from 0 to 4 min at 15 % methanol, followed by a linear gradient from 5 to 25 min at 15 % to 100 % methanol and isocratic from 25 to 30 min at 100 % methanol. Fractions were collected every two minutes and immediately stored at −20 °C.

2.3. Chemical identification

The extracts and fractions were analyzed by liquid chromatography-high resolution mass spectrometry (LC—HRMS) following similar procedures as described by Silva et al. [33]. Untargeted analysis was performed for raw extracts and targeted analysis of steroids applied to both, raw extracts, and fractions. The LC—HRMS system was a Thermo Scientific™ UltiMate™ 3000 UHPLC, coupled to an Orbitrap Elite (Thermo Scientific, Waltham, MA, USA) mass spectrometer with a Heated Electro-Spray Ionization source (HESI-II; Thermo Scientific, Waltham, MA, USA). A Thermo Scientific Accucore RP-18 column (2.1 × 100 mm, 2.6 μm) and a mobile phase composed of water (A) and acetonitrile (B), both with 0.1 % formic acid, were used for both untargeted and targeted analysis. In the former, the gradient (in *v/v* %) started with 100 % for two minutes. The ratio of B/A increased linearly to 30 % B over 13 min, then to 100 % B over 16 min, and then stayed at 100 % B for four minutes. The mobile phase then returned to 100 % A for 1 min and the column was stabilized at 100 % A for four minutes before the next run. Separation was performed at a flow of 0.3 ml/min.

Targeted analysis was performed in the same conditions but using a flow of 0.5 ml/min and the following gradient composition: started with 20 % B and then increase to 100 % B over three minutes, remained at 100 % B for two minutes and then returned to the initial composition in 20 % B during 2 min. This mobile phase composition was then stabilized for 3.5 min before the next run. The first fractions obtained after fractionation of raw extracts were also analyzed using an HILIC column to separate the polar compounds. The column was a ACQUITY Premier BEH Amide (2.1 × 100 mm, 1.7 μm) column (Waters, USA) at 35 °C. The mobile phase was composed of water with 0.1 % formic acid and 10 mM ammonium formate (A) and acetonitrile with 0.1 % formic acid and 2 % 10 mM ammonium formate solution (B). The gradient (in *v/v* %) started with 5 % B and increased linearly to 95 % over 11 min. This composition was maintained for 1 min and then returned to 5 % B over one minute and remained at this composition for two minutes before the next run [34]. The flow rate was 0.3 ml/min and the injection volume was 10 μL.

Data were acquired under positive and negative polarity (separate runs) using the following parameters: spray voltage, 3.8 kV; sheath gas, 40 arbitrary units; auxiliary gas, five arbitrary units; heater temperature, 300 °C; capillary temperature, 350 °C; S-Lenses RF level, 64.9 %. The untargeted analysis was performed in data-dependent mode by selecting the three most intense ions under dynamic exclusion and collision-induced dissociation (CID) activation. The scan range was 100–1500

m/z. Targeted analysis of cholic acid (CA) and taurocholic acid (TCH) were performed under MRM using the following CID transitions: CA – MS2 (407→342–347); TCH – MS2 (514→352–354, 411–413, 495–497). Scan range was 100–600 *m/z*.

LC-MS data analysis was performed using Xcalibur v4.1 Qual Browser (Thermo Scientific). LC-MS profiles were also processed using Compound Discoverer 3.3 (Thermo Scientific, Waltham, MA, USA). Profiles were processed using the Max ID workflow. Compound annotation was based on the mzCloud results. Identification of CA and TCH was confirmed by comparison to authentic standards.

Quantification was performed by preparing calibration curves from peaks of chromatograms obtained under targeted analysis, after injection of concentrations ranging from 0.01 to 1 μM. When necessary, samples were diluted so that the concentration of targeted molecules fell within the range of the calibration curve. The limits of detection were ~ 1 ng/ml.

2.4. Electro-olfactogram

The electro-olfactogram (EOG) response to C18-SPE and HPLC fractions, as well as non-treated faeces samples, were recorded as previously described [35]. Briefly, male tilapia were anesthetized with 100 mg/L MS-222 (3-aminobenzoic acid ethyl ester, Sigma-Aldrich) and placed in a padded V-clamp. Aerated water containing 50 mg/L 3-aminobenzoic acid ethyl ester (MS222) was pumped over the gills. The right olfactory rosette was exposed by removing the skin surrounding the nostril. The recording electrode was positioned close to the centre of the olfactory rosette, and the reference electrode positioned lightly on the skin outside the nostril. The perfusion tube for delivering the stimuli was placed over the olfactory epithelium. The stimulus solutions were fed, *via* gravity, through a three-way solenoid valve (in 4 s pulses). The EOG signal was pre-amplified using a DC pre-amplifier and head-stage (NL102, Digitimer Ltd, UK) and filtered above 50 Hz (NL125, Digitimer Ltd), amplified (x100; NL106, Digitimer Ltd), digitized (DigiData 1440A, Molecular Devices, San Jose, USA) and recorded on a PC running Axoscope v10.6 (Molecular Devices). C18-SPE methanol fractions were diluted 1:1000, and HPLC fractions diluted 1:100, in dechlorinated tap-water prior to recording EOG. The olfactory responses of tilapia to the bile acids (5α-cyprinol sulphate, cholic acid, taurocholic acid taurodeoxycholic acid and chenodeoxycholic acid) were recorded to generate the concentration–response curves. Briefly, the olfactory epithelium of tilapia was exposed to an increasing concentration of each bile acid from 10^{-10} M to 10^{-5} M with 60 s intervals to ensure EOG return to a baseline state before the next exposure. The EOG responses to L-serine at 10^{-5} M were used to normalize EOG responses of test stimuli.

2.5. EOG cross-adaptation and binary-mixture tests

EOG cross-adaptation tests and binary mixture tests were used to assess whether bile acids are detected by the same receptor(s). In the cross-adaptation tests, the response amplitude of one bile acid ('test' stimulus) is recorded before and during adaptation to a second bile acid ('adapting' stimulus). If the test stimulus shares a common receptor with the adapting stimulus, the response to the test stimulus during adaptation will be reduced. The bile acids were purchased from Sigma Aldrich Chemical Co. (Madrid, Spain), except for 5α-cyprinol sulphate (CYP-S) which was a gift from A.F. Hofmann and L.R. Hagey (University of California, U.S.A.). The cyprinol sulphate has been used in the present study as it has sulfated conjugation in its chemical structure. Therefore, it was a good indicator to see whether cholic acid (i.e., as a free bile acid) and taurocholic acid (i.e., with taurine conjugation) acting via a shared or independent olfactory receptors. The adapting bile acid (1×10^{-7} M for CYP-S, 10^{-6} M for other bile acids) was perfused for 30 s over the olfactory epithelium until the voltage stabilized. Next, the response to the adapting bile acid (10^{-6} M adapting bile acid in 10^{-6} M adapting bile acid) was recorded (cross adaptation blank). Test solutions were then administrated as 4 s pulses, beginning with adapting bile acid (the self-adapted control at 2×10^{-7} M for CYP-S and 2×10^{-6} M for the other bile acids). The amino acid L-serine at 10^{-5} M was used as a control as it is understood amino acids act via different olfactory receptors [36]. The EOG responses to the bile acids before adaptation were blank subtracted using the response to the blank water (the water used to dilute the stimulus). EOG responses to the test stimulus during adaptation were then blank-subtracted using the response to the 1×10^{-6} M (1×10^{-7} M for CYP-S) adapting bile acid blank. Finally, EOG responses to the test bile acids during adaptation were converted to a percentage of the initial (unadapted) response (%R_1).

In the binary mixture tests, if the two odorants act through independent olfactory mechanisms, the EOG response to a mixture of the two odorants is expected to be close to the sum of EOG responses of the individual odorants. In contrast, the EOG response to the two odorants is expected to be much smaller than the sum of the individual responses if they are detected through a shared olfactory mechanism. The EOG binary mixture test in the present study started by recording the EOG responses to the bile acids at the same concentration as those used in the cross-adaptation tests. In the beginning, the EOG responses to bile acids A and B were recorded and their respective EOG amplitudes R_A and R_B were obtained. Then EOG response to bile acids A and B were recorded at twice the concentration. Finally, olfactory epithelium was exposed to a mixture of A and B to produce R_{A+B} response.

The amplitude of responses to these binary mixture tests were used to calculate an independent component index (I_{CI}) and a mixture discrimination index (I_{MD}). The I_{CI} (Eq. (1)) of two odorants that are detected through independent receptor mechanism is expected to be around 1 and in contrast, the I_{CI} is smaller than 1. The I_{MD} (Eq. (2)) is predicted to be around 1 in the case of a shared receptor mechanism, and greater than 1 (approximately 1.5) if there is receptor independence [37, 38].

$$I_{CI} = \frac{R_{A+B}}{(R_A + R_B)} \tag{1}$$

$$I_{MD} = \frac{R_{A+B}}{0{\cdot}5(R_{2A} + R_{2B})} \tag{2}$$

2.6. Mirror assay

The mirror assay was conducted to test the behavioural effect(s) of the HPLC fractions that showed differences in EOG responses between dominant and subordinate males (fractions 2 and 9), and between pre-ovulatory and post-spawning females (fractions 2, 14, and 15) [7]. Briefly, focal males were randomly chosen from the mixed-sex tanks and transferred to the experimental aquaria where they were maintained isolated for five days until the experiments. A camera was set in front of the 30 L aquarium to register the behaviour and aeration valves were closed to avoid possible air bubble disturbance of a clear mirror image to focal males. A peristaltic pump was used to apply the odorant at a flow rate of 40 ml/min. On the day of experiment, 1 ml of each stimulus separately was diluted 1:100 *v/v* in distilled water, and randomly assigned to each aquarium. An opaque plate covering the mirror was lifted, and immediately after the first reaction of the focal male to its own image, the stimulus was applied in five 30 s pulses, separated by 30 s intervals followed by a five-minute interval without stimulus. After each trial, the fish were transferred back to the stock tanks and the assay system was washed with hot water (>80 ºC). The recorded videos were viewed with KMplayer 64X v. 2020,06.9.40 (www.kmplayer.com). For each fish were measured: 1) latency, defined as the time from the removal of the opaque plate until the first reaction of the focal male to his mirror image, and 2) number of bites performed against the mirror for 5 min. The males that did not react to their mirror image after 15 min were removed from analysis. Of the 69 males exposed to their mirror image, 60 % reacted to it.

2.7. Preference experiments

The setup for the preference experiments is depicted in Fig. 1. The preference tank (56 × 40 × 40 cm; ca. 90 l) was divided lengthwise by glass into three sections. The central compartment (neutral zone) had an S-shaped design to each lateral compartment (preference zone). Neutral and preference zones had aeration, a sandy substrate, and water kept at 27 °C. The plastic tubes of a multi-channel peristaltic pump were fixed approximately 2 cm below the water surface, on the corner of each preference zone to inject different stimuli simultaneously. A food colorant was used to estimate the time that the stimuli take to reach the neutral zone (approximately 30 min with aeration inside the neutral zone). Accordingly, the flow rate of the peristatic pump was set at 10 ml. min^{-1}.

In the first preference experiment dominant males were individually exposed to (1) conditioned water of pre-ovulatory female versus dechlorinated tap-water as control, (2) conditioned water of post-spawning female versus tap-water control, (3) feaces extract of pre-ovulatory females versus tap-water control, (4) 10^{-3} M CA versus tap-water control, and (5) 10^{-5} M TCH versus tap-water control. The conditioned waters from pre-ovulatory females and post-spawning females were collected according to Miranda et al. [21]. Female tilapia (pre-ovulatory and post-spawning) were isolated individually in 20 l of dechlorinated tap-water for two hours, before collection of faeces by gently squeezing the abdomen. Faeces were weighed (0.35 ± 0.04; $n = 7$) and vortexed after adding distilled water (1:1 w/*v*). After centrifugation, the supernatant was collected and used in the experiment. The stock solutions of CA and TCH in methanol were prepared to reflect the concentration of bile acids in the faeces of females at 10^{-1} M and 10^{-3} M, respectively, and 1.5 ml aliquots were stored at −20 °C. Before the preference trial with bile acids, an aliquot was diluted 1:100 *v/v* in distilled water. The concentrations of CA and TCH in the preference zones after 15 min of injection were estimated at 10^{-6} M and10^{-8} M, respectively.

In the second experiment, the preference of pre-ovulatory females and dominant males was assessed when exposed to dechlorinated tap-water (control) versus a mixture of amino acids identified in HPLC fraction 2 from dominant males and pre-ovulatory females. The stock solution consisted of a pool of 10^{-3} M serine, phenylalanine, arginine, threonine, proline, valine, methionine, asparagine, tryptophan, and leucine in distilled water, divided into 1.5 ml aliquots. Before the preference experiment, the aliquots were thawed, and diluted 1:100 *v/v* in distilled water.

Each set of experiments consisted of a 15 min stimulus injection period followed by 25 min post injection. The time (s) spent by focal fish in each preference and neutral zone was calculated over 40 min. Furthermore, the number of nest-digging behaviours (a common courtship behaviour in tilapia; [30,39], was also quantified.

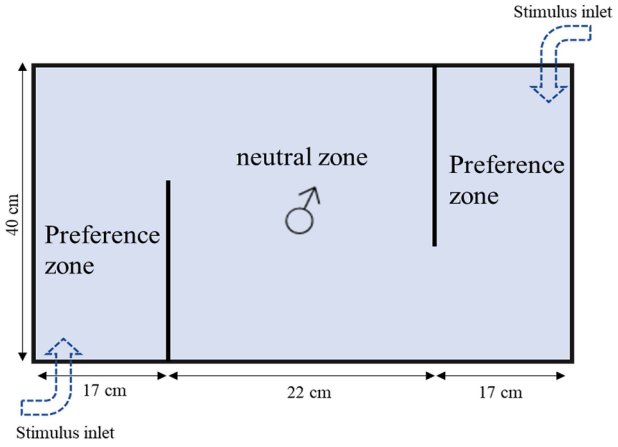

Fig. 1. Preference test aquarium design. Males were housed for 24 h in the test aquarium prior to testing. During testing, males were placed in the neutral zone and exposed to different stimuli that were injected simultaneously through the stimulus inlet into each preference zone. Each test consisted of a 15 min stimulus-delivery period followed by 25 min observation.

2.8. Statistical analysis

A two-way analysis of variance (ANOVA) was used to compare the EOG amplitude in response to SPE and HPLC fractions normalized using the square root transformation, with social rank and fraction as factors for males, and ovulation status and fractions as factors for females, followed by the Holm-Sidak *post hoc* test. The two-tailed Students *t*-test was used to compare log-transformed faecal bile acid concentrations according to social status (males) and ovulation status (females). The threshold of detection of bile acids was assessed by linear regression of log-transformed data and calculating the intercept on the *x*-axis [40]. One-way ANOVA was used to compare the thresholds of detection of bile acids, followed by the Holm-Sidak post hoc test. One-way ANOVA was used to compare the cross-adaptation measurements, followed by the Holm-Sidak multiple comparisons versus SAC as the control group. One-way ANOVA was used to compare the independent component and mixture discrimination indices of binary mixture tests, followed by the Holm-Sidak multiple comparisons versus the combination of cholic acid and taurocholic acid as the control group. The effects of the different stimuli on the number of bites and digs was determined using one-way ANOVA followed by the Holm-Sidak post hoc test. Student's *t*-test was used to compare the time the males and females spent in each preference zone. The statistical analysis was conducted using SigmaPlot 14.0 (Systat Software, Inc., San Jose, CA, USA) and data is presented as mean ± standard error of the means (S.E.M.).

3. Results

3.1. Olfactory responses of males to faeces and their fractions

The amplitudes of EOG responses to untreated faeces showed no significant differences between dominant and subordinate males, as well as pre-ovulatory and post-spawning females. (Fig. 2a,b). However, the EOG amplitude in response to C18-SPE methanol eluate or filtrate fractions of dominant male faeces was higher than the corresponding fractions of subordinate males. In contrast, only the C18-SPE methanol eluate, not the filtrate, from pre-spawning females evoked a higher EOG amplitude in males compared to the corresponding extracts from post-spawning females.

HPLC fractions 2 and 9 of the C18-SPE methanol eluate from dominant males evoked higher EOG amplitudes in males compared to the corresponding fractions from subordinate males (Fig. 3a). In females, HPLC fractions 2, 14, and 15 of the C18-SPE methanol eluate from pre-ovulatory females evoked significantly higher EOG amplitudes than those from post-spawning females (Fig. 3b).

3.2. Compounds identified in HPLC fractions

LC–MS analyses of HPLC fraction 2 from dominant males and pre-ovulatory females putatively identified the amino acids listed in Table 1.

LC–MS analyses detected CA and TCH in HPLC fractions 9 (male extracts) and 14–15 (mainly 15, females) and identities were confirmed against authentic standards. Quantification by LC-MS revealed higher CA concentrations in raw faeces of dominant and pre-spawning females compared, respectively, to subordinate males (two-tailed Students *t*-test, $n = 10$, $p = 0.03$; Fig. 4a) and post-spawning females (two-tailed Students *t*-test, $n = 7$, $p < 0.001$, Fig. 4c). The same samples contained also higher concentrations of TCH in dominant males and pre-spawning females compared, respectively, to subordinate males (two-tailed Students *t*-test, $n = 10$, $p < 0.001$; Fig. 4b) and post-spawning females (two-tailed Students *t*-test, $n = 7$, $p < 0.001$, Fig. 4d).

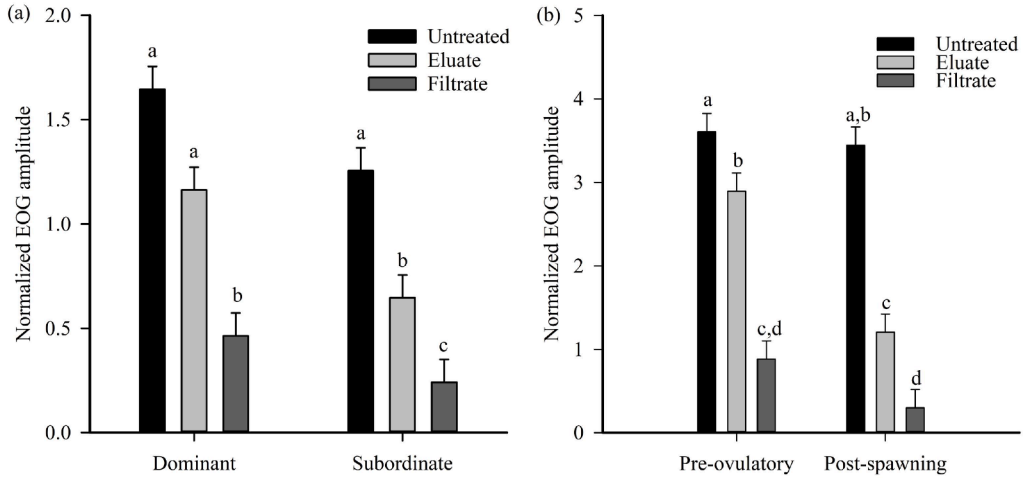

Fig. 2. Normalized EOG amplitude in response to untreated (raw) faeces and respective C18-SPE fractions from (a) males ($n = 10$) and (b) females ($n = 7$). EOG responses were obtained from males ($n = 7$; L_S: 11.8 ± 0.5 cm; weight: 46.4 ± 5.4 g) Different letters indicate significant differences ($p < 0.05$) within the same social (dominant and subordinate males) and reproductive (pre-ovulatory and post-spawning females) status.

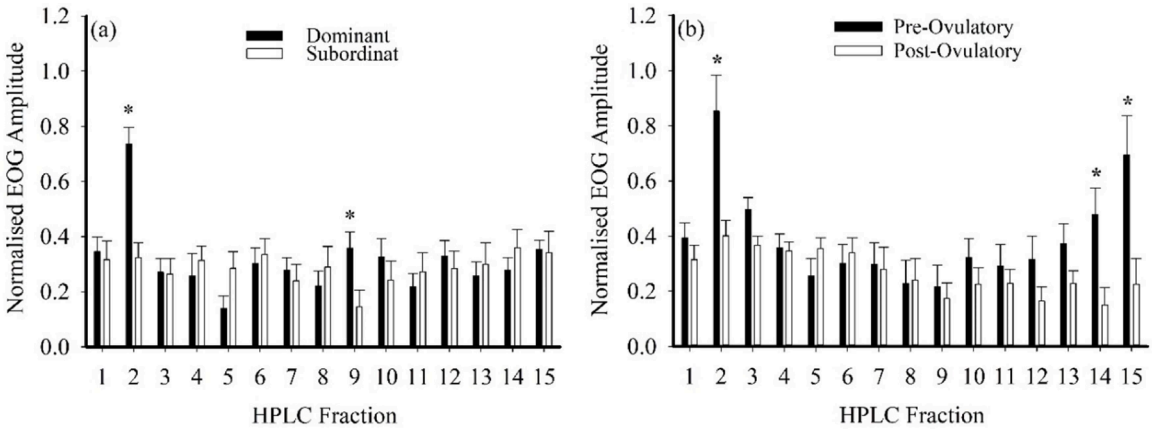

Fig. 3. Normalized EOG responses of tilapia olfactory epithelia to HPLC fractions of the C18-SPE eluate from (a) males ($n = 10$) and (b) females ($n = 7$). EOG responses were obtained were obtained from males (for male faeces $n = 8$; L_S: 10.9 ± 0.3; weight: 42.0 ± 2.4 g; for females faeces $n = 6$; L_S: 12.6 ± 0.5 cm; weight: 45.3 ± 6.2 g) The asterisk (*) indicates statistical difference ($p < 0.05$) between fractions of different social (dominant and subordinate males) and reproductive (pre-ovulatory and post-spawning females) status.

Table 1
Compounds annotated in fraction 2 showing mzCloud match ≥ 85 %.

Compound	Formula	*m/z*	ion	mzCloud match	EOG sensitivity
Choline	$C_5H_{13}NO$	104.107	$[M+H]^{+1}$	99.8	n.s.
Creatine	$C_4H_9N_3O_2$	132.077	$[M+H]^{+1}$	90.9	n.s.
Betaine	$C_5H_{11}NO_2$	118.086	$[M+H]^{+1}$	97.6	n.s.
Serine	$C_3H_7NO_3$	106.050	$[M+H]^{+1}$	92.2	10^{-7}*
Proline	$C_5H_9NO_2$	116.071	$[M+H]^{+1}$	95.3	10^{-6}*
Glutamine	$C_5H_{10}N_2O_3$	147.077	$[M+H]^{+1}$	88.4	10^{-7}*
Histidine	$C_6H_9N_3O_2$	156.077	$[M+H]^{+1}$	98.8	10^{-7}*
Arginine	$C_6H_{14}N_4O_2$	175.119	$[M+H]^{+1}$	99	10^{-8}*

Note: n.s. EOG responses not significantly different from blank water at 10^{-4} M.
* threshold values from [41].

3.3. Olfactory potency of identified compounds

The EOG responses to identified compounds of fraction 2 from dominant males and pre-ovulatory females (Table 1) showed that the olfactory epithelium of tilapia was insensitive to choline, creatine and betaine except for amino acids, as reported by Kutsyna et al. [41].

The olfactory epithelium of tilapia had sensitivity to CA and TCH with calculated threshold of detection of $10^{-9.26\pm0.10}$ M and $10^{-9.38\pm0.05}$ M, respectively (Fig. 5). Also, tilapia responded to taurodeoxycholic acid (TDC), chenodeoxycholic acid (CDCA), and CYP-S with a threshold of detection of $10^{-10.12\pm0.14}$ M, $10^{-9.71\pm0.11}$ M, and $10^{-10.34\pm0.20}$ M, respectively. There were no significant differences in the threshold of detection between CA and TCH (one-way ANOVA, $F_{4,25} = 10.446$, $P = <0.001$).

3.4. Cross-adaptation tests

Cross-adaptation tests showed that CYP-S and L-Ser act via independent receptor mechanisms (Fig. 6). Furthermore, adaptation to TCH did not reduce the EOG responses to CA, suggesting that these bile acids are acting through separate receptor mechanisms. However, the two taurine-conjugated bile acids, TDC and TCH, showed a strong

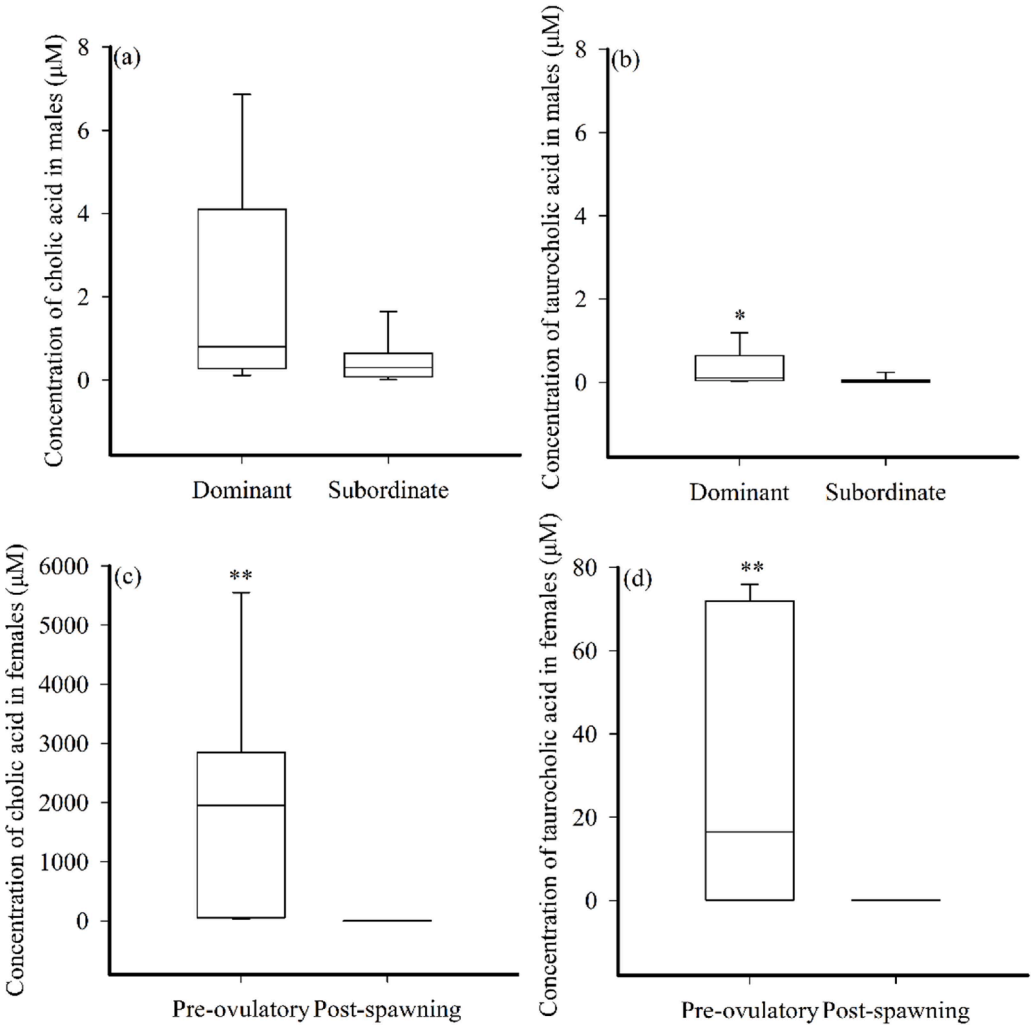

Fig. 4. Boxplot of the concentration of cholic acid and taurocholic acid (μM) in faeces from males (a and b, $n = 10$) and females (c and d, $n = 7$). The upper and lower edges of the box indicate the 25th and 75th percentile of the data set, the line in the box is the median. The asterisk (*) indicates statistical difference between different social and ovulation status. *, $p < 0.01$; **, $p < 0.001$.

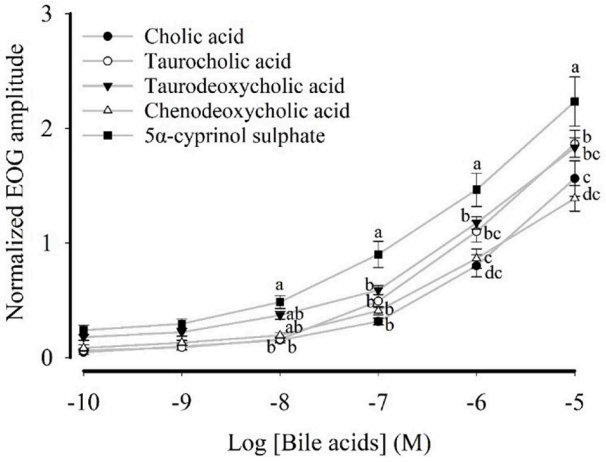

Fig. 5. Normalized EOG concentration–response curves to different concentrations of cholic acid, taurocholic acid, taurodeoxycholic acid, chenodeoxycholic acid, and 5α-cyprinol sulphate. Different letters indicate significant differences at each concentration ($p < 0.05$).

reciprocity in their capacity to reduce each-others' EOG responses to a point that they were not significantly different from the self-adapted control (SAC). This suggests that they are acting via (a) common receptor(s).

3.5. Binary mixture tests

The binary mixture tests showed similar results to the cross-adaptation tests (Fig. 7). The I_{CI} and I_{MD} of the binary mixture of TCH and TDC were around the expected values 0.5 and 1, respectively, when the components of a mixture are detected by a shared receptor. The mean I_{CI} and I_{MD} values for CA mixed with CDCA exceeded the expected values of 0.5 and 1, respectively. Furthermore, the I_{CI} and I_{MD} of CA mixed with TCH were close to 1 (I_{CI}) or 1.5 (I_{MD}) and significantly different from the mixture of CA+CDCA and TCH+TDC, confirming our hypothesis that TCH and CA are detected through independent receptors.

3.6. Behavioural responses

Fractions 2 and 9 from male faeces had no significant effects on the biting behaviour of males towards the mirror image in comparison to the water control (one-way ANOVA, $F_{2,15} = 0.536$, $p = 0.596$). Similarly, fractions 2, 14, and 15 from female faeces had no significant aggression-

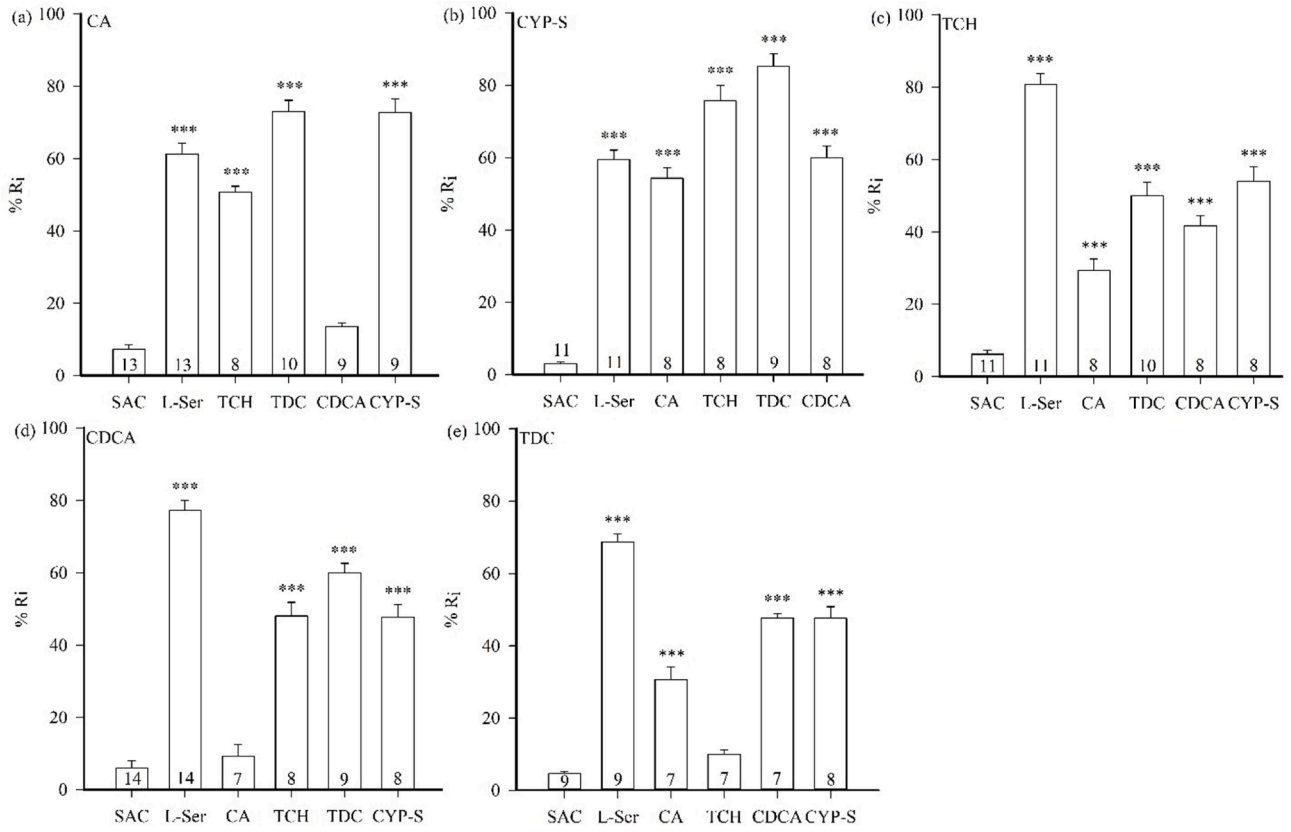

Fig. 6. Results of EOG cross-adaptation experiment expressed as a percentage of the initial unadapted response (% Ri) to the same bile acid delivered before cross-adaptation. Numbers in bars denote the number if independent replicates. The asterisks (***) indicate significant difference from the self-adapted control SAC ($p < 0.001$).

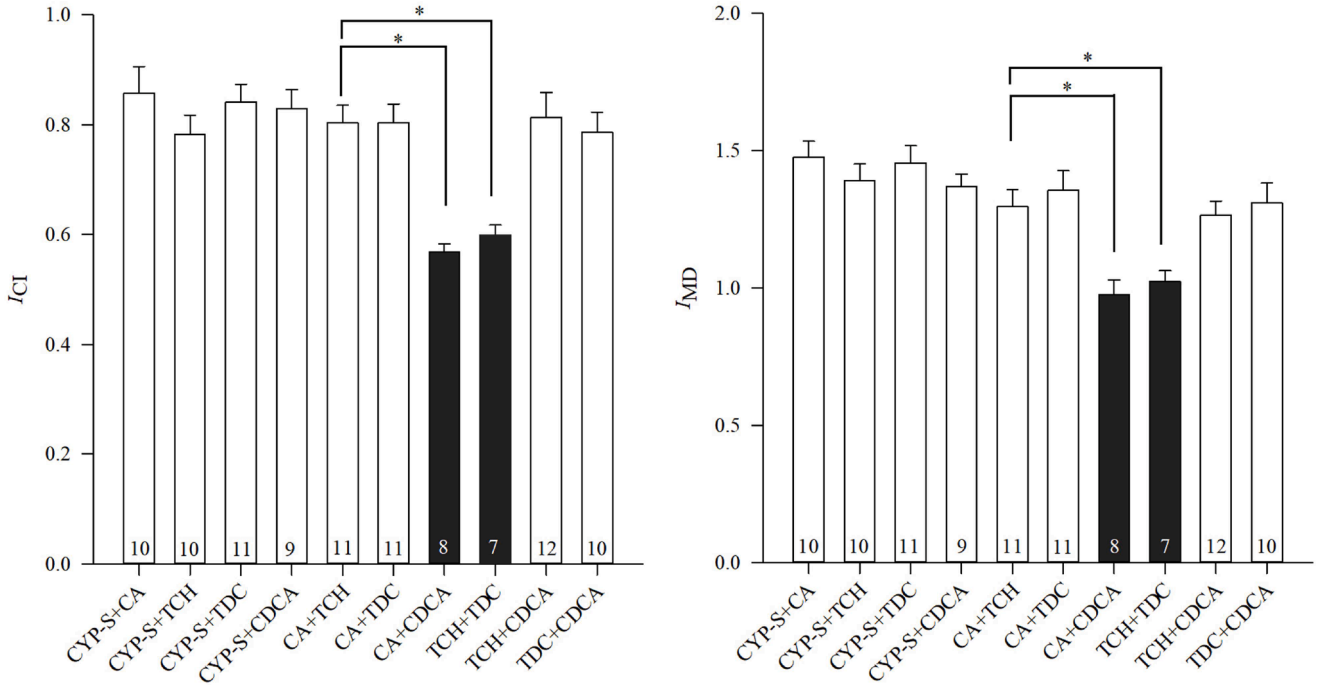

Fig. 7. Independent component and mixture discrimination indices from binary mixture test. Open bars indicate an interaction with a shared receptor mechanism. Black bars indicate interaction with an independent receptor mechanism. Numbers in bars denote sample size. The asterisk (*) indicates a significant difference from the combination of cholic acid and taurocholic acid ($p < 0.01$).

reduction effects on males (one-way ANOVA, F 3,20 = 0.394, p = 0.759).

In the preference test, dominant males showed a preference for the zones receiving pre-ovulatory female-conditioned water over control water and post-spawning females conditioned water (Fig. 8a–c). Furthermore, males also preferred the compartment receiving the female faeces extract compared to the control water compartment. Although, males had no preference towards a mixture of amino acids (Fig. 9), they preferred the zones receiving CA or TCH compared to those receiving water control (Fig. 8c,d).

Quantification of digging behaviour (nest-digging) during the preference experiment was significantly higher in the zone receiving pre-ovulatory conditioned water and faeces, as well as CA and TCH in comparison to the zone receiving post-spawning conditioned water (Fig. 10). However, focal males showed no digging behaviour towards the mixture of amino acids.

4. Discussion

The present study shows that tilapia has high olfactory sensitivity towards conspecific faeces and faeces components extracted by SPE-C18, which include amino acids and bile acids. The lower EOG response to the non-retained SPE-C18 filtrate indicates that either tilapia releases a low amount of polar compounds via their faeces and/or do not have olfactory sensitivity to them. On the other hand, the high EOG responses to the SPE-C18 methanol eluate is consistent with the relative levels of bile acids contained in faeces fractions in males and females. The behavioural preference of male tilapia for water conditioned by pre-ovulatory females, their faeces, and the two bile acids contained therein is suggestive of a possible pheromonal role for CA and TCH carried by faeces related to reproduction.

Dominant males and pre-ovulatory females release faeces with higher olfactory potency than subordinates and post-spawned females, respectively, because of the higher content of bile acids and amino acids. Based on the mirror assay, none of the faeces fractions from males and females, nor the amino acids or bile acids tested had aggression-reduction effect on focal males. This supports the hypothesis that the urine of dominant males is solely responsible for reducing aggression during interactions between rival males [7,42]. In contrast, the fact that pre-ovulatory female faeces, and in particular the bile acids CA and TCH identified in the faeces, attract males to a similar extent as pre-ovulatory female conditioned water and significantly more than post-spawning female conditioned water, suggest these bile acids may be part of a chemical bouquet related to reproductive behaviour prior to spawning. During mouthbrooding, Mozambique tilapia females do not feed and consequently produce less faeces than pre-ovulatory females [43]. Since the concentration of bile acids in the intestinal fluid is affected by diet, the higher levels of bile acids in the faeces of pre-ovulatory females may be perceived by males as indicative of female health status and reproductive potential. However, the higher concentrations of bile acids in the faeces of pre-ovulatory females cannot be solely linked to feeding, as males have lower levels of bile acids in their faeces and typically have higher food consumption rates [44]. Studies in mammals reported that the concentration of faecal bile acids among males and females can differ [45,46] and physiological factors such as hormonal changes can contribute to variation in excreted levels of bile acids between the sexes [47]. How bile acid release would be regulated is not yet clear; some evidence suggests that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) may restrict bile acid release [48], but whether there is a specific interaction with the reproductive system is not known. Interestingly, it has been shown recently

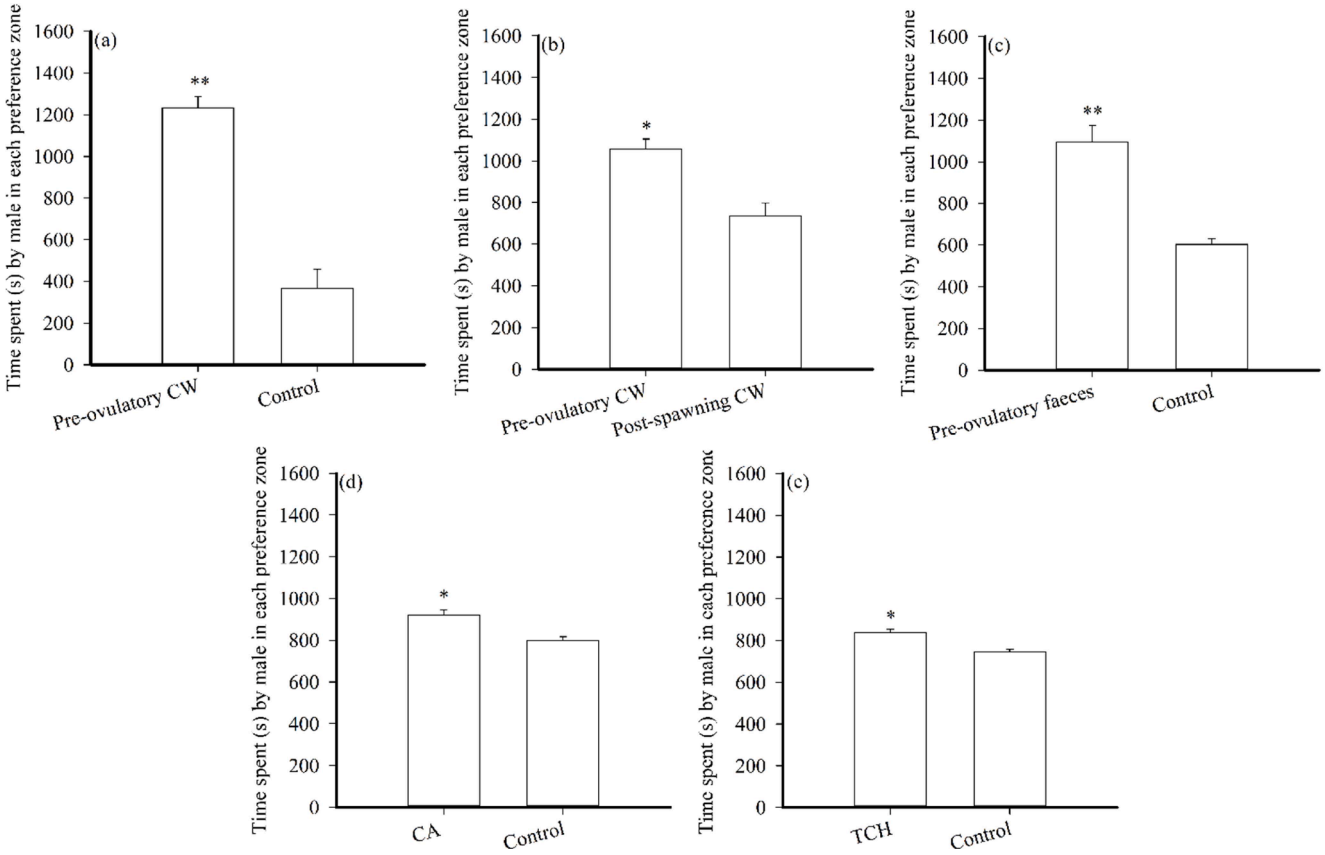

Fig. 8. The preference responses of focal males to: (a) conditioned water of pre-ovulatory female versus control; (b) conditioned water of post-spawning female versus control; (c) faeces extract of pre-ovulatory females versus control; (d) 10^{-3} M CA versus control; (e) 10^{-5} M TCH versus control. The asterisk (*) indicates statistical difference between different preference zones: * $p < 0.01$; ** $p < 0.001$.

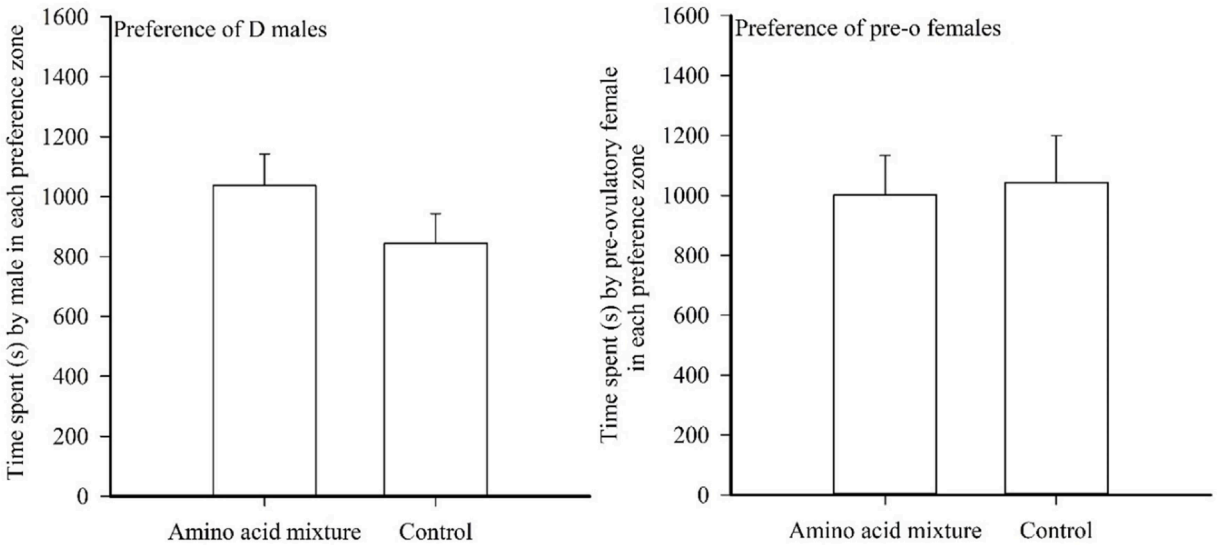

Fig. 9. The preference responses of dominant males ($n = 9$) and pre-ovulatory females ($n = 9$) to the mixture of amino acids.

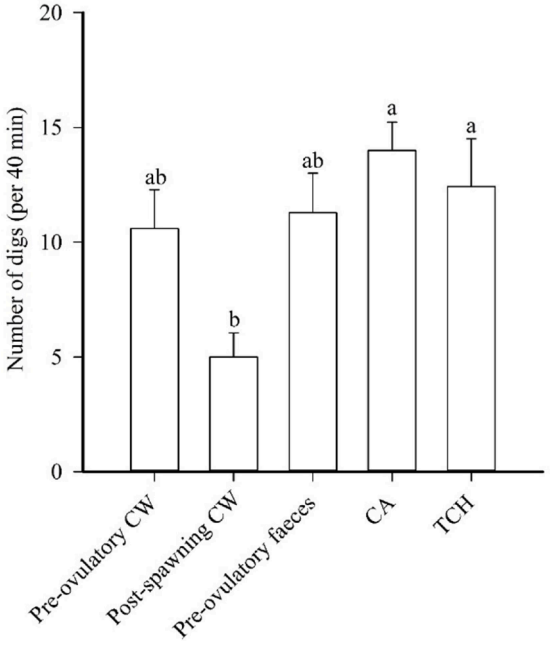

Fig. 10. The number of digging behaviours in focal males exposed to pre-ovulatory female-conditioned water ($n = 5$), post-spawning conditioned water ($n = 5$), pre-ovulatory faeces extract ($n = 7$), 10^{-1} M CA ($n = 7$), and 10^{-3} M TCH ($n = 7$) over 40 min of preference experiment. Different letters indicate a significant difference ($p < 0.05$).

that despite lacking sensitivity to $PGF_{2\alpha}$, *A. burtoni* males are attracted by females injected with $PGF_{2\alpha}$ and by fertile females, and this preference could be abolished by injection of PGF synthesis inhibitor [49]. Since the mechanism does not involve the hormonal receptor Ptgfr [49], it is tempting to speculate that bile acids, in particular CA and TCH, released by females under the regulation of $PGF_{2\alpha}$, are the chemical cues responsible for the preference of males by pre-ovulatory (fertile) females in Mozambique tilapia and perhaps other cichlids. Further studies are required to confirm this hypothesis.

CA and TCH are detected through independent olfactory receptor sites and, when applied in the preference experiment, males showed a strong preference for each of them. Consistent with our findings, Zhang et al. [50] showed that lake char also releases large quantities of bile acids, including TCH, through their faeces to which they are highly sensitive [51]. More recently, Zhu et al. [52] studied the behavioural preference of large yellow croaker (*Larimichthys crocea*) to faecal bile acids and found that not only intestinal fluid can cause attraction, and that CA can evoke similar behavioural responses. In contrast to the present study, however, their cross-adaptation experiments revealed that CA and TCH are detected through a shared olfactory receptor. The likely explanation for these differences is that tilapia, and perhaps other cichlids, have evolved (a) specific olfactory receptor(s) for taurine-conjugated bile acids. Furthermore, in pre-ovulatory faeces of tilapia only CA and TCH were differentially detected, but croaker faeces contained three other bile acids: taurochenodeoxycholic acid, chenodeoxycholic acid, and taurodeoxycholic acid [52].

Although olfactory sensitivity to bile acids is widespread in fish [reviewed by 25], only in the sea lamprey they have been established to have a pheromonal role, including as sex pheromones [e.g., 53–55]. In contrast, in teleost fishes, only two classes of reproductive pheromones, steroids and prostaglandins, have been demonstrated, who also play a fundamental role as hormones during the reproductive cycle, and readily produce polar metabolites highly soluble in water [9]. Thus, it appears that pheromone candidates tend to have high water solubility and are a product or by-product of a physiological state of social relevance open the possibility of chemical communication [9]. However, it is still poorly understood how fish have evolved to use bile acids as chemical cues, but probably involved externalization of bile acid receptors to the olfactory epithelium [25].

Fraction 2 contained strong odorants which were identified largely as amino acids. The existence of amino acids in the faeces of tilapia could be related to their feeding behaviour in which dominant males normally have higher accessibility to food than subordinates [56,57]. This explanation could be true also for female tilapia; the mandatory fasting of females during mouthbrooding could be associated with lower amounts of faecal odors such as amino acids. This could explain how social and ovulation status influence the composition of odours in the faeces. However, the present study did not show any evidence of preference of either male or female tilapia for amino acids.

In conclusion, we have shown that it is likely that pre-ovulatory females release bile acids CA and TCH in much higher concentration *via* faeces and speculate they may be signaling their health and fertility status to territorial males [21]. Furthermore, the release of bile acids could be under regulation of $PGF_{2\alpha}$ produced in reproductive females, thus explaining the high CA and TCH levels. Taken together, these findings are consistent with a role for faeces - and the bile acids therein -

in chemical communication during reproduction, at least in Mozambique tilapia, but possibly other cichlids and even among teleosts.

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Declaration of Competing Interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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