Behavioral Ecology and Sociobiology (2025) 79:48
https://doi.org/10.1007/s00265-025-03591-7

ORIGINAL ARTICLE

Behavioural responses to visual and chemical cues in Mozambique tilapia (*Oreochromis mossambicus*): a putative pheromonal activity for 17β-estradiol 3-glucuronate

Samyar Ashouri[1,2] · **Adelino V. M. Canário**[1] · **Peter C. Hubbard**[1]

Received: 4 September 2024 / Revised: 13 March 2025 / Accepted: 26 March 2025

Abstract

In reproduction of Mozambique tilapia (*Oreochromis mossambicus*), chemical communication mainly relies on urinary steroidal glucuronides (sex pheromones) that dominant males release to prime the final ovulation in pre-ovulatory females and increase reproductive readiness. However, the role of steroids in chemical communication in females has received less attention. Here, we aimed to determine whether 17β-estradiol 3-glucuronate released by pre-ovulatory females functions as a reproductive signal towards males. We investigated the preference of focal males to visual, chemical (female conditioned water), and a combination of both stimuli from pre-ovulatory or post-spawning females, as well as 10^{-9} M 17β-estradiol 3-glucuronate. Compared to the control zone, where no stimuli were present, males were more responsive (time spent near the stimuli, digging behaviour, and urination frequency) in the visual and visual + chemical zones than in the chemical-only zone. Males preferred pre-ovulatory to post-spawning female-conditioned water. Interestingly, the time spent by focal males near the source of 17β-estradiol 3-glucuronate was similar to pre-ovulatory conditioned water. However, there was no significant difference in digging and urine pulses in response to 17β-estradiol 3-glucuronate compared to the control group. We suggest that male tilapia recognise the ovulation status of females using visual and chemical cues, and 17β-estradiol 3-glucuronate is part of the odorant content released by pre-ovulatory females to communicate their reproductive status.

Significance

Fishes interact with their social environment using multiple sensory channels, and in the Mozambique tilapia (*Oreochromis mossambicus*), vision and chemical communication play vital roles. Males release urine containing pheromones to maintain social hierarchies, attract females, and stimulate their endocrine system to initiate reproductive behaviour. The male sex pheromones are 3-glucuronidated progestogens. However, how females signal their reproductive readiness remains unknown. In a multiple-choice maze, males prefer both pre-ovulatory females as a visual stimulus or their conditioned water to the same stimuli from postspawning females. Furthermore, males have high olfactory sensitivity to and prefer 17β-estradiol 3-glucuronate equally to pre-ovulatory female-conditioned water. Since this metabolite is released to the water at the end of vitellogenesis, our findings indicate that it plays a role in females signalling to mature males the approach of ovulation, contributing to spawning synchrony in this species.

Keywords Social hierarchy · Cichlid · Chemical cues · Visual cues · Conjugation · Behaviour

---

Communicated by I. Hamilton.

✉ Samyar Ashouri
Samyar_ashouri@yahoo.com

[1] Centro de Ciências do Mar (CCMAR), Universidade do Algarve, Campus de Gambelas, Faro 8005- 139, Portugal

[2] Departamento de Biologia Animal and cE3c - Center for Ecology, Evolution and Environmental Changes & CHANGE - Global Change and Sustainability Institute, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, Lisboa 1749-016, Portugal

Introduction

Among teleost fishes, the largest and most diverse vertebrate taxon, several species establish social hierarchies and use multiple sensory channels, including visual, acoustic, and chemical, to transfer information about their social status (Escobar-Camacho and Carleton 2015). Fishes often live in a dark environment, and for this have evolved a highly sensitive olfactory system that allows them to receive and process chemosensory cues released by conspecifics and heterospecifics, which can elicit a variety of physiological and behavioural responses (Kasumyan 2004; Bowers et al. 2023). If these conspecific-released compounds benefit both the sender and the receiver, they are called pheromones (Sorensen and Stacey 2004). Various chemical compounds with pheromonal activity have been identified in fish, including steroids, bile acids, prostaglandins, fatty acids, and amino acids (Dulka et al. 1987; Sorensen et al. 1988; Kobayashi et al. 2002; Li et al. 2002, 2018, 2023; Yambe et al. 2006; Keller-Costa et al. 2014b).

Steroids are vital regulators of many biological processes, such as sex determination, embryonic development, and sexual maturation. Steroid hormones are derived from the common precursor cholesterol and are classified into C18, C19, C21, and C24 based on the number of carbon atoms in their chemical structure (Miller 2013; Penning et al. 2019). In teleost fishes, steroid hormones and their metabolites, free or conjugated with glucuronide or sulphate, have been identified as sex pheromones (Scott and Sorensen 1994; Keller-Costa et al. 2014b; Stacey 2014). These hormonally derived steroids have been selected to have a pheromonal function in fishes for several reasons. As hormones, they are biologically active at very low concentrations. Furthermore, they provide information about the physiological state of the senders and, when released, they provide a correlate of reproductive readiness. Furthermore, their conjugates (i.e., sulphates or glucuronides), have increased water solubility. The hormonal-pheromone system of goldfish (*Carassius auratus*) is a good example of the pheromonal role of free steroids and their conjugates in a teleost fish, where 4-pregnen-17,20β dihydroxy-3-one (17,20β-P, the maturation-inducing steroid) is released into the water by ovulated females both in its free form and conjugated with glucuronic acid or sulphate. The preovulatory pheromonal complex promotes spawning synchrony and stimulates milt production in males (Stacey and Sorensen 2002; Sorensen and Stacey 2004).

The Mozambique tilapia (*Oreochromis mossambicus*), is a maternal mouthbrooder cichlid fish, native of flowing rivers of southeastern and central Africa (Simbine et al. 2014). The mating system of Mozambique tilapia is characterised by polygyny, where females spawn asynchronously (Rocha and Reis-Henriques 1996; Russell et al. 2012). Dominant males in their social hierarchies defend a territory associated with the areas where they dig, nest, and shelter. During spawning, dominant, black males use urinary signals to attract ovulated females. These urinary signals contain a mixture of two epimeric steroids, 20α- and 20β-pregnanetriol 3α-glucuronate (P3Gs), acting as sex pheromones and priming final maturation and ovulation in pre-ovulatory females (Huertas et al. 2014; Keller-Costa et al. 2014b). After spawning, females brood the eggs in their buccal cavity until they are hatched and ready to swim independently. Like other members of the cichlid family, the Mozambique tilapia is a good model for investigating how sensory modalities such as olfaction and vision can mediate the behavioural changes under different social such as reproduction and aggression (Barata et al. 2007; Maruska 2014).

Many fishes, including the Mozambique tilapia, also rely on visual and sound cues to interact with conspecifics, such as during the formation of social hierarchies and mate selection (Barata et al. 2007; Carleton 2009; Tave 2023). In *Pseudotropheus zebra*, the inferior lobes of the hypothalamus are involved in visual object recognition and memory formation (Calvo et al. 2023). The male visual pattern (redness) in the male Lake Victoria cichlid (*Pundamilia nyererei*) is the key visual signal for females to choose mates (Maan et al. 2004). In Mozambique tilapia, visual and chemical signals together yield a stronger behavioural response than each signal separately (Barata et al. 2007; Butler and Maruska 2016).

Studies on chemosensory communication in Mozambique tilapia have primarily focused on the role of males, largely because of their complex behaviours that they show within dominance hierarchies. Therefore, the potential role of females in these hierarchies has not been investigated directly. However, it has been shown that pre-ovulatory females release urine and faeces with higher olfactory potency in comparison to post-spawning females (Almeida et al. 2005; Miranda et al. 2005; Ashouri et al. 2023). Moreover, male tilapia can distinguish the ovulation state of females based on odours, and increase their urine pulses in the presence of pre-ovulatory females (Miranda et al. 2005). In the goldfish, plasma E_2 during vitellogenesis can stimulate the urinary release of an unidentified chemical cue that acts as a male attractant to initiate spawning synchrony (Kobayashi et al. 2002). Furthermore, it has been proposed that E_2 released by female zebrafish, may act as a sex pheromone and attract the male conspecifics (van den Hurk and Lambert 1983; van den Hurk and Resink 1992). 17β-estradiol (E_2) is produced by the granulosa cells of secondary oocytes and its production decreases at the end of vitellogenesis prior to oocyte final maturation and ovulation (Babin et al. 2007; Zohar et al. 2010; Servili et al. 2020).

Based on the reproductive role of E_2 during vitellogenesis and oocyte growth and studies showing fish olfactory sensitivity to E_2, it could be hypothesised E_2 or its conjugates could act as a chemical cue released by Mozambique tilapia pre-ovulatory females, signalling their reproductive development. Huertas et al. (2014) showed that pre-ovulatory female tilapia releases a higher concentration of E_2 through the urine (50–150 ng kg^{-1} h^{-1}) than post-spawn females. Urine from preovulatory Mozambique tilapia females also contains large quantities (100–150 ng ml^{-1}) of E_2 (3 and/or 17)-glucuronate (Keller-Costa et al. 2014a). Males are highly sensitive to E_2 3-glucuronate which is detected by a specific receptor (Keller-Costa et al. 2014a). Other teleosts, such as the round gobby (*Neogobius melanostomus*) also have olfactory sensitivity towards E_2 and E_2 3-glucuronate, and the latter causes increased ventilatory rate in females (Murphy et al. 2001).

Here, we aimed to establish the mate preference of Mozambique tilapia males exposed to chemical and visual cues from females with different ovulation status and to determine whether E_2 3-glucuronate can increase the preference of focal males to visual signals.

Materials and methods

Mozambique tilapia

Sexually mature male and female tilapia used in the present study were obtained from a broodstock maintained at the University of Algarve, Faro, Portugal. Tilapia were divided into 15 separate 300 L tanks (family tanks with 2 males to 6 females) with a glass front, sandy substrate, and an undergravel filter. Twenty-five per cent of the water in the family tanks was exchanged daily with aerated, dechlorinated tap water. The water temperature was kept at 27 °C and the photoperiod was 12 h light and 12 h dark. Fish were hand-fed once a day in the morning with a commercial cichlid diet (Sparos Lda., Olhão, Portugal). Females in each family tank were dorsal muscle tagged using coloured plastic labels (T-Bar anchor FD94, Floy Tag, Seattle, WA, USA). The fish were housed under these conditions for two months to follow the reproductive cycle length of the females. Recording the spawning date allowed us to predict the next spawning date and collect the samples according to the female ovulation state. The average cycle length of regularly spawning females was 18 days (mean ± standard deviation; 18.0 ± 4.2) (Miranda et al. 2005; Ashouri et al. 2023). One to two days before the predicted spawning date, the females were considered pre-ovulatory and a day after spawning, they were considered post-spawning. After each spawning, eggs were removed from the females' mouths to maintain their cycle.

Preference experiment

The experimental aquarium for evaluating the focal males' preference for either visual stimulus, chemical stimulus, or both is shown in Fig. 1. It consisted of five parts: two chemically isolated chambers at each end of the tank to provide a visual-only stimulus and an S-shaped compartment containing a central zone (neutral zone) and two zones to which the chemical stimuli were added (preference zones) in front of

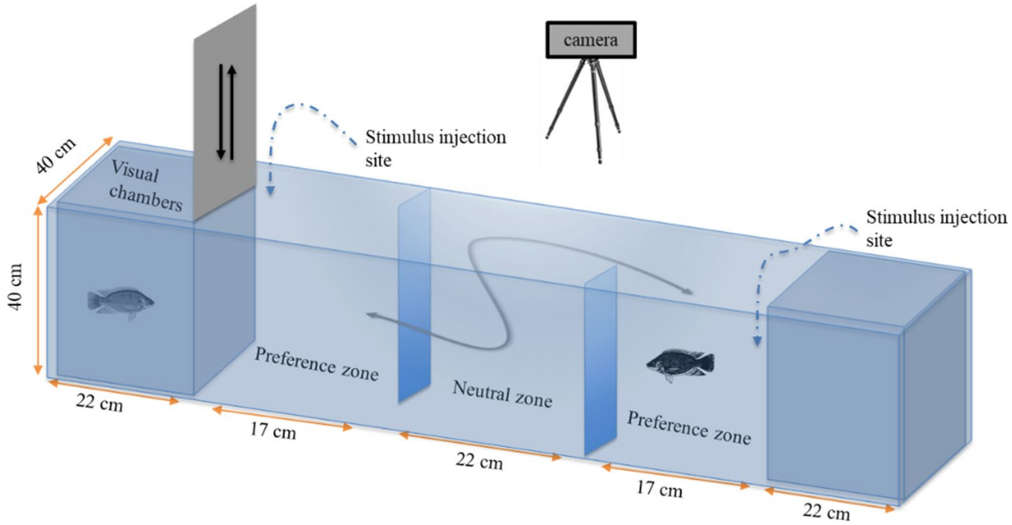

Fig. 1 Preference aquarium. Focal males were housed in the preference aquarium for 24 h in prior to recording the behaviours. In each trial, focal males were exposed to different stimuli provided through a peristaltic pump (for chemical stimuli) and/or visual chambers (for visual stimuli) in each preference zone. The experimental period consisted of a 15-minute stimulus injection followed by 25 min without injection, during which it was recorded the time spent by focal males in preference zones, as well as the frequency of urination and digging behaviour

the visual chambers. The visual chambers were concealed with a movable opaque plate. The temperature in the preference tanks was kept at 27 °C, and the photoperiod was 12 h light and 12 h dark. The experimental aquarium had a sandy substrate, and the two visual chambers and neutral zones had air stones to provide an adequate oxygen supply. Two vinyl tubes were connected separately to two channels of a peristaltic pump, and the outlet of each vinyl tube was fixed about two centimetres below the water surface in each preference zone to deliver the chemical stimuli at the flow rate of 10 $ml.min^{-1}$ (Ashouri et al. 2023). The preference time and behaviour of focal males were filmed using an iPhone 13 (Model number, MLPF3QL/A) placed in front of the experimental tank and synchronised to a PC through the Elgato Camera Hub software for desktop and the EpocCam version 2024.1 (Corsair Memory, Inc.) application for IOS to remotely control the recording. The flow rate of the chemical stimulus inside the tank was estimated by recording the time a food dye would take from delivery to appearing in the neutral zone in the middle of the preference tank. Accordingly, the experimental period was divided into a period of injections of the chemical stimulus of 15 min followed by 25 min without injection (post-injection). The visual stimulus was also provided alone for 40 min. In treatments with visual and chemical stimuli together, the exposure of focal males to both stimuli started simultaneously. After each experimental period, the preference tank was siphoned from the bottom to remove chemicals and solid waste.

Experimental protocol

A day before the experiment, a focal male was taken from a family tank and anaesthetised with 200 $mg.L^{-1}$ 3-aminobenzoic acid ethyl ester (MS222; Sigma-Aldrich), injected intramuscularly with 100 μL of phenol red (50 mg mL^{-1} dissolved in 0.9% saline) to visualise urination and housed in the preference tank (Barata et al. 2007; Ashouri et al. 2024). The preference of focal males, urination and digging behaviour frequencies were recorded in response to the following treatments: (1) female pre-ovulatory conditioned water versus control (males, $N=9$, weight (W): 200.1 ± 25.3 g [mean ± standard error of the mean], standard length (L_s): 18.8 ± 0.8 cm; females, $N=9$, W: 86.5 ± 8.8 g, L_s: 14.2 ± 0.4 cm), (2) female post-spawning conditioned water versus control (males, $N=9$, W: 257.3 ± 9.1 g, L_s: 20.5 ± 0.3; females, $N=9$, W: 117.0 ± 6.6 g, L_s: 15.7 ± 0.3 cm), (3) female pre-ovulatory visual stimulus versus control (males, $N=10$, W: 232.8 ± 18.3 g, L_s: 19.6 ± 0.5 cm; females, $N=10$, W: 128.3 ± 16.7 g, L_s: 15.4 ± 0.7 cm), (4) female post-spawning visual stimulus versus control (males, $N=9$, W: 234.3 ± 12.4 g, L_s: 20.1 ± 0.3 cm; females, $N=9$, W: 87.3 ± 7.4 g, L_s: 14.5 ± 0.4 cm), (5) female pre-ovulatory conditioned water + visual stimulus versus control (males, $N=9$, W: 255.0 ± 11.1 g, L_s: 21.0 ± 0.6 cm; females, $N=9$, W: 106.3 ± 8.1 g, L_s: 13.9 ± 0.5 cm), (6) female post-spawning conditioned water + visual stimulus versus control (males, $N=9$, W: 253.2 ± 11.8 g, L_s: 20.8 ± 0.6 cm; females, $N=9$, W: 103.5 ± 7.7 g, L_s: 13.8 ± 0.6 cm), (7) female pre-ovulatory conditioned water + visual stimulus (males, $N=9$, W: 251.1 ± 7.0 g, L_s: 20.6 ± 0.3 cm; females, $N=9$, W: 75.3 ± 16.2 g, L_s: 14.3 ± 0.4 cm) versus post-spawning conditioned water + visual stimulus (females, $N=9$, W 90.4 ± 11.9 g, L_s 14.0 ± 0.6 cm), (8) 17β estradiol 3-glucuronate + visual stimulus (males, $N=9$, W: 238.2 ± 12.2 g, L_s: 20.1 ± 0.5 cm; females, $N=9$, W: 113.6 ± 10.6 g, L_s: 14.9 ± 0.4 cm) versus visual stimulus (females, $N=9$, W: 103.2 ± 10.6 g, L_s: 14.8 ± 0.4 cm).

At the end of each set of experiments, experimental fish (focal males and females), were transferred to stock tanks and the preference aquarium was siphoned, washed in warm water, and filled with new freshwater for the next set of experiments. The vinyl tubes of peristaltic pump were also washed with warm water to remove any residues.

The control refers to that side of the preference tank receiving neither visual nor chemical stimuli. In the trials with only visual stimuli, the females of different ovulation statuses were taken from a different family tank from that of the focal male and isolated in the visual chambers with the opaque plate in place. In the trials with only chemical stimuli, the visual chambers remained empty, and only conditioned water obtained from females of different ovulation statuses was pumped into the preference zones. For visual and chemical stimuli, the females that produced the conditioned water being tested were used for both. A total of 92 focal males were used, of which only 73 (W: 240.1 ± 5.3 g; L_s: 20.2 ± 0.2 cm) reacted to visual and chemical stimuli. Almost 21% of focal males remained inactive and immobile without swimming in the experimental tank for more than 20 min. These males were removed from the analysis. The data was not recorded blind because our study involved testing the preference of focal fish in the experimental tanks.

Preparation of visual and chemical stimuli

The visual stimuli were provided directly by housing pre-ovulatory and post-spawning females individually in the visual chambers. The chemical stimuli (pre-ovulatory and post-spawning females' conditioned waters) were obtained according to Miranda et al. (2005). Briefly, the females with different ovulation statuses were isolated in buckets filled with 20 L aerated, dechlorinated tap water for 2 h; the water was collected and used immediately. EOG experiments have shown that the Mozambique tilapia threshold of detection for 17β-estradiol 3-glucuronate was about

10^{-10} M (Keller-Costa et al. 2014a). The concentration of 17β-estradiol 3-glucuronate (Sigma Aldrich Co. Ltd., St. Louis, USA) delivered to the preference zones was estimated to be 10^{-9} M from a solution of 10^{-5} M in distilled water made from a stock solution of 10^{-2} M in methanol; tilapia cannot detect methanol up to a concentration of 1:1000 *v*/*v* (Miranda et al. 2005).

Statistical analysis

In the present study, treatments 1–6, which were compared to control (no visual or chemical stimulus), were analyzed separately from treatments 7 and 8. For treatments 1–6, the time that focal males spent in each preference zone (dependent variable) in response to different stimuli was analyzed by three-way analysis of variance (ANOVA) with independent variables the reproductive state of females (pre-ovulatory and post-spawning), type of stimulus (chemical and/or visual), and the preference zones (control and stimulus). Since digging behaviour and urine pulses were not seen in the control zone of treatments 1–6, only behaviours in the stimulus zones were compared and a two-way ANOVA was employed. The digging data was transformed using square root transformation and the urine pulse data were transformed by adding one as a positive constant to meet the assumptions of ANOVA. For treatments 7 and 8, a one-way ANOVA was used to compare the effects of the stimuli on the time spent by focal males in each preference zone, digging and urine pulses. The Holm-Sidak post hoc was used to test all-pairwise comparisons between levels of independent variables. The data were analysed using SigmaPlot 14.0 (Systat Software, Inc., San Jose, CA, USA). Data are presented as mean ± standard error of the mean (SEM).

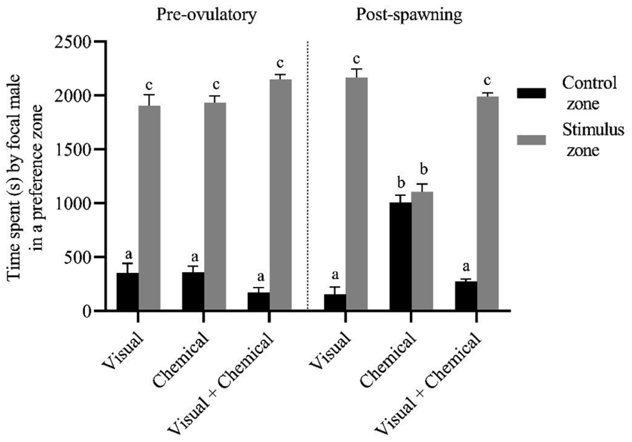

Fig. 2 Time (s) spent by focal males in a preference zone– control side (black bar) or stimulus side (grey bar) - in response to visual, chemical, or visual + chemical stimulus from pre-ovulatory and post-spawning females. Bars are mean ± SEM. Different letters represent statistically significant differences ($P < 0.05$)

Results

There was a statistically significant three-way interaction between the reproductive state of females, stimulus type and preference zone ($F_{(2,98)} = 54.746$, $P < 0.001$) (Fig. 2). There were significant two-way interactions between stimulus type and preference zone ($F_{(2,98)} = 69.836$, $P < 0.001$) and reproductive state of females and preference zone ($F_{(1,98)} = 28.409$, $P < 0.001$) but not between stimulus type and reproductive state of females ($F_{(2,98)} = 0.624$, $P = 0.538$). The time spent by focal males in the stimulus preference zone, irrespective of stimulus or female type, was approximately 5 to 10 times the time spent in the preference zone of the control ($F_{(1,98)} = 1486.904$, $P < 0.001$). The exception was males spending similar time in the preference zone of post-ovulatory females conditioned water and control water. Males showed a stronger chemical attraction to pre-ovulatory females compared to post-spawning females ($P < 0.05$). Although focal males showed similar attraction to pre-ovulatory females through both visual and chemical stimuli, they were less attracted to the chemical stimulus of post-spawning females compared to their visual stimulus ($P < 0.05$).

No digging was observed in the control zone, indicating that a visual or chemical stimulus is necessary. There was a significant effect of the stimulus type ($F_{(2,49)} = 102.337$, $P < 0.001$) and female stage on digging ($F_{(1,49)} = 12.633$, $P < 0.001$) (Fig. 3 left). Digging by focal males was higher when the stimulus was visual than when it was chemical ($P < 0.001$), with no additional effect of adding the chemical stimulus to the visual stimulus ($P = 0.138$). Digging was similar for pre-ovulatory and post-spawning females if the stimulus was visual ($P = 0.058$) or visual + chemical ($P = 0.069$) but significantly higher for pre-ovulatory females when the stimulus was chemical ($P = 0.023$).

As with digging, there was no urination in the control zone. Urination frequency by focal males was affected by the type of stimulus ($F_{(2,49)} = 64.036$, $P < 0.001$) and the female reproductive stage ($F_{(1,49)} = 4.900$, $P = 0.032$) (Fig. 3 right). Focal males urinated less frequently with a chemical than a visual stimulus, irrespective of the reproductive stage of the females ($P < 0.05$). The reproductive stage only influenced focal male urination when chemical (difference of means = 1.333, $t = 0.803$, $P = 0.426$) and visual (difference of means = 0.867, $t = 0.536$, $P = 0.595$) stimuli were present simultaneously (difference of means = 4.111, $t = 2.477$, $P = 0.017$) and was lower in response to post-spawning females ($P < 0.05$).

There was a significant effect of chemical treatments compared to the control visual cue only ($F_{(3,\ 32)} = 35.07$; $P < 0.001$) (Fig. 4). Focal males spent significantly more time near a chemical + visual stimulus, in particular, if the

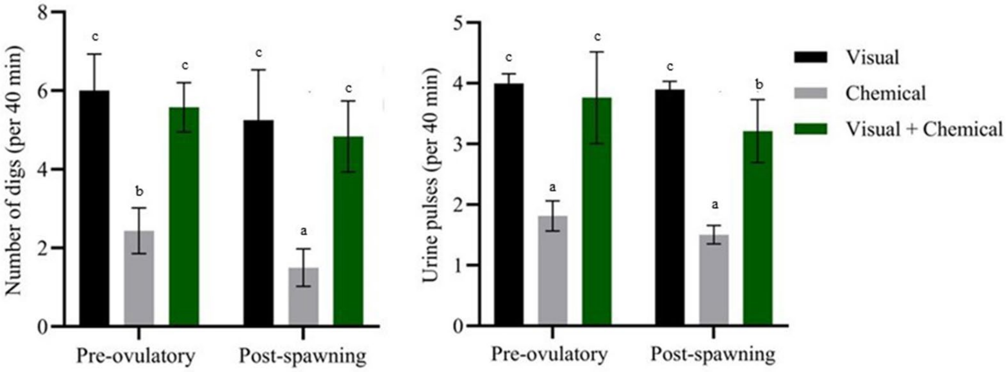

Fig. 3 Number of digs (left) and urine pulses (right) in response to visual, chemical, or visual + chemical stimulus from pre-ovulatory and post-spawning females. Bars are mean ± SEM. Different letters represent statistically significant differences ($P < 0.05$)

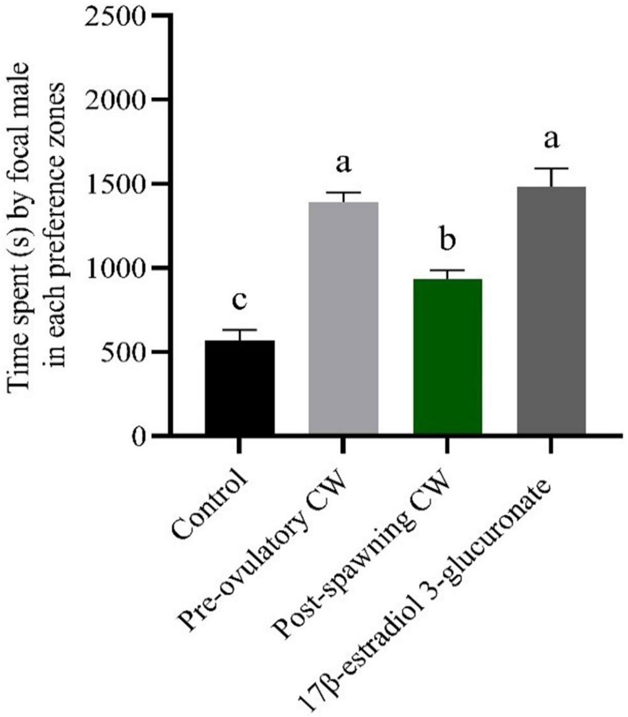

Fig. 4 Time (s) spent by focal males in preference zones receiving visual stimulus only (control) and a combination of visual and chemical stimuli (pre-ovulatory conditioned water [CW], post-spawning conditioned water, and 17β-estradiol 3-glucuronate). Bars are mean ± SEM. Different letters represent statistically significant differences ($P < 0.05$)

stimuli were from pre-ovulatory females, than near a visual stimulus only as control ($P < 0.001$). Furthermore, estradiol-17β glucuronate had the same effect on the time spent by males in the preference zone as conditioned water from pre-ovulatory females ($P < 0.05$).

There was also a significant effect of chemical treatments on digging ($F_{(3, 32)} = 17.30$; $P < 0.001$) (Fig. 5 left). However, only focal males exposed to pre-ovulatory conditioned water showed significantly higher digging activity than the control visual treatment ($P < 0.001$). There were no significant differences between the digging of focal males exposed to post-spawning conditioned water and 17β-estradiol 3-glucuronate ($P = 0.176$).

There was no significant effect of any of the chemical treatments on urination of focal males compared to visual treatment ($P = 0.342$) (Fig. 5 right).

Discussion

The present study showed that male tilapia prefer the zones receiving visual, chemical, and their combination from pre-ovulatory females in comparison to control zones. Furthermore, focal males showed significant attraction to the preference zones receiving visual and visual + chemical stimuli from post-spawning females in comparison to control zones.

Previous studies have shown that conditioned water from pre-ovulatory female tilapia provide stronger olfactory cues to males than post-spawning females, indicating that potent odorants contained in the conditioned water pre-ovulatory females are responsible for the higher attraction of focal males in comparison to post-spawning females' conditioned water (Miranda et al. 2005). Our study arrived at a similar conclusion as pre-ovulatory conditioned water was more attractive for focal males than post-spawning conditioned water. It seems that male tilapia discriminate between chemical stimuli from pre-ovulatory and post-spawning females, but are attracted to visual signals alone, independently of reproductive status, which indicates that both stimuli play a crucial role in mate choice and recognition of ovulation status.

Visual cues could include female colouration, body shape, body posture, and behaviour (Rowland 1999; Escobar-Camacho and Carleton 2015). However, colour changes in the females are not obvious to the human observer. Several studies have shown how visual stimuli can influence

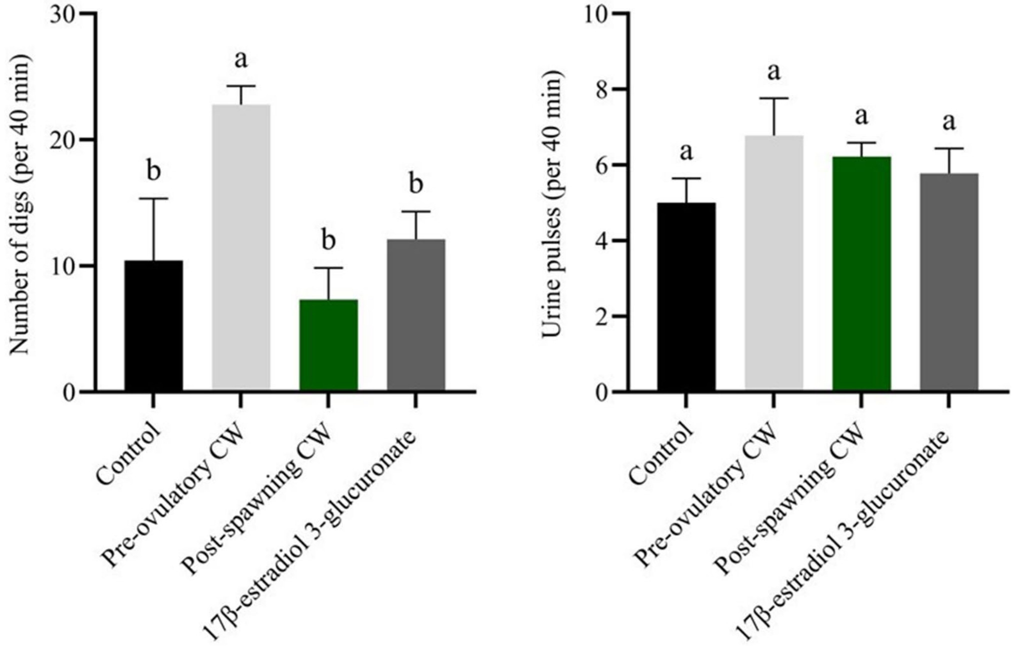

Fig. 5 Number of digs (left) and urine pulses (right) by focal males in preference zones receiving visual stimulus only (control) and a combination of visual and chemical stimuli (pre-ovulatory conditioned water [CW], post-spawning conditioned water [CW], and 17β-estradiol 3-glucuronate). Bars are mean ± SEM. Different letters represent statistically significant differences ($P < 0.05$)

the effect of olfactory stimuli. In round gobies (*Neogobius melanostomus*) an imitation of a blank-coloured model of a reproductive male (visual stimulus) attracted reproductive females more easily than olfactory cues (urine) (Yavno and Corkum 2010). In Nile tilapia (*Oreochromis niloticus*), which possess a highly sensitive visual system, female visual cues alone are sufficient to evoke reproductive behaviours and affect gonadal development (Castro et al. 2009). However, recognising a potential mate in a social hierarchy may not rely only on visual or chemical cues alone. Swordtail (*Xiphophorus nigrensis*) females use both visual and chemical cues to distinguish conspecifics from heterospecifics (Crapon de Caprona and Ryan 1990). Exposure of male guppies to both visual and chemical stimuli of virgin females has confirmed that chemical cues are primarily used by males to assess and discriminate the reproductive status of females (Guevara-Fiore et al. 2009). In a social hierarchy, different sensory modalities are likely to be used by conspecifics to guide them towards the potential reproductive mate. Since chemical cues are dissolved in the water, they may orient the fish from longer distances followed by visual cues for further and detailed assessment of mate in closer vicinity (Crapon de Caprona and Ryan 1990); in other words, mating preference in Mozambique tilapia can be mediated through the multiple cues rather than relying on only one signal. This likely increases the accuracy of mate assessment and eventually reduces the cost of mate choice (Candolin 2003).

Recently, we have shown that focal males increase their nest-digging behaviour in response to conditioned water and faeces extract from pre-ovulatory females compared to post-spawning females' conditioned water (Ashouri et al. 2023). In the present study, the number of digs and urine pulses, both as courtship behaviour in male tilapia (Baerends and Baerends-van Roon 1950; Barata et al. 2007) were quantified and focal males showed higher number of digs in response to conditioned water from pre-ovulatory than post-spawning females. This might be explained by a higher concentration of odorants contained in the pre-ovulatory female-conditioned water, which could include bile acids, amino acids and/or steroids (Ashouri et al. 2023). Body fluids such as urine may be responsible for evoking higher nest-digging behaviour by focal males since urine from pre-ovulatory has higher olfactory potency than post-spawning females (Miranda et al. 2005). However, the amount of male digging behaviour was not distinguishable in the presence of chemical and visual stimuli sent by pre-ovulatory and post-spawning females. One possible explanation is that the experimental design did not allow focal males to assess the females' reproductive status through courtship as if they were allowed to interact. Male tilapia typically show a wide range of courtship behaviours to attract and orient the pre-ovulatory females to their nest, including chasing and nipping, together with urinary sex pheromones (Baerends and Baerends-van Roon 1950). Furthermore, digging and courtship behaviours may convey different information about the readiness and maturity of focal males to the females that likely initiate their ovulation cycle and make them ready for the next spawning. This can be further explained by the energy-consuming maternal care of females after spawning, and females prefer to invest and evaluate dominant males through the visual courtship signals (Maruska and Butler 2021).

Physical assessment, also known as tactile interaction, has been seen in other species to mediate anti-predator responses within a group-based hierarchy (Riley et al. 2019). Other species such as the Mexican molly (*Poecilia sphenops*) have a moustache-like structure used by males to attract females and provide tactile information during the nipping (Schlupp et al. 2010).

In the current study, the comparison between treatments 7 and 8 (set 2) showed that focal males were more attracted to pre-ovulatory female-conditioned water + visual stimulus than to a visual stimulus alone (control) or a visual stimulus plus post-spawning conditioned water. These results are different from when focal males showed no differences in preference between visual plus chemical stimulus from pre-ovulatory and post-spawning females (treatments 1 to 6 of set 1). Having a choice of stimuli may be more discriminatory and better highlight the differences between stimuli.

A visual stimulus plus E_2 3-glucuronate was just as attractive as a visual stimulus plus pre-ovulatory conditioned water. This suggests that E_2 3-glucuronate is a major component of pre-ovulatory female-conditioned water that acts as an attractant to males. Given the release of significant amounts of E_2 3-glucuronate by pre-ovulatory females and the high olfactory sensitivity of male tilapia to 10 pmol.l^{-1} E_2 3-glucuronate (Ellis et al. 2013; Huertas et al. 2014), this hormonal metabolite appears to convey information about the females' reproductive readiness and to attract male conspecifics. Interestingly, tilapia males are also highly sensitive and are attracted to faecal cholic and taurocholic acids from pre-ovulatory females (Ashouri et al. 2023) although the behavioural response appears to be smaller than E_2 3-glucuronate. Together, this suggests a female pheromone signalling reproductive status to males in which E_2 3-glucuronate is likely to play a major role. *Haplochromis burtoni* has olfactory sensitivity to E_2 sulphate (Cole and Stacey 2006), suggesting that estrogens and their metabolites could have a similar role in other cichlids. Thus, as with the goldfish (Poling et al. 2001), in tilapia, male and female hormones and bile acids appear to communicate reproductive status and facilitate spawning synchrony.

However, unlike the effect on attraction, digging behaviour was only increased in pre-ovulatory females in conditioned water, as previously shown (Ashouri et al. 2023), and no differential effect on urination was observed for any of the treatments. This indicates that pre-ovulatory females likely produce an additional component other than E_2 3-glucuronate that stimulates digging behaviour and that urination results only from a visual stimulus. The component that stimulates digging could be another sexual steroid perhaps linked to final oocyte maturation (Tokarz et al. 2015). In addition, providing only one component from a complex stimulus such as conditioned water in which can include urine, faeces, ovarian fluid, skin mucus, etc., may disrupt the multiple signalling where both sexes in the interaction convey information about their reproductive and/or social status (Hebets and Papaj 2005; Hebets and Rundus 2010).

Mozambique tilapia is a member of the African Pseudocrenilabrinae subfamily, the only one of the four cichlid subfamilies sensitive to sex steroids (Stacey 2014). It belongs to the Oreochromini tribe, the only one that has a receptor exclusive for estrogen 3-glucuronides (Stacey 2014). The Oreochromini are also the only exclusive maternal mouth brooders of the Pseudocrenilabrinae, with females receiving no help from males (Balshine-Earn and Earn 1998). It is tempting to speculate that the E_2 3-glucuronate signal evolved to provide males with an honest signal of female reproductive quality, which they use together with visual appearance. Due to the critical role of females in brood success, males are likely under strong selective pressure to evaluate female quality and therefore rely on multiple cues (Maruska and Butler 2021).

In conclusion, our study confirms the synergistic effect of multisensory signals on Mozambique tilapia behaviour and provides suggestive evidence that the appearance of a receptor for estrogen 3-glucuronides in the Oreochromini may be linked to a mechanism for assessing female reproductive status.

Author contributions SA, PCH, and AVMC conceived the study. SA carried out experiments and data analysis. SA wrote the first draft. PCH and AVMC edited the manuscript.

Funding Open access funding provided by FCT|FCCN (b-on).
This study was funded by the Science and Technology Foundation (FCT), Portugal [projects ID UIDB/04326/2020, https://doi.org/10.54499/UIDB/04326/2020; UIDP/04326/2020, https://doi.org/10.54499/UIDP/04326/2020; LA/P/0101/2020, https://doi.org/10.54499/LA/P/0101/2020]. SA received a PhD fellowship from FCT [reference number, 2020.08404.BD].

Data availability Supporting data are available at https://doi.org/10.5281/zenodo.13684901.

Declarations

Ethical approval Fish maintenance and the experimental procedures were carried out in accordance with Directive 2010/63/EU on protecting animals used for scientific purposes under license number 0421/000/000/2020 granted by the Directorate-General for Food and Veterinary of Portugal. All applicable international, national, and/or institutional guidelines for the use of animals were followed.

Conflict of interest The authors declare no conflict of interest.

References

Almeida OG, Miranda A, Frade P, Hubbard PC, Barata EN, Canário AV (2005) Urine as a social signal in the Mozambique tilapia (*Oreochromis mossambicus*). Chem Senses 30:i309–i310

Ashouri S, Da Silva JP, Canário AV, Hubbard PC (2023) Bile acids as putative social signals in Mozambique tilapia (*Oreochromis mossambicus*). Physiol Behav 272:114378

Ashouri S, Hubbard PC, Canário AVM (2024) Minimizing the time to evaluate pheromone-mediated reduction of aggressive behavior in Mozambique tilapia (*Oreochromis mossambicus*). J Fish Biol 105:691–698

Babin PJ, Cerdà J, Lubzens E (2007) The fish oocyte: from basic studies to biotechnological applications. Springer Science & Business Media, Dordrecht

Baerends GP, Baerends-van Roon J (1950) An introduction to the study of the ethology of the cichlid fishes. Behav Suppl: 1–243

Balshine-Earn S, Earn DJ (1998) On the evolutionary pathway of parental care in mouth–brooding cichlid fishes. Proc R Soc Lond B 265:2217–2222

Barata EN, Hubbard PC, Almeida OG, Miranda A, Canário AV (2007) Male urine signals social rank in the Mozambique tilapia (*Oreochromis mossambicus*). BMC Biol 5:54

Bowers JM, Li C-Y, Parker CG, Westbrook ME, Juntti SA (2023) Pheromone perception in fish: mechanisms and modulation by internal status. Integr Comp Biol 63:407–427

Butler JM, Maruska KP (2016) The mechanosensory lateral line system mediates activation of socially-relevant brain regions during territorial interactions. Front Behav Neurosci 10:93

Calvo R, Hofmann M, Schluessel V (2023) Brain areas activated during visual learning in the cichlid fish Pseudotropheus zebra. Brain Struct Funct 228:859–873

Candolin U (2003) The use of multiple cues in mate choice. Biol Rev 78:575–595

Carleton K (2009) Cichlid fish visual systems: mechanisms of spectral tuning. Integr Zool 4:75–86

Castro A, Gonçalves-de-Freitas E, Oliveira C (2009) Visual communication stimulates reproduction in nile tilapia, *Oreochromis niloticus* (L). Braz J Med Biol Res 42:368–374

Cole TB, Stacey NE (2006) Olfactory responses to steroids in an African mouth-brooding cichlid, *Haplochromis burtoni*(Günther). J Fish Biol 68:661–680

Crapon de Caprona M-D, Ryan MJ (1990) Conspecific mate recognition in swordtails, *Xiphophorus nigrensis* and *X. pygmaeus* (Poeciliidae): olfactory and visual cues. Anim Behav 39:290–296

Dulka J, Stacey N, Sorensen P, Kraak GVD (1987) A steroid sex pheromone synchronizes male–female spawning readiness in goldfish. Nature 325:251–253

Ellis T, Sanders M, Scott A (2013) Non-invasive monitoring of steroids in fishes. Vet Med Austria 100:255–269

Escobar-Camacho D, Carleton KL (2015) Sensory modalities in cichlid fish behavior. Curr Opin Behav Sci 6:115–124

Guevara-Fiore P, Skinner A, Watt P (2009) Do male guppies distinguish Virgin females from recently mated ones? Anim Behav 77:425–431

Hebets EA, Papaj DR (2005) Complex signal function: developing a framework of testable hypotheses. Behav Ecol Sociobiol 57:197–214

Hebets EA, Rundus A (2010) Chemical communication in a multimodal context. In: Breithaupt T, Thiel M (eds) Chemical communication in crustaceans. Springer, New York, pp 335–354

Huertas M, Almeida OG, Canário AV, Hubbard PC (2014) Tilapia male urinary pheromone stimulates female reproductive axis. Gen Comp Endocrinol 196:106–111

Kasumyan A (2004) The olfactory system in fish: structure, function, and role in behavior. J Ichthyol 44:S180–S223

Keller-Costa T, Canário AVM, Hubbard PC (2014a) Olfactory sensitivity to steroid glucuronates in Mozambique tilapia suggests two distinct and specific receptors for pheromone detection. J Exp Biol 217:4203–4212

Keller-Costa T, Hubbard PC, Paetz C, Nakamura Y, da Silva JP, Rato A, Barata EN, Schneider B, Canario AV (2014b) Identity of a tilapia pheromone released by dominant males that primes females for reproduction. Curr Biol 24:2130–2135

Kobayashi M, Sorensen PW, Stacey NE (2002) Hormonal and pheromonal control of spawning behavior in the goldfish. Fish Physiol Biochem 26:71–84

Li W, Scott AP, Siefkes MJ, Yan H, Liu Q, Yun S-S, Gage DA (2002) Bile acid secreted by male sea lamprey that acts as a sex pheromone. Science 296:138–141

Li K, Brant CO, Huertas M, Hessler EJ, Mezei G, Scott AM, Hoye TR, Li W (2018) Fatty-acid derivative acts as a sea lamprey migratory pheromone. P Natl Acad Sci USA 115:8603–8608

Li C-Y, Lawrence K, Merlo-Coyne J, Juntti SA (2023) Prostaglandin $F_{2\alpha}$ drives female pheromone signaling in cichlids, revealing a basis for evolutionary divergence in olfactory signaling. P Natl Acad Sci USA 120:e2214418120

Maan ME, Seehausen O, Söderberg L, Johnson L, Ripmeester EA, Mrosso HD, Taylor MI, van Dooren TJ, van Alphen JJ (2004) Intraspecific sexual selection on a speciation trait, male coloration, in the lake Victoria cichlid Pundamilia nyererei. Proc R Soc Lond B 271:2445–2452

Maruska KP (2014) Social regulation of reproduction in male cichlid fishes. Gen Comp Endocrinol 207:2–12

Maruska KP, Butler JM (2021) Reproductive-and social-state plasticity of multiple sensory systems in a cichlid fish. Integr Comp Biol 61:249–268

Miller WL (2013) Steroid hormone synthesis in mitochondria. Mol Cell Endocrinol 379:62–73

Miranda A, Almeida OG, Hubbard PC, Barata EN, Canário AVM (2005) Olfactory discrimination of female reproductive status by male tilapia (*Oreochromis mossambicus*). J Exp Biol 208:2037–2043

Murphy CA, Stacey NE, Corkum LD (2001) Putative steroidal pheromones in the round goby, *Neogobius melanostomus*: olfactory and behavioral responses. J Chem Ecol 27:443–470

Penning TM, Wangtrakuldee P, Auchus RJ (2019) Structural and functional biology of aldo-keto reductase steroid-transforming enzymes. Endocr Rev 40:447–475

Poling KR, Fraser EJ, Sorensen PW (2001) The three steroidal components of the goldfish preovulatory pheromone signal evoke different behaviors in males. Comp Biochem Physiol B 129:645–651

Riley RJ, Gillie ER, Savage JL, Boogert NJ, Manica A, Jungwirth A (2019) The role of tactile interactions in flight responses in the bronze Cory catfish (*Corydoras aeneus*). Ethology 125:810–820

Rocha M, Reis-Henriques M (1996) Plasma and urine levels of C18, C19 and C21 steroids in an asynchronous fish, the tilapia Oreochromis mossambicus (Teleostei, Cichlidae). Comp Biochem Physiol 115:257–264

Rowland WJ (1999) Studying visual cues in fish behavior: a review of ethological techniques. Environ Biol Fish 56:285–305

Russell D, Thuesen P, Thomson F (2012) A review of the biology, ecology, distribution and control of Mozambique tilapia, *Oreochromis mossambicus* (Peters 1852)(Pisces: Cichlidae) with particular emphasis on invasive Australian populations. Rev Fish Biol Fish 22:533–554

Schlupp I, Riesch R, Tobler M, Plath M, Parzefall J, Schartl M (2010) A novel, sexually selected trait in poeciliid fishes: female preference for mustache-like, rostral filaments in male *Poecilia sphenops*. Behav Ecol Sociobiol 64:1849–1855

Scott A, Sorensen P (1994) Time course of release of pheromonally active gonadal steroids and their conjugates by ovulatory goldfish. Gen Comp Endocrinol 96:309–323

Servili A, Canario AV, Mouchel O, Muñoz-Cueto JA (2020) Climate change impacts on fish reproduction are mediated at multiple levels of the brain-pituitary-gonad axis. Gen Comp Endocrinol 291:113439

Simbine L, Viana da Silva J, Hilsdorf A (2014) The genetic diversity of wild O reochromis mossambicus populations from the M ozambique Southern watersheds as evaluated by microsatellites. J Appl Ichthyol 30:272–280

Sorensen PW, Stacey NE (2004) Brief review of fish pheromones and discussion of their possible uses in the control of non-indigenous teleost fishes. N-Z J Mar Freshw Res 38:399–417

Sorensen PW, Hara TJ, Stacey NE, Goetz FW (1988) F prostaglandins function as potent olfactory stimulants that comprise the postovulatory female sex pheromone in goldfish. Biol Reprod 39:1039–1050

Stacey N (2014) Hormonally derived pheromones in teleost fishes. In: Sorensen PW, Wisenden BD (eds) Fish pheromones and related cues. Wiley, Hoboken, pp 33–88

Stacey N, Sorensen P (2002) Hormonal pheromones in fish. In: Pfaff DW (ed) Hormones, brain and behavior. Elsevier, Amsterdam, pp 375–434

Tave D (2023) Effect of female body color on mate selection by male Mozambique tilapia (Oreochromis mossambicus). J Appl Aquacult 35:45–50

Tokarz J, Möller G, de Angelis MH, Adamski J (2015) Steroids in teleost fishes: A functional point of view. Steroids 103:123–144

van den Hurk R, Lambert J (1983) Ovarian steroid glucuronides function as sex pheromones for male zebrafish, *Brachydanio rerio*. Can J Zool 61:2381–2387

van den Hurk R, Resink JW (1992) Male reproductive system as sex pheromone producer in teleost fish. J Exp Zool 261:204–213

Yambe H, Kitamura S, Kamio M, Yamada M, Matsunaga S, Fusetani N, Yamazaki F (2006) L-Kynurenine, an amino acid identified as a sex pheromone in the urine of ovulated female Masu salmon. P Natl Acad Sci USA 103:15370–15374

Yavno S, Corkum L (2010) Reproductive female round gobies (*Neogobius melanostomus*) are attracted to visual male models at a nest rather than to olfactory stimuli in urine of reproductive males. Behaviour 147:121–132

Zohar Y, Muñoz-Cueto JA, Elizur A, Kah O (2010) Neuroendocrinology of reproduction in teleost fish. Gen Comp Endocrinol 165:438–455