J Chem Ecol (2016) 42:173–182
DOI 10.1007/s10886-016-0668-0

A Multi-Component Pheromone in the Urine of Dominant Male Tilapia (*Oreochromis mossambicus*) Reduces Aggression in Rivals

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Received: 21 August 2015 / Revised: 5 January 2016 / Accepted: 17 January 2016 / Published online: 4 February 2016

Abstract Males often use scent to communicate their dominance, and to mediate aggressive and breeding behaviors. In teleost fish, however, the chemical composition of male pheromones is poorly understood. Male Mozambique tilapia, *Oreochromis mossambicus*, use urine that signals social status and primes females to spawn. The urinary sex pheromone directed at females consists of 5β-pregnane-3α,17α,20β-triol 3-glucuronate and its 20α-epimer. The concentration of these is positively correlated with male social rank. This study tested whether dominant male urine reduces aggression in receiver males, and whether the pregnanetriol 3-glucuronates also reduce male-male aggression. Males were allowed to fight their mirror image when exposed to either: i) water control or a chemical stimulus; ii) dominant male urine (DMU); iii) C18-solid phase (C18-SPE) DMU eluate; iv) C18-SPE DMU eluate plus filtrate; v) the two pregnanetriol 3-glucuronates (P3Gs); or vi) P3Gs plus DMU filtrate. Control males mounted an increasingly aggressive fight against their image over time. However, DMU significantly reduced this aggressive response. The two urinary P3Gs did not replicate the effect of whole DMU. Neither did the C18-SPE DMU eluate, containing the P3Gs, alone, nor the C18-SPE DMU filtrate to which the two P3Gs were added. Only exposure to reconstituted DMU (C18-SPE eluate plus filtrate) restored the aggression-reducing effect of whole DMU. Olfactory activity was present in the eluate and the polar filtrate in electro-olfactogram studies. We conclude that P3Gs alone have no reducing effect on aggression and that the urinary signal driving off male competition is likely to be a multi-component pheromone, with components present in both the polar and non-polar urine fractions.

Keywords Social behavior · Aggression · Chemical communication · Urine · Mirror · Cichlid

Introduction

In many animals, including teleost fishes, males use scent to signal social rank and mediate agonistic and breeding behaviors (reviewed in Wyatt 2014). Fish pheromones may consist of a single compound as, for example, the sex pheromone L-kynurenine of female masu salmon (Yambe et al. 2006), or a mixture of different substances as, for example, the female goldfish pheromone (Stacey and Sorensen 2009). In this context, research in fish has focused mainly on the role of reproductive pheromones (reviewed by Stacey and Sorensen 2009; Stacey 2015). Fishes with complex social structures also actively release chemical cues that advertise social status and mediate male-male aggression. Sexually mature male goldfish, *Carassius auratus*, for example, release large amounts of androstenedione (AD) into the water, which increases aggressiveness and pushing behavior (a male hitting another male with his head) in conspecific males, suggesting that AD functions as a male pheromone in this species (Poling et al. 2001; Sorensen et al. 2005). Studies on African cichlids (Barata et al. 2007; Keller-Costa et al. 2012; Maruska and Fernald 2012) and the fathead minnow (Martinovic-Weigelt et al. 2012) have provided evidence for the existence of dominance pheromones that signal social rank. However, the identity and complexity of these signals, as well as their regulation and precise action, remains unknown.

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Male Mozambique tilapia, *Oreochromis mossambicus* Peters 1852, use urinary cues for multiple purposes (reviewed in Keller-Costa et al. 2015) and are, therefore, a useful model for chemical communication studies. In their natural habitat, males establish social hierarchies in leks and aggressively defend a territory, while visiting females mate preferentially with the dominant males near the center of the lek (Bruton and Boltt 1975). Male urine is a potent olfactory stimulus for conspecifics (Frade et al. 2002), and its potency increases with the social rank of the donor (Barata et al. 2008). The muscular wall of the urinary bladder of dominant males is thicker than that of subordinates (Keller-Costa et al. 2012), which allows expansion and storage of large volumes of urine and control of its release in different social contexts (Barata et al. 2007; Miranda et al. 2005). Dominant males increase urination frequency when courting females (Barata et al. 2008), and exposure of females to male urine stimulates their endocrine system to produce the oocyte maturation-inducing hormone 17,20β-dihydroxypregn-4-en-3-one (17,20β-P; Huertas et al. 2014). Recently, we identified 5β-pregnane-3α,17α,20β-triol 3-glucuronate (20β-P-3-G) and 5β-pregnane-3α,17α,20α-triol 3-glucuronate (20α-P-3-G) as the male tilapia sex pheromone that stimulates 17,20β-P production in females, priming them for spawning (Hubbard et al. 2014; Keller-Costa et al. 2014b). Interestingly, the urinary concentrations of the two pregnanetriol 3-glucuronates are tightly and positively correlated with male social rank (Keller-Costa et al. 2014b), suggesting that they may serve a second function as mediators of male aggression.

Dominant male tilapia also increase urination frequency during aggressive disputes with rivals. In contrast, a submissive male never releases urine towards its opponent (Barata et al. 2007). In male pairs where urination is prevented, interactions escalate more frequently and rapidly into highly aggressive behaviors and mouth-to-mouth fights than in unrestricted controls (Keller-Costa et al. 2012). Furthermore, in control pairs, the male displaying the first aggressive behavior usually wins the subsequent fight; this is not the case in males prevented from urinating (Keller-Costa et al. 2012). This strongly suggests that chemical signals present in a rival's urine are a key regulator of social interactions; they allow prediction of the outcome of a subsequent fight, and prevent males from engaging in energy-demanding escalating battles (Ros et al. 2006) if they discern the opponent to be of higher rank (Keller-Costa et al. 2012). It thus is likely that the dominance signal demonstrates a high resource holding potential (RHP; Parker 1974) of a male. Such a mechanism ultimately contributes to the overall stability of the social group.

Another way of testing aggressive responses in fish is by using mirrors. Fishes are unable to recognize their mirror image, and attack it as if it were a rival (Dijkstra et al. 2012; Oliveira et al. 2005; Riebli et al. 2011). An advantage of this approach is that only a visual stimulus is presented to the fish; addition of any other stimulus (e.g., chemical) is under the control of the experimenter. Here, we used this assay to test two hypotheses: 1) that dominant male urine reduces aggressiveness in males fighting their mirror image; and 2) that the two pregnanetriol 3-glucuronates identified as a sex pheromone to females are mediators of male aggressive behavior. The effect of pregnanetriol 3-glucuronates, dominant male urine (DMU) and its C18 solid phase extraction (C18-SPE) fractions (the nonpolar C18-SPE eluate of DMU or its polar C18-SPE filtrate), and combinations, were applied in the mirror test, and behaviors were quantified. The relationship between dominance status and olfactory sensitivity to urine and the same urinary C18-SPE fractions was assessed by using the electro-olfactogram (EOG).

Methods and Materials

Ethical Statement Fish care and experimentation complied with the national legislation for the use of laboratory animals under a Group-1 license issued by the Veterinary General Directorate of the Ministry of Agriculture, Rural Development and Fisheries of Portugal.

Experimental Animals Mozambique tilapia were raised in captivity from a brood-stock maintained at the University of Algarve (Faro, Portugal). Mature males and females were kept together in several 500 l stock tanks with a sand substratum in aerated recirculating freshwater at 25–27 °C and 12 L: 12D photoperiod until used either to set up social groups for urine collection or in behavioral or electrophysiological studies. Fish were fed once per day with commercial cichlid feed.

Assessment of Social Status and Collection of Urine Each social group consisted of five females and five males; males were of similar standard length (SL, mm; Table 1) and body mass (coefficient of variation of body-mass < 5 %; Table 1). Males were color tagged (T-Bar anchor FD94, Floy Tag Inc., Seattle, WA, USA) for systematic daily focal observation of their behavior, as previously described (Barata et al. 2007; Keller-Costa et al. 2012). Briefly, the frequency of submissive behaviors (submissive displays, flight) during agonistic interactions or absence of dark coloration without social interaction, and dominant behaviors such as aggression (biting, chasing, lateral displays, circling, or mouth-to-mouth fights), nest digging, courtship towards females, or dark coloration without social interaction were recorded over five min for each male. A daily and 5 d average dominance index (DI), ranging from zero to one, was calculated for each male as the ratio between the sum of dominant behaviors and the sum of dominant and subordinate behaviors (Barata et al. 2007; Keller-Costa et al. 2012).

Urine was collected from each male after each daily observation by gently squeezing the area above and anterior to the

Table 1 | Stimulus types and characteristics of recipient males: DMU - dominant male urine; Control – control water; Steroids - synthetic mixture of the two pregnanetriol 3-glucuronates (20α-P-3-G and 20β-P-3-G); DMU eluate - C18-SPE eluate of dominant male urine; DMU filtrate – aqueous C18-SPE filtrate of dominant male urine (data are given as mean ± s.e.m)

Stimulus	N^a	SL (mm)[b]	BM (g)[c]	Latency MA (min)[d]	Total MA[e]
Set 1					
DMU	11	108 ± 5.1	43.1 ± 5.6	6.0 ± 1.7	59 ± 23 *a*
Control	11	109 ± 3.5	39.5 ± 3.2	2.3 ± 0.8	121 ± 15 *b*
Steroids	10	119 ± 6.8	43.3 ± 4.0	5.4 ± 1.8	181 ± 43 *b*
Set 2					
DMU eluate	12	112 ± 3.2	40.5 ± 2.5	4.4 ± 1.5	121 ± 30 *b*
Steroids + DMU filtrate	11	112 ± 3.8	43.6 ± 4.1	4.3 ± 1.2	123 ± 19 *b*
DMU eluate + filtrate	11	108 ± 3.1	–	8.5 ± 1.8	29 ± 12 *a*

[a] Number of male replicates

[b] Standard body length

[c] Body mass

[d] Latency until first mouth attack (MA); one-way ANOVA; Set 1: $P = 0.196$; Set 2: $P = 0.100$, no significance n.s

[e] Total number of mouth attacks during experiment; letters (a,b) behind values indicate significant differences; one-way ANOVA followed by Tukey-test; Set 1: $F_{2,29} = 4.511$, $P = 0.02$; Set 2: $F_{2,31} = 5.706$, $P = 0.008$

urogenital papilla and stored at −20 °C until use. From this urine, two large urine pools then were prepared and used as test stimuli. One pool (~30 ml) was derived from 30 mature dominant males (body-mass = 81.8 ± 40.5 g, mean ± SD) with a 5 d average dominance-index greater than 0.5 and was partitioned into 1 ml aliquots that served as the dominant male urine (DMU) stimulus. The second pool (~34 ml) was derived from 18 mature dominant males (body mass = 194.8 ± 58 g, mean ± SD) with a 5 d average dominance-index greater than 0.5, and was solid-phase extracted to provide the DMU eluate and DMU filtrate stimuli (see below). Due to the limited amount of urine available and the time taken to collect sufficient urine for all behavioral assays, experiments were structured into two sets: a first set in which we tested the effect of dominant male urine (urine pool 1) vs. a water control and a mixture of the pregnanetriol-3-glucuronates (sex pheromone); then, a second set in which we tested the effect of the solid-phase extracted fractions (i.e., DMU eluate and DMU filtrate derived from urine pool 2) and their combination. Because of the limited availability of equally-sized mature test males and the time-consuming preparation, we had to waive the inclusion of a common stimulus (e.g., DMU) in the two experimental sets.

Preparation of Stimuli for the Mirror Assay The following stimuli (Table 1) were prepared for the mirror assay (see below).

(1) Water control (collected at the beginning of each experimental day from the recirculating assay system (Fig. 1) at an outlet after the filter passage).
(2) Synthetic pregnanetriol 3-glucuronate mixture consisting of a 500 μM solution of a 4:1 mixture of 5β-pregnane-3α,17α,20β-triol 3-glucuronate (20β-P-3-G; 400 μM) and 5β-pregnane-3α,17α,20α-triol 3-glucuronate (20α-P-3-G; 100 μM). The same ratio and concentration were found previously in a pool of dominant male tilapia urine and shown to be active in females (Keller-Costa et al. 2014b). Nevertheless, the ratio may not be critical as both pregnanetriol 3-glucuronates are detected by the same olfactory receptor(s) (Keller-Costa et al. 2014a). One ml aliquots of the synthetic steroid mixture were stored in glass vials at −20 °C until use.
(3) Dominant male urine (DMU) collected from 30 mature dominant males (body mass = 81.8 ± 40.5 g, mean ± SD) with a 5 d average dominance-index greater than 0.5; a total of 30 ml urine was collected and partitioned into 1 ml aliquots.
(4) DMU eluate, the solid-phase extracted methanol eluate of a 34 ml pool of urine collected from 18 mature dominant males (body mass = 194.8 ± 58 g, mean ± SD).
(5) DMU aqueous filtrate from (4) plus synthetic steroid mix as in (2).
(6) DMU eluate from (4) plus DMU filtrate from (4); this stimulus represents the reconstituted dominant male urine.

The C18-SPE cartridges (500 mg C18 sorbent cartridges, 6 ml glass reservoir, Isolute®, Biotage) were prepared by activation with 5 ml methanol and subsequent washing and wetting with 5 ml ultra-pure water under the use of a vacuum pump. Each aliquot of dominant male urine (5 ml) from pool 2 was passed through a fresh cartridge, and the resulting aqueous DMU filtrate (~5 ml) containing the highly polar compounds was collected. The non-polar (steroid containing) urine fraction (DMU eluate, **4, 6**) then was eluted with 5 ml of methanol. Thus, in total, seven separate SPE extractions of about 5 ml of urine were each performed to extract urine pool 2 of ca 34 ml. The resulting seven DMU filtrates (5 ml each) were pooled together before being aliquoted (1 stimulus =1 ml aliquot). Likewise the seven DMU eluates (5 ml each) were

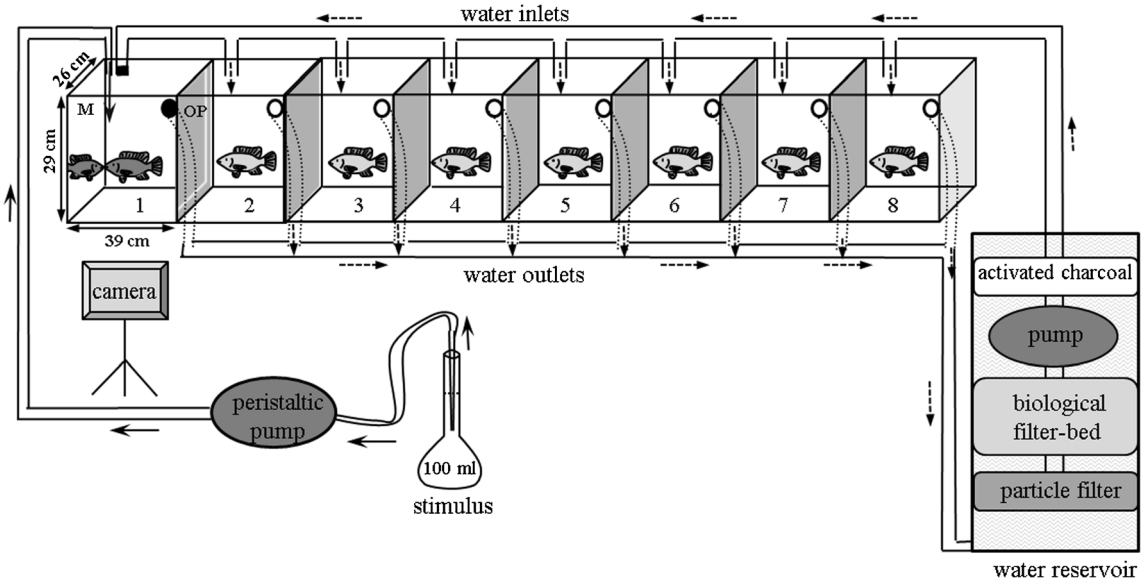


Fig. 1 Experimental set-up to study stimulus dependent mirror-elicited behavior in male tilapia. Eight aquaria, connected to a recirculating freshwater system, allowed consecutive testing of eight male replicates on the same day. Males were socially isolated in their aquaria without visual contact with each other, for 7 d before the experiment. During this period, the mirror was concealed by an opaque plate (OP). The water in- and outlet of the aquarium was closed before each trial, and the mirror (M) exposed. Each test male was given up to 20 min time to approach the mirror and/or show a first reaction towards it. As soon as this happened, a chemical stimulus was delivered close to the mirror image in 5 one-min pulses separated by one-min intervals. The animal's behavior was video recorded for 15 min

pooled together and then aliquoted (1 stimulus =1 ml aliquot). All 1 ml aliquots were stored in glass vials at −20 °C until use.

Immediately before the start of a behavioral trial, an aliquot was thawed and diluted 1:100 *v*/v in water collected from the recirculating assay system at an outlet after the filter passage. The same water also was used as the water control stimulus (**2**). A preliminary test showed that the 1:100 dilution of 1 ml of pure methanol in the water of the recirculation system had no effect on male behavior.

Behavioral Assays All males for the behavioral assays were of similar size (Table 1; one-way ANOVA on standard length (SL): $F_{60,65} = 0.875$; $P = 0.504$) but smaller than the urine donor males; this was due to size constraints of the experimental set-up (Fig. 1) and to minimize the volume of stimuli used. None of the males that performed the mirror trial contributed as a urine donor to one of the urine pools used as chemical stimulus in this study. Furthermore, none of the mirror trial-performing males had any previous contact with the urine donor males; mirror trial-performing males and urine donor males derived from separate stock tanks. This was to avoid any effect of recognition of a familiar male competitor, or self-recognition, by smelling familiar urine. Each male performed the mirror trial only once. Before transfer to the mirror aquarium, each male was housed for 7 d with 3–5 females in a 200 l tank to minimize possible effects from previous intra-sexual competitions. All males were reproductively active; spawning occurred spontaneously, but eggs were removed from the female's mouth to stimulate the initiation of a new ovulatory cycle. Males did not express high aggression towards females; however, they occasionally chased-away non-ovulatory females from the nesting-side.

Eight glass aquaria (39 × 26 × 29 cm; ca. 29 l) were connected to a closed water circuit (Fig. 1). De-chlorinated tap-water at 27 °C was pumped through a three-step filtration system (mechanical, biological, and chemical/activated charcoal) before returning to the assay aquaria. Each aquarium had aeration, a sandy bottom, the sides covered with opaque polystyrene plates (except the frontal observer side), and a sliding opaque plate concealing a mirror attached to the inner right side. Each male was socially isolated in its assay aquarium for 7 d to further standardize the (social) environment before testing, and to allow habituation to the test environment (Oliveira et al. 1996). This set-up allowed testing eight males consecutively on the same day without moving or disturbing the animals. At the start of the assay, the water inlet and outlet of the assay tank were closed to avoid carry-over of the test-stimulus from one assay-tank to the next. Upon completion of an assay, the tank remained disconnected from the re-circulating system until all assays in all other tanks had been performed on the respective experimental day.

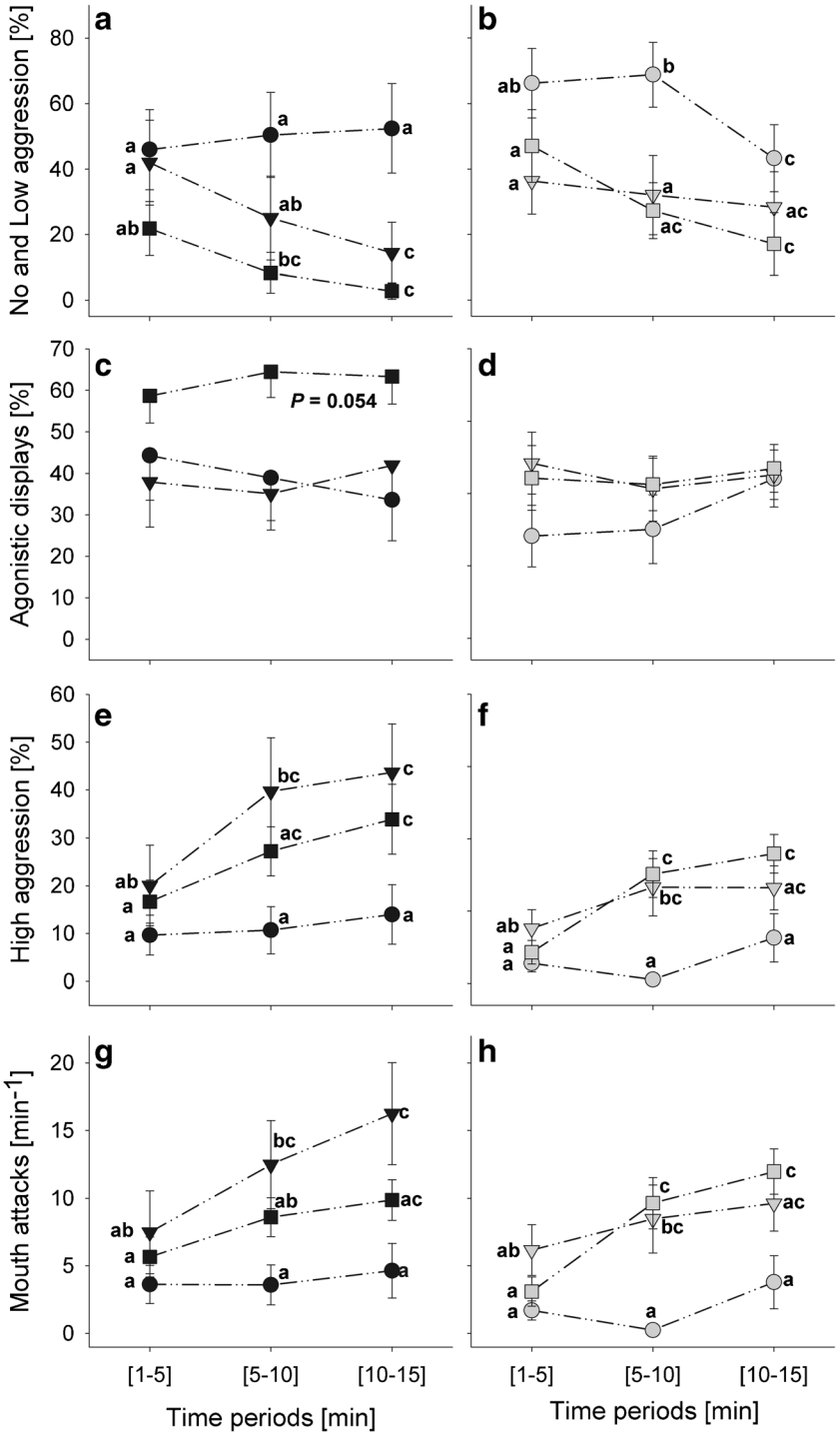
After the closure of the inlet and outlet, the sliding plate was lifted, exposing the mirror. Each male was allowed up to 20 min to approach the mirror and/or show a first reaction towards it. Although the latency to initiate the first interaction with the mirror varied among males, the mean latency until the

first reaction between the different treatment groups was statistically similar (one-way ANOVA, $P = 0.328$). Immediately after the first approach/reaction, the chemical stimulus was applied via a silicon tube and peristaltic pump, close to the mirror, in 5 one-min pulses (20 ml.min^{-1}), separated by one-min intervals. Male behavior was recorded by a digital camera for 15 min after starting stimulus delivery. The experimenter, who was not visible to the male, was able to follow the fish's behavior in real time on a small display in order to control the stimulus delivery and camera. After each trial, the stimulus delivery system and video camera were discreetly moved to another randomly selected aquarium to start a new test. At the end of the experimental day, aquaria, filters, pumps, and tubing were thoroughly cleaned, and water in the circuit exchanged before the next set of males moved in. Ten to 12 valid replicates (i.e., males showing an aggressive response to their mirror image) were obtained for each stimulus type.

Male behaviors recorded on digital video were quantified using The Observer XT software v. 8 (Noldus Information Technologies, Wageningen, The Netherlands). Behaviors and percentage of time in each of the following states were scored: *non-aggressive* (hovering in the water column, fins not erect, grey coloration) together with *low aggression* (approaching and/or staying in front of the mirror with erect dorsal fin, change to darker coloration); *aggressive displays* (lateral and frontal, tail beating, expansion of mouth and/or opercula); *high aggression* (frontal attacks; duration of consecutive mirror bites). In addition, mouth attacks (mirror bites) per min were counted (as single events). Although behavioral analysis was not analyzed blind (experimental design made this difficult), observations were validated by a second independent observer. The experiments were carried out in two series. First, the effect of the two pregnanetriol 3-glucuronates was investigated in comparison with whole DMU and no chemical stimulus (water control). Second, having established that the two pregnanetriol 3-glucuronates alone did not replicate the aggression-reducing effect of whole DMU, the effect of the eluate alone, and the filtrate plus the two pregnanetriol 3-glucuronates were compared with reconstituted DMU (eluate plus filtrate). One-way ANOVA followed by Tukey's test (when significant) was used to compare the effect of the different stimuli on total number of mouth attacks, latency until the first mouth attack following application of the stimulus, and total percentage of time dedicated to each state of aggression. Two-way repeated measures ANOVA (including a one-factor repetition with time as repeated factor) was used to compare the effects of the different chemical stimuli (independent variable) and time (dependent variable) on aggressive behaviors. The data for each test stimulus (i.e., control water, DMU, steroid mix etc.) were considered as independent because each male was tested only once and only with one type of stimulus. However, the data for time (i.e., 0–5 min vs. 5–10 min and 10–15 min) were considered dependent for each male replicate because the behavior of a male during the first five min will naturally affect his behavior during the following minutes of observation. The test for homogeneity of variance (Levene's test) was passed successfully for all two-way repeated measures ANOVAS applied here ($P \geq 0.263$ for all tests). The Tukey's test was used as the *post-hoc* method in all two-way repeated measures ANOVAs. The significance level was 5 %.

Electro-Olfactogram (EOG) Recordings Preparation of animals and recording of the EOG was carried out as previously described (Frade et al. 2002). The DC voltage signal was pre-amplified, then filtered (low-pass 50 Hz) and amplified (Neurolog NL102, Digitimer Ltd., Welwyn Garden City, UK), digitized (Digidata 1322 A, Axon Instruments, Inc., now Molecular Devices, Sunnyvale, CA, USA), and stored on a PC running Axoscope software (version 9.1). The olfactory potency of urine and respective C18-SPE urinary eluates and filtrates collected from dominant (DI $\geq$ 0.8; N = 6; mean ± SD, body mass = 150 ± 31 g; SL = 168 ± 11.7 mm), intermediate (DI $\geq$ 0.2 but $\leq$0.5; N = 5; mean ± SD, body mass = 156 ± 26.9 g; SL = 171 ± 12.3 mm), and subordinate (DI $\leq$ 0.16; N = 6; mean ± SD, body mass = 150 ± 42.5 g; SL = 170 ± 13.5 mm) males were assessed. Urine donors for the EOG were different from the donor males contributing to the urine pools used in the mirror assay; extraction of urine samples was carried out as described above.

The electro-olfactogram was recorded from six adult fish: three tilapia males (mean ± SD, body mass = 157.9 ± 19.1 g) and three females (mean ± SD, body mass = 110.3 ± 15.4 g) at a dilution of 1:10 000 in water (*v*/v). EOG data were normalized to the response to 10^{-5} M L-serine standard. Statistical analyses were performed on pooled data from both sexes, since EOG amplitudes of male and female responses were statistically similar (Wilcoxon signed rank test; DMU $P = 0.196$; DMU eluate $P = 0.523$; DMU filtrate $P = 1.00$). Each EOG test fish (male or female) was exposed sequentially to all three types of test stimuli (i.e., 6 DMU samples, 6 DMU eluate samples and 6 DMU filtrate samples). The order in which the stimuli were applied was varied among fish. One-way repeated measures ANOVA followed by Tukey's *post-hoc* test were used to compare olfactory responses to urine, urine eluate, and urine filtrate. Data were normally distributed and of equal variance. Further, to assess the relationship between olfactory responses to urine filtrate samples and the social rank (dominance index) of the urine donor males, a Spearman correlation analysis was performed. The olfactory potency of urine from 17 donor males (6 dominants, 5 intermediates, and 6 subordinates; see above) was assessed using the same six adult fish (three males and three females). The significance level was 5 % for all analyses.



Results

Male Aggressive Behavior In total, 176 males were exposed to their mirror image (Fig. 1), of which 78 (44 %) approached and/or reacted to it. The other 98 males (56 %) remained immobile at the bottom of the aquarium or hovered in the water column. Twelve reactive males (10 exposed to control water and 2 to the pregnanetriol 3-glucuronate mixture) showed clear mating behavior (i.e., courtship, nest digging, deep black coloration), instead of aggression, during the entire observation period and were excluded from further analysis. Males reacting aggressively (as expected) usually first approached the mirror with slow movements and an inconspicuous light grey coloration. At the mirror, some males immediately erected their fins and positioned themselves lateral to the image, exhibiting a posture of low aggression. Their color changed progressively into darker shades of grey, and they exhibited lateral and frontal displays that - with time -

◀ **Fig. 2 Aggressive behaviors of receiver males over time**. Relative duration of non- or low aggression (**a**, **b**), aggressive display (**c**, **d**), and highly aggressive (**e**, **f**) states, and frequency of mouth attacks (**g**, **h**) of males exposed to their mirror image and exposed to the pregnanetriol 3-glucuronate mix (black triangles, $N = 10$), water control (black squares, $N = 11$), or dominant male urine (DMU, black circles, $N = 11$) (**a**, **c**, **e**, **g**); or with C18-SPE DMU eluate (grey triangles, $N = 12$), C18-SPE DMU filtrate plus pregnanetriol 3-glucuronate mix (grey squares, $N = 11$), or reconstituted DMU, i.e., eluate plus filtrate (grey circles, $N = 11$) (**b**, **d**, **f**, **h**). All values are means ± SEM of time (%) or frequency (min^{-1}) observed in each of the 3 five min periods starting at the onset of chemical stimulation. Two-way repeated measures ANOVA (with 'time' as repeated factor) followed by the *post-hoc* Tukey-test when significant were used to compare stimuli within each five min period and over time. Different letters next to mean data points indicate significant differences. (**a**) stimulus: $F = 3.952$, $P = 0.003$; time: $F = 5.703$, $P = 0.005$; interaction between stimulus and time: $F = 3.12$, $P = 0.022$; (**b**) stimulus: $P = 0.065$ n.s.; time: $F = 9.44$, $P < 0.001$; interaction: $P = 0.123$ n.s.; (**c**) stimulus: $P = 0.054$ n.s.; time: $P = 0.982$ n.s. interaction: $P = 0.508$ n.s.; (**d**) stimulus: $P = 0.454$ n.s.; time: $P = 0.258$ n.s.; interaction: $P = 0.534$ n.s.; (**e**) stimulus: $F = 4.275$, $P = 0.024$; time: $F = 6.132$, $P = 0.004$; interaction: $P = 0.384$ n.s.; (**f**) stimulus: $F = 4.724$, $P = 0.016$; time: $F = 13.914$, $P < 0.001$; interaction: $F = 4.135$, $P = 0.005$; (**g**) stimulus: $F = 4.511$, $P = 0.02$; time: $F = 7.397$, $P = 0.001$; interaction: $P = 0.150$ n.s.; (**h**) stimulus: $F = 5.706$, $P = 0.008$; time: $F = 15.13$, $P < 0.001$; interaction: $F = 4.314$, $P = 0.004$

escalated into high aggression (biting their mirror-image). Other males instead first spent up to two minutes exploring the mirror, swimming up and down and along it while nudging it gently with their mouth closed, before they would assume color changes and a similar behavior pattern as described above.

Some variation in activity between the two experimental sets could be observed in that the focal males of the second experimental set seemed to react slightly less aggressively towards their own mirror image than those of the first. This might be due to the two different urine pools used in the two experimental sets, although the mean dominance index of the urine donors was similar (pool 1 DI = 0.78 ± 0.14; pool 2 DI = 0.76 ± 0.39, mean ± SD).

The various chemical stimuli within one experimental set affected aggressiveness of receiver males differently. However, the latencies (Table 1) and aggressive behaviors within the first five minutes of observation were similar (Fig. 2). In males exposed to dominant male urine (DMU), the percentage of time allocated to the different behavioral states (Fig. 2a, c and e) and the frequency of mouth attacks (Fig. 2g) remained relatively constant, whereas in control males, non- and low-aggression behavior decreased, and highly aggressive behavior increased (Fig. 2a, e). Moreover, males spent less time being highly aggressive when exposed to DMU (mean ± SEM, 11.4 ± 4.6 %) compared to the pregnanetriol 3-glucuronate mix (34.4 ± 8.3 %) or water control (25 ± 3.3 %; one-way ANOVA, $F_{2,29} = 4.239$, $P = 0.024$). The total number of mouth attacks was lower in DMU exposed males (mean ± s.e.m., 59 ± 23, $P = 0.02$) than control water (121 ± 15) and pregnanetriol 3-glucuronate mix exposed males (181 ± 43, Table 1). Additionally, the percentage of time allocated to non- and low-aggression behavior was lower in the pregnanetriol 3-glucuronate mix (mean ± SEM, 27.2 ± 11.4 %) and decreased with time, as compared to DMU exposed males (49.6 ± 14.8 %), whereas percentage of time spent in highly aggressive behavior and frequency of mouth attacks increased with time (Fig. 2e, g). However, there was no difference in agonistic displays between control males and DMU or pregnanetriol 3-glucuronate exposed males, although the *P*-value ($P = 0.054$) was close to the significance limit (5 %).

Because aggression was not reduced by the pregnanetriol 3-glucuronate mix alone, we then investigated whether additional components in dominant male urine are necessary to elicit the aggression-reducing effect. Indeed, DMU eluate combined with the DMU filtrate (representing 'reconstituted' DMU) had the lowest number of mouth attacks (Table 1), while the overall pattern of aggressive behavior was similar to that of DMU alone (Fig. 2). The aggression-reducing effect of reconstituted DMU confirms that the elution of active components from the C18-SPE cartridges was efficient and suggests that no active compounds were chemically altered or lost. In contrast, males exposed to only the DMU eluate, or the DMU filtrate combined with the pregnanetriol 3-glucuronates, were more aggressive (one-way ANOVA, $F_{2,31} = 6.13$, $P = 0.006$) with a higher frequency of mouth attacks during the last 10 min ($P = 0.003$, Fig. 2h) than males exposed to reconstituted DMU.

Olfactory Responses to Male Urine and its C18-SPE Fractions As expected, male urine was the most potent olfactory stimulus in the EOG, followed by C18-SPE urine eluate. However, there was also olfactory activity in the C18-SPE urine filtrate (Fig. 3a, b). Interestingly, as previously found for the C18-SPE urine eluate and the pregnanetriol 3-glucuronates (see Introduction), the olfactory potency of the urine filtrate increased with ascending social ranks of the donor males (Fig. 3c).

Discussion

The present study shows that conspecific dominant male urine reduces aggressive behavior during male-male interactions in tilapia, and that the chemical signal consists of both polar and non-polar components. Males fighting their own mirror image react significantly less aggressively when they are exposed to DMU than control males. However, the study also shows that a pregnanetriol 3-glucuronate mix comprising the previously identified male tilapia pheromone (Keller-Costa et al. 2014b), directed at females, is not responsible alone for the

aggression-reducing properties of DMU, thus rejecting our initial hypothesis. Furthermore, neither the C18-SPE DMU eluate on its own, nor the C18-SPE DMU filtrate combined with the pregnanetriol 3-glucuronate mix reduced aggressive escalation towards the mirror. Only reconstituted DMU (i.e., DMU C18-SPE eluate plus DMU C18-SPE filtrate) reduced aggression to the same level as raw DMU. Together, our results strongly suggest that multiple urinary components, present in both the polar (DMU filtrate) and non-polar (DMU eluate) urine fractions, are responsible for the modulation of male aggression.

Often, pheromones affecting animal behavior are odor blends rather than only one or two substance(s) (Wyatt 2014). When the multiple pheromone components are combined in a particular ratio, they then act in synergy. For example, 2,3-dehydroxy-exo-brevicomin and 2-s-butyl-4,5-dohydrothiazole in male mice, *Mus musculus*, urine act synergistically and elicit aggression in receiver males. They are inactive on their own, i.e., when simply added to the water, and only become active when added to urine of castrated animals which, in turn, is inactive alone (Novotny et al. 1985). Both male and female tilapia have olfactory sensitivity to the polar DMU C18-SPE filtrate, and responses were positively correlated with the donor's social status. Moreover, although most olfactory activity in the non-polar DMU C18-SPE eluate is attributed to the pregnanetriol 3-glucuronates (Keller-Costa et al. 2014b), there is evidence that other compounds are present in the DMU C18-SPE eluate that elicit olfactory responses as well. For example, the olfactory response to the whole DMU eluate is larger than the olfactory response to the steroid-containing fraction of the DMU C18-SPE eluate (see Fig. 1D in Keller-Costa et al. 2014b). Those additional non-polar odorants present in the DMU eluate and the polar odorants present in the DMU filtrate likely are important for modulating inter-male aggression.

In the recent literature, there is an increasing discussion on the putative functions of conspecific hydrophilic odorants in various types of social interactions in fishes. However, so far, the only evidence that hydrophilic urinary components, probably trimethylamine, play a role in the communication of social status comes from a report in the fathead minnow (Martinovic-Weigelt et al. 2012). Small major histocompatibility complex (MHC) peptides also have been shown to influence mate choice decision in stickleback (Milinski et al. 2005, 2010) and olfactory imprinting on kin in zebrafish (Hinz et al. 2013). The polar fraction of ovulated female carp holding water synergizes the attraction of prostaglandin to males; these polar products may confer species-specific information, and amino acids as putative constituents have been hypothesized (Lim and Sorensen 2011). Species-specific

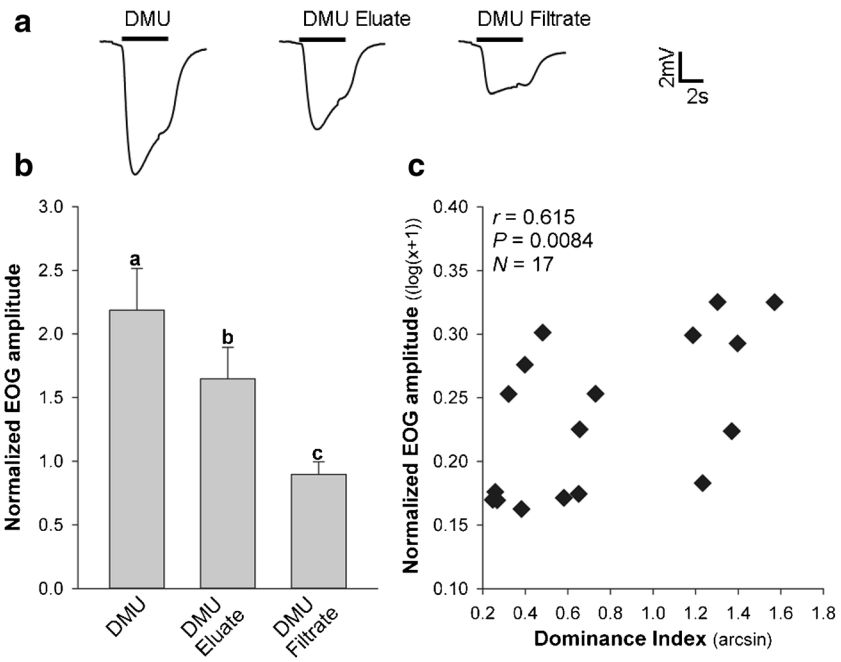


Fig. 3 Olfactory responses to male urine and its C18-SPE fractions at 1:10 000 v/v dilution in water. **a** Typical electro-olfactograms recorded in response to a urine sample and its C18-SPE urine eluate and C18-SPE urine filtrate from a dominant donor male. **b** EOG responses (mean + SEM; normalized to 10^{-5} M L-serine standard) of 6 tilapia (3 males, 3 females) to raw urine, C18-SPE urine eluate and filtrate samples obtained from 6 dominant tilapia males. Urine was the most potent olfactory stimulus, followed by the urine eluate and urine filtrate (one-way RM ANOVA followed by the *post-hoc* Tukey test; $F_{2,10} = 29.423$, $P < 0.001$). **c** Mean EOG responses (normalized to 10^{-5} M L-serine standard; log(x + 1)-transformed) of the same 6 tilapia (3 males, 3 females) to C18-SPE urine filtrate samples (black diamonds) obtained from 17 tilapia males of different social rank (6 dominants, 5 intermediates, 6 subordinates). EOG responses are plotted over the donor male's social status (dominance index in arcsin-transformed values). Olfactory responses to the urine filtrate were positively correlated with social rank (Spearman correlation, $N = 17$, $r = 0.615$, $P = 0.008$)

polar metabolites similarly have been discussed also for the goldfish (Levesque et al. 2011). Circumstantial data implicate urinary amino acids, L-arginine and L-glutamate, in this role in tilapia (Kutsyna et al. 2016). However, further research is necessary to shed light on the identity and function of additional chemical cues released into the urine of tilapia males. Future investigations should clarify whether the function of two identified pregnanetriol 3-glucuronates is restricted to a priming role in females, and if their presence in the urine is relevant to male aggressiveness, or whether they still play a role but in a blend with additional, as yet unidentified, compounds, that act, when combined at a certain ratio, in synergy. The tight positive correlation of the urinary concentration of these steroids to the social rank of the donor male (Keller-Costa et al. 2014b) suggests the latter. Since this study did not test the DMU filtrate alone but in combination with the pregnane 3-triol-glucuronates (or the DMU eluate), the added pregnane 3-triol-glucuronates might have had some kind of inhibitory action on the DMU filtrate. However, this seems unlikely given that both pregnanetriol 3-glucuronates are natural constituents of dominant male urine, and thus always co-occur with the active compound(s) present in the DMU filtrate.

In this study, not all the test individuals reacted to their mirror image. A similar observation was made in another African cichlid, *Pundamilia sp*. (Dijkstra et al. 2012), although the reason(s) remains unclear and could be manifold (see Balzarini et al. 2014 for discussion). It may echo a lower aggressive motivation, a different stress coping style (Øverli et al. 2004), or 'shyness' (Coleman and Wilson 1998; Wilson et al. 1993). Moreover, there is recent evidence that mirror images may not elicit exactly the same behaviors and hormonal or brain responses as when encountering a real opponent (Desjardins and Fernald 2010; Elwood et al. 2014; Oliveira et al. 2005), suggesting that the focal fish recognize something unusual about the mirror image. The mirror image, for example, lacks the possibility for a head-to-tail orientation which many cichlids, including tilapia, show naturally during lateral displays and 'circle-fights' with a real opponent, and this lack could alter the response of the focal animal (Elwood et al. 2014). In the convict cichlid, Elwood et al. (2014) showed that real opponents elicited more displays in the focal male than did mirror images, suggesting that fish respond more to initiatives of a real opponent, one that can be smelled and heard as well as seen. As the mirror image does not initiate moves, the focal fish only moves when it is ready to change its position; this may well explain why several test males did not approach or attack the mirror.

Interestingly, 45 % of control males showed clear mating behavior (courtship, nest digging, black coloration) towards their mirror image. Courting seems non-adaptive in this context, since the mirror image of the focal individual displays a male rather than a female. However, male-male courtship has been described in the Mozambique tilapia and suggested to be a 'side-effect' of high sexual motivation, making males less discriminatory (Oliveira and Almada 1998). In circumstances of high competition, dominant males are more likely to attract any neutral or light colored individual that looks like a potential mate, and leave discrimination to a later stage (Oliveira and Almada 1998). This may explain the courtship observed in some of the mirror-stimulated males, since when approaching the mirror for the first time, tilapia males usually adopt a light grey (female-like) coloration before changing to a darker (male-like) shade. All responding males built and attended nests in their aquarium prior to experiments, despite social isolation, which may indicate their high sexual motivation. In contrast, courting behavior rarely was observed in males exposed to the synthetic steroid mix and *never* in males exposed to male urine or urinary fractions. This strengthens the emerging evidence that chemical cues facilitate discrimination of conspecifics and their interactions in this cichlid (Almeida et al. 2005; Barata et al. 2007, 2008; Keller-Costa et al. 2012; Miranda et al. 2005).

We conclude that dominant male urine reduces aggression in conspecifics, which helps to prevent males from engaging in highly risky, energy-demanding escalating conflicts with rivals. However, the two urine-derived pregnanetriol 3-glucuronates, a sex pheromone stimulating the female reproductive system, are - alone - insufficient to reduce male-male aggression. The urinary signal that mediates male aggression is a multi-component pheromone consisting of compounds from both polar and non-polar urine fractions. Thus, male-male aggression is reduced by a chemical signal different from that of ovulation priming in females.

Acknowledgments We are grateful to Drs C. Paetz, Y. Nakamura, and B. Schneider (Max Planck Institute of Chemical Ecology, Jena, Germany) for synthesizing the steroid-conjugates 20α-P-3-G and 20β-P-3-G. We also thank João Reis and Miguel Viegas (University of Algarve, Faro, Portugal) for help in rearing and maintaining tilapia.

This work received national funds through FCT - Foundation for Science and Technology, Portugal, through project UID/Multi/04326/2013 and fellowships SFRH/BD/46192/2008 to T.K.-C. and SFRH/BPD/67008/2009 to J.L.S.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

Almeida OG, Miranda A, Hubbard PC, Frade P, Barata EN, Canário AVM (2005) Urine as a social signal in the Mozambique tilapia (*Oreochromis mossambicus*). Chem Senses 30 (supplement 1):i309-i310.

Balzarini V, Taborsky M, Wanner S, Koch F, Frommen JG (2014) Mirror, mirror on the wall: the predictive value of mirror tests for measuring aggression in fish. Behav Ecol Sociobiol 68:871–878

Barata EN, Fine JM, Hubbard PC, Almeida OG, Frade P, Sorensen PW, Canário AVM (2008) A sterol-like odorant in the urine of Mozambique tilapia males likely signals social dominance to females. J Chem Ecol 34:438–449

Barata EN, Hubbard PC, Almeida OG, Miranda A, Canário AVM (2007) Male urine signals social rank in the Mozambique tilapia (*Oreochromis mossambicus*, Peters 1852). BMC Biol 5

Bruton MN, Boltt RE (1975) Aspects of the biology of *Tilapia mossambica* Peters (pisces: cichlidae) in a natural freshwater lake (Lake Sibaya, South Africa). J Fish Biol 7:423–445

Coleman K, Wilson DS (1998) Shyness and boldness in pumpkinseed sunfish: individual differences are context-specific. Anim Behav 56: 927–936

Desjardins JK, Fernald RD (2010) What do fish make of mirror images? Biol Lett 6:744–747

Dijkstra PD, Schaafsma SM, Hofmann HA, Groothuis TGG (2012) 'Winner effect' without winning: unresolved social conflicts increase the probability of winning a subsequent contest in a cichlid fish. Physiol Behav 105:489–492

Elwood R, Stoilova V, McDonnell A, Earley RL, Arnott G (2014) Do mirrors reflect reality in agonistic encounters? A test of mutual cooperation displays. Anim Behav 97:63–67

Frade P, Hubbard PC, Barata EN, Canário AVM (2002) Olfactory sensitivity of the Mozambique tilapia to conspecific odours. J Fish Biol 61:1239–1254

Hinz C, Namekawa R, Behrmann-Godel J, Oppelt C, Jaeschke A, Muller A, Friedrich RW, Gerlach G (2013) Olfactory imprinting is triggered by MHC peptide ligands. Scientific Reports 3.

Hubbard PC, Mota V, Keller-Costa T, da Silva JP, Canário AVM (2014) Chemical communication in tilapia: a comparison of *Oreochromis mossambicus* with *O. niloticus*. Gen Comp Endocrinol 207:13–20

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

Keller-Costa T, Canário AVM, Hubbard PC (2015) Chemical communication in cichlids: a mini-review. Gen Comp Endocr 221:64–74

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, Canário AVM (2014b) Identity of a tilapia pheromone released by dominant males that primes females for reproduction. Curr Biol 24:2130–2135

Keller-Costa T, Lopes OS, Almeida O, Hubbard PC, Iacovella A, Lima M, Barata EN, Canário AVM (2012) Muscular hypertrophy of urinary bladders in dominant tilapia facilitates the control of aggression through urinary signals. Behaviour 149:953–975

Kutsyna O, Velez Z, Canário AVM, Keller-Costa T, Hubbard PC (2016) Variation in urinary amino acids in the Mozambique tilapia: a potential signal of dominance or individuality? In: Schulte B, Goodwin T, Ferkin M (eds) Chemical signals in vertebrates, vol 13. Springer International Publishing, pp 189–203

Levesque HM, Scaffidi D, Polkinghorne CN, Sorensen PW (2011) A multi-component species identifying pheromone in the goldfish. J Chem Ecol 37:219–227

Lim H, Sorensen PW (2011) Polar metabolites synergize the activity of prostaglandin F-2 alpha in a species-specific hormonal sex pheromone released by ovulated common carp. J Chem Ecol 37:695–704

Martinovic-Weigelt D, Ekman DR, Villeneuve DL, James CM, Teng Q, Collette TW, Ankley GT (2012) Fishy aroma of social status: urinary chemo-signalling of territoriality in male fathead minnows (*Pimephales promelas*). PLoS One 7:e46579

Maruska KP, Fernald RD (2012) Contextual chemosensory urine signaling in an African cichlid fish. J Exp Biol 215:68–74

Milinski M, Griffiths S, Wegner KM, Reusch TBH, Haas-Assenbaum A, Boehm T (2005) Mate choice decisions of stickleback females predictably modified by MHC peptide ligands. Proc Natl Acad Sci U S A 102:4414–4418

Milinski M, Griffiths SW, Reusch TBH, Boehm T (2010) Costly major histocompatibility complex signals produced only by reproductively active males, but not females, must be validated by a 'maleness signal' in three-spined sticklebacks. P Roy Soc B-Biol Sci 277: 391–398

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

Novotny M, Harvey S, Jemiolo B, Alberts J (1985) Synthetic pheromones that promote inter-male aggression in mice. Proc Natl Acad Sci U S A 82:2059–2061

Oliveira RF, Almada VC (1998) Mating tactics and male-male courtship in the lek-breeding cichlid *Oreochromis mossambicus*. J Fish Biol 52:1115–1129

Oliveira RF, Almada VC, Canário AVM (1996) Social modulation of sex steroid concentrations in the urine of male cichlid fish *Oreochromis mossambicus*. Horm Behav 30:2–12

Oliveira RF, Carneiro LA, Canário AVM (2005) No hormonal response in tight fights. Nature 437:207–208

Øverli Ø, Korzan WJ, H glund E, Winberg S, Bollig H, Watt M, Forster GL, Barton BA, Øverli E, Renner KJ, Summers CH (2004) Stress coping style predicts aggression and social dominance in rainbow trout. Horm Behav 45:235–241

Parker GA (1974) Assessment strategy and the evolution of fighting behaviour. J Theor Biol 47:223–243

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 129B:645–651

Riebli T, Avgan B, Bottini A-M, Duc C, Taborsky M, Heg D (2011) Behavioural type affects dominance and growth in staged encounters of cooperatively breeding cichlids. Anim Behav 81:313–323

Ros AFH, Becker K, Oliveira RF (2006) Aggressive behaviour and energy metabolism in a cichlid fish, *Oreochromis mossambicus*. Physiol Behav 89:164–170

Sorensen PW, Pinillos M, Scott AP (2005) Sexually mature male goldfish release large quantities of androstenedione into the water where it functions as a pheromone. Gen Comp Endocr 140:164–175

Stacey N (2015) Hormonally derived pheromones in teleost fishes. In: Sorensen PW, Wisenden BD (eds) Fish pheromones and related cues. John Wiley & Sons, Inc., Ames, pp. 33–88

Stacey N, Sorensen P (2009) Hormonal pheromones in fish. In: Pfaff DW, Arnold AP, Etgen AM, Fahrbach SE, Rubin RT (eds) Hormones, brain and behavior., vol 1. 2nd edn. Academic Press, San Diego, pp 639–681

Wilson DS, Coleman K, Clark AB, Biederman L (1993) Shy bold continuum in pumpkinseed sunfish (*Lepomis gibbosus*) - an ecological study of a psychological trait. J Comp Psychol 107:250–260

Wyatt TD (2014) Pheromones and animal behavior. Chemical signals and signatures. 2nd Edition edn. Cambridge University Press, Cambridge

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. Proc Natl Acad Sci U S A 103:15370–15374