CHEMICAL IDENTIFICATION OF DOMINANCE PHEROMONES IN MOZAMBIQUE TILAPIA MALES

URINARY PHEROMONES IN TILAPIA

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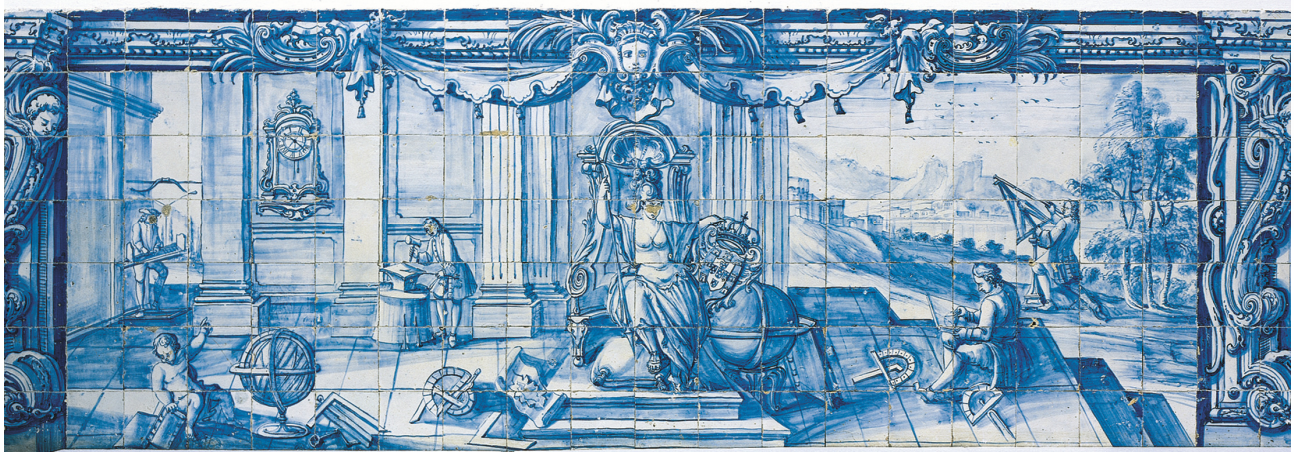

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Abstract

Fishes use pheromones (intraspecific chemical messages) to coordinate reproduction, migration and social interactions but their identity is rarely known. In the Mozambique tilapia (*Oreochromis mossambicus*), a social, lek-breeding cichlid, reproduction and male aggression are mediated through urinary cues released by dominant males. The main goal of this thesis was the chemical identification of such pheromones and establishment of their function(s). Two steroid glucuronates (5β-pregnane-3α,17,20β-triol 3-glucuronate and its 20α-epimer) were identified as the most potent odorants in male urine. Both steroids act, *via* a specific olfactory receptor mechanism, on the females' endocrine axis, stimulating oocyte maturation. However, in contrast to dominant male urine, these steroids on their own do not reduce male aggression in receivers, suggesting that multiple, as yet unidentified, compounds are likely responsible for this effect. In conclusion, dominant tilapia males release pregnanetriol glucuronates *via* their urine as a sex pheromone, likely to synchronize spawning and enhance reproductive success.

Resumo

Indentificação química das feromonas de dominância dos machos da tilápia moçambicana

Os peixes usam feromonas (mensagens químicas intra-específicas) para coordenar a reprodução, migrações e interações sociais, cuja identidade é pouco conhecida. Na tilápia moçambicana (*Oreochromis mossambicus*), um ciclídeo social que se reproduz em agregações, a reprodução e agressão entre machos são mediados por odores libertados pela urina de machos dominantes. O objetivo principal desta tese é determinar a identidade e função destes. Os esteróides 5β-pregnane-3α,17,20β-triol 3-glucurónido e o seu epímero 20α foram identificados como os compostos mais potentes presentes na urina de machos. Ambos atuam no eixo endócrino reprodutor das fêmeas através de um recetor olfativo específico e estimulam a maturação dos ovócitos. Ao contrário da urina dos machos, estes esteróides por si só não reduzem a agressão dos machos recetores, sugerindo a presença de múltiplos compostos, ainda por identificar, responsáveis por este efeito. Conclui-se que os machos dominantes da tilápia libertam uma feromona sexual através da urina que sincroniza a reprodução e melhora o seu sucesso reprodutor.

Zusammenfassung

Chemische Identifizierung der Dominanz-Pheromone männlicher Weißkehl-Buntbarsche

Fische nutzen Pheromone (chemische Botenstoffe, die innerhalb einer Art Nachrichten vermitteln), um wichtige Aspekte ihres Lebens zu koordinieren, z.B. Fortpflanzung, Migration sowie verschiedene soziale Wechselbeziehungen. Häufig kennen wir jedoch die Identität dieser Moleküle nicht und somit bleibt unser Verständnis über die genaue Funktionsweise von Pheromonen begrenzt. Der aus Afrika stammende Weißkehl-Buntbarsch (L. *Oreochromis mossambicus*; oft auch Tilapia genannt) gehört in die Gruppe der Barschverwandten und weist ein hoch komplexes Sozialverhalten vor. In sogenannten Balzarenen stellen die Männchen Rangordnungen auf. Die Dominantesten verteidigen ein Nest in der Arenamitte und werden von fortpflanzungsbereiten Weibchen bevorzugt. Über den Urin geben dominante Männchen Pheromone als wirksame Geruchsstoffe ab, um aggressive Auseinandersetzungen mit anderen Männchen zu schlichten, aber auch um Weibchen anzulocken und zum Laichen zu stimulieren. Das Hauptziel dieser Doktorarbeit war es, die chemische Identität dieser Geruchsstoffe aufzuklären und ihre Funktion als Pheromon zu überprüfen. Zwei mit Glucuronat konjugierte Steroide (5β-Pregnan-3α,17α,20β-triol 3-Glucuronat und sein 20α-Epimer) wurden als Hauptkomponenten im Urin des Weißkehl-Buntbarsches identifiziert. Beide Substanzen werden sowohl vom weiblichen als auch männlichen Geruchssinn mit hoher Sensitivität und über einen spezifischen olfaktorischen Rezeptormechanismus wahrgenommen. Darüber wird das Hormonsystem der Weibchen angeregt, was die Endreifung der Oozyten bewirkt. Im Gegensatz zum Urin dominanter Männchen, vermindern diese zwei Steroide allein jedoch nicht Aggressionen zwischen Männchen. Höchstwahrscheinlich bedarf es dazu eine komplexere Mischung von noch nicht identifizierten Substanzen aus dem Urin. Aus dieser Arbeit lässt sich schlussfolgern, dass dominante Weißkehl-Buntbarschmännchen ein Sexpheromon, bestehend aus zwei konjugierten Steroiden, über ihren Urin ausscheiden, um die Laichabgabe mit den Weibchen abzustimmen und somit die externe Befruchtung zu koordinieren und den Fortpflanzungserfolg zu erhöhen.

List of abbreviations used in this thesis

General

DC	direct current
DI	dominance index
DMU	dominant male urine
EOG	electro-olfactogram
HPLC	high-performance liquid chromatography
MS	mass spectrometry
NMR	nuclear magnetic resonance spectroscopy
ORN	olfactory receptor neuron
SPE	solid phase extraction

Chemicals

3K-ACA	3-keto-allocholic acid
3K-PS	3-keto-petromyzonol 24-sulphate
11K-ETIO-3-G	11-keto-etiocholanolone 3-glucuronate
11K-T	11-keto-testostone
15K-PGF2α	15-keto-prostaglandin F2α
17,20α-P	17α,20α-dihydroxy-4-pregnen-3-one
17,20β-P	17α,20β-dihydroxy-4-pregnen-3-one
17,20β-P-S	17α,20β-dihydroxy-4-pregnen-3-one 20-sulfate
20α-P-3-G	5β-pregnane-3α,17α,20α-triol 3-glucuronate
20β-P-3-G	5β-pregnane-3α,17α,20β-triol 3-glucuronate
20one-P-3-G	5β-pregnane-3α,17α-dihydroxy-20-one 3-glucuronate
AD	androstenedione
CDC	chenodeoxycholic acid
E2	17β-estradiol
E2-3-G	17β-estradiol 3-glucuronate
ETIO-3-G	etiocholanolone 3-glucuronate
FA	formic acid
MeOH	methanol
PGF2α	prostaglandin F2α
T	testosterone
TCD	taurochenodeoxycholic acid

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Justification, objectives and thesis outline

Justification of the work

Pheromones are excreted chemical messages that mediate communication between members of the same species and influence key aspects of their lives, such as reproduction, migration, aggregation or territory marking. Although fishes represent by far the largest group of vertebrates, to date only in three species, namely the sea lamprey (Li and others 2002; Sorensen and others 2005), an ancient jawless fish, the goldfish (Appelt and Sorensen 2007; Dulka and others 1987), and the masu salmon (Yambe and others 2006) have pheromones been chemically identified and their biological roles clearly defined. Thus, many questions on the mechanism of action of pheromones are still to be answered and much knowledge to be gained on the chemical identities of the compounds involved and their spatial-temporal patterns of release. Pheromones may contribute to the invasive success of some fish species, and identification of their structure and function may provide methods to control invaders (Sorensen and Stacey 2004). The Mozambique tilapia (henceforth named tilapia) is both an aquaculture and invasive species in many parts of the world. It shows complex social structuring and behaviours that can be modulated by chemical signals. It thus represents a good model for studying chemical communication in teleost fishes. Tilapia males establish dominance hierarchies; the most dominant are chosen as mates by females (Bruton and Boltt 1975). Dominant males store large urine volumes in their urinary bladders and aggressive interactions among males and female reproduction are modulated by tactical release of urinary odorants by dominants (Barata and others 2008; Barata and others 2007). One urine fraction was shown to evoke particularly large olfactory responses (Barata and others 2008), yet its chemical nature remains unknown.

Objectives

The chief aims of this study were to identify the pheromone(s) released by dominant Mozambique tilapia males via their urine, and advance our knowledge on the mechanisms of chemical signaling in this species.

Several specific objectives were formulated in the course of this study to accomplish these goals:

1) Establish whether the ability of dominant males to store and release large urine volumes is linked to physiological (urine production) and/or morphological (bladder, kidney) differences.
2) Isolate and elucidate the structure of the putative pheromonal compound(s) present in the most active male urine fraction.
3) Characterise the olfactory sensitivity and receptor specificity of males and females to the identified compound(s) and structurally related substances.
4) Investigate the biological, i.e. pheromonal function of the identified compound(s) on female and male receivers.

Thesis outline

This thesis is organized into six chapters, starting with a general introduction chapter, followed by four experimental chapters in the form of scientific manuscripts and a last chapter with a final discussion.

Chapter I is divided into three sections and provides the background for the topic, thesis aims and methodological approach. Firstly, an introduction into chemical communication and short review on the diversity and function of chemical signals/pheromones in fish is given. Secondly, the Mozambique tilapia as a model for studying chemical communication in teleosts is presented. Thirdly, the methodological approach and the main techniques of the study are introduced.

Chapter II addresses objective **1)**, by comparing primary urine production, urinary bladder and kidney morphology between the sexes and between males of different social status. It is revealed that urine production is independent of sex and social status, but dominant males have more muscular urinary bladders, likely to facilitate storage of larger urine volumes and control of urination frequency during social interactions. Behavioural results suggest that male aggression is modulated by urine release and that males can predict the outcome of a fight by smelling their rival's urine. This chapter was published in a special issue on water-borne chemical signals in *Behaviour*.

Chapter III unveils the chemical identity of the (olfactory) most active compounds present in tilapia male as two epimeric pregnanetriol 3-glucuronates; objective **2)**. Both compounds were synthesized and their olfactory activity is demonstrated; objective **3)**. Moreover

it is shown that the two steroids stimulate the endocrine system of female tilapia, establishing their function as sex pheromones; objective **4)**. The contents of this chapter were published in *Current Biology*.

Chapter IV gives more insights into the perception of the identified, as well as related, steroids on the olfactory level and addresses objective **3)** in more detail. It is established that the Mozambique tilapia has evolved high olfactory sensitivity and specificity to 3-glucuronidated steroids through two distinct olfactory receptors. One receptor detects the steroids identified from male urine, while the other receptor detects estradiol 3-glucuronate, a possible chemical signal released by female tilapia. This chapter was submitted to *The Journal of Experimental Biology* for publication.

Chapter V investigates if the two urinary steroids identified as sex pheromone for females are also the major constituents mediating male-male aggression; objective **4)**. It is shown that the two steroids alone do not evoke the same aggression-reducing effect as dominant male urine, and that only reconstitution of all urine fractions is able to restore this effect. This suggests that the chemical signal driving off competition is different from the female sex pheromone and likely a more complex mixture of compounds. This chapter was submitted to *Hormones and Behavior* for publication.

Chapter VI integrates the major findings from the individual chapters into a final discussion and overall conclusion. It also delineates unanswered questions giving an outlook into future research.

Chapter I

General introduction

In this chapter, firstly, an introduction into chemical communication and short review on the diversity and function of chemical signals/pheromones in fishes is given. Secondly, the Mozambique tilapia as a model for studying chemical communication in teleosts is presented. Thirdly, the methodological approach and the main techniques of the study are introduced.

General Introduction

1. Chemical communication and pheromones

It is conceivable that all living entities communicate. Communication implies the exchange of information between organisms, benefiting both sender and receiver. Information is transferred via one or different kinds of signals and perceived via different, non-mutually exclusive, sensory channels; thus, communication often is multi-modal. For humans, the most familiar way of communicating is via acoustic signals; with 6000-7000 languages worldwide, human linguistic diversity and verbal capability is truly outstanding. Visual displays are another well-known form of signaling, such as the colourful courtship plumage of many birds, the enigmatic play of colour in coral reef fishes and the multiplicity of animal behaviours, from the display of distinct body parts and movements to complex facial expressions. Perhaps less noticed are tactile, vibrational (e.g. in insects or elephants; O'Connell-Rodwell 2007; Virant-Doberlet and Cokl 2004) or electric (e.g. in electric fishes; Hopkins 1999; Lissmann 1958) signals.

However, the most prevalent and ancient way of exchanging information is chemical communication. Single-celled bacteria and archaea exchange information on the cell density of their local population using chemical signals, through a process named quorum sensing, to coordinate certain behaviours such as biofilm formation, bioluminescence or host colonization (Taga and Bassler 2003; Zhang and others 2012). When attacked by herbivores, various plants release volatiles to attract predators or parasites of the herbivore or to 'warn' their neighbouring plants, which will then mount a defense response (Paré and Tumlinson 1999). Chemical communication is widespread also in the animal kingdom, wherein chemical signals act mostly via the sense of smell or, to a lesser extent, taste.

Chemical signals used for communication between individuals of the same species are called pheromones. Peter Karlson and Martin Lüscher defined pheromones as "substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behaviour or a developmental process" (Karlson and Lüscher 1959; in Wyatt 2009). The specific reaction does not require learning and should be always also beneficial to the sender. A pheromone may consist of a single compound or a mixture of several substances which, when combined in a certain ratio, act in synergy (Wyatt 2003b). Some examples from aquatic animals are elaborated in the following section.

1.1 Water-borne chemical signals

Pheromones play an important role in intraspecific communication of aquatic animals in key aspects of their lives, such as reproduction, migration, aggregation or territory marking. To be effective, the odor molecule needs to physically travel from the sender to the receiver. Water currents can carry chemical messages over long distances to reach far conspecifics (Wyatt 2003a). While volatility and thus a relatively low molecular weight is an important feature of most (but not all) pheromones in terrestrial animals, solubility is an important characteristic of many pheromones in aquatic systems. To increase hydrophilic properties, water-borne chemical signals frequently contain numerous functional groups which raise electronegativity, hydrogen bonding abilities and the dipole moment of the molecule, e.g. carboxyl-, hydroxyl-, keto-, amino-, or sulfo-groups. Less crucial is the molecular weight, which can be quite large as seen for polypeptide pheromones from marine invertebrates (Zimmer and Butman 2000). Many molluscs, such as sea slugs *(Aplysia* spp.*)*, use proteins as chemical attractants to facilitate reproduction (Susswein and Nagle 2004). Cuttlefish are naturally solitary animals but aggregate when spawning. Sexually mature individuals are attracted to conspecific eggs (Boal and others 2010) and some peptides, including the tetrapeptide 'ILME' were identified as chemical messengers (Enault and others 2012; Zatylny and others 2000). The sea anemone *Anthopleura elegantissima* responds with contraction and withdrawal of tentacles to an alarm pheromone named Anthopleurine, a quarternary ammonium ion, released by wounded conspecifics (Howe and Sheikh 1975). Lobster and crayfish males use chemical signals in their urine to recognise the social rank of their opponent, mediate agonistic interactions and establish dominance hierarchies (Breithaupt and Atema 2000; Breithaupt and Eger 2002; Thomas Breithaupt 2011; some more information can be found in introduction of chapter II). Fish pheromones have been reviewed several times in book chapters and journal articles (Stacey and Sorensen 1991; Stacey and Sorensen 2002; Stacey and Sorensen 2005; Stacey and others 2013) though knowledge remains still restricted because for only a few species is the chemical nature of the pheromone known. The following section will illuminate some examples after giving a more general insight into the olfactory perception and transduction of chemical signals in fish.

1.2 Chemical signaling in fishes

1.2.1 Olfactory perception and transduction of pheromone signals in fish

The key sense for vertebrate animals to detect pheromones is olfaction. But, unlike many terrestrial vertebrates, fish do not possess a vomeronasal organ, the auxiliary olfactory organ for the perception of pheromones and social chemosensory stimuli (Hansen and Zielinski 2005). The olfactory organ in jawed (gnathostome) fishes is a paired structure consisting of two cavities, usually one on each side dorsally of the forehead, containing the olfactory epithelium. The cavity may open to the outside through one (e.g. cichlids) or, more commonly, two openings (Zeiske and Hansen 2005). On the base of the cavity lies the olfactory rosette, consisting of several olfactory lamellae which contain the sensory epithelium; rosette shape and lamellae number vary between different species (Figure 1). The sensory olfactory epithelium comprises basal cells, supporting cells and olfactory receptor cells (i.e. olfactory receptor neurons, ORNs; Zeiske and Hansen 2005). ORNs are nerve cells with one end exposed to the external environment and the other end terminating directly in the brain (Wyatt 2003c). Three different types of ORNs can be distinguished in teleost fish: ciliated, microvilli and crypt cells, which project to distinct and segregated regions in the olfactory bulb (Hansen and others 2003). It is as yet not very clear what the exact functions of the different cell types are. However, some studies suggest that the different cell types may be detecting distinct odorant classes. For example, amino acids have been suggested to be detected mainly by microvilli but also by ciliated cells (Hansen and others 2003; Lipschitz and Michel 2002), whereas bile acids seem to be detected only by ciliated cells (Doving and others 2011; Hansen and others 2003; Kolmakov and others 2009). The pheromonal candidate etiocholan-3α-ol-17-one 3-glucuronate as well as conspecific urine was able to activate ciliated cells in rainbow trout (Sato and Suzuki 2001). Similarly, olfactory disruption experiments in goldfish suggested that the sex pheromone 17,20β-dihydroxypregn-4-en-3-one 20-sulphate (17,20β-P-S) is detected via ciliated cells (Kolmakov and others 2009). This suggests that microvilli cells are more specific for amino acids, whereas ciliated cells may detect a broader odorant spectrum, possibly social stimuli and pheromones. Interestingly, crypt cells were discussed recently to be involved in the detection of chondroitin

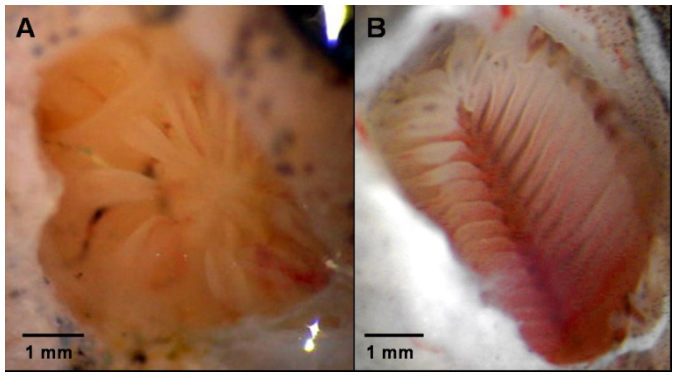

Figure 1 | Photograph taken of the olfactory rosette of **A)** the Mozambique tilapia, *Oreochromis mossambicus* and **B)** the European eel, *Anguilla anguilla*; pictures by Dr P Hubbard and Dr M Huertas.

fragments, an alarm signal of zebrafish (Mathuru and others 2012). While some scientists propose that each ORN may only express one single receptor gene (Ngai and others 1993), others suggest that in fish, there may be more than one receptor expressed per cell (Caprio and others 1989), something that still awaits elucidation. But what is becoming increasingly clear is that fish receptor specificity is very high for some odorants, and several independent receptor sites for the distinction of various pheromonal molecules can be present (Sorensen and others 1998), an aspect that is further discussed in chapter IV of this thesis.

When an odorant molecule binds to a receptor protein on the outer membrane surface of an olfactory receptor neuron (ORN), a cascade of enzymatic reactions is activated, leading to the production of secondary messengers in the inner membrane which cause depolarization of the neuronal membrane (Hara 2005). This triggers an action potential which transports the information about the activation of the specific receptor to the brain. The axons of the ORNs join together, to form the olfactory nerve. The olfactory nerve projects directly to the olfactory bulb, a structure of the forebrain and the initial olfactory processing center where the ORNs terminate and synapse with second order olfactory neurons (mitral cells). The connections of the nerves form so called glomeruli, in which integration of the olfactory signal presumably occurs (Laberge and Hara 2001). From the glomeruli, the secondary olfactory neurons, project to higher brain regions (Wyatt 2003c). However, there is evidence from the lake whitefish (*Coregonus clupeaformis*; Salmonidae) that putative reproductive pheromone signals (F-type prostaglandins) are integrated through a distinct neuron population located at the ventromedial brain area at the transition between olfactory bulb and telencephalon, segregated from the olfactory bulb areas where responses to other odorant classes, e.g. amino acids, are usually processed (Laberge and Hara 2003b). The authors suggest that this brain area may represent an olfactory subsystem in fish, something similar to what is seen in insects and perhaps a precursor to the vomeronasal organ of terrestrial vertebrates (Laberge and Hara 2003b). However, variability between schemes encoding pheromonal information may exist between different fish species. Clearly, much research still needs to be done to achieve a more comprehensive understanding of pheromone coding. This holds true also for the processing and integration of pheromonal information in higher brain regions of the fish and the translation into behavioural responses, whereof hardly any studies exist. In the following subsections, examples of different pheromone types and their functions in fishes are discussed.

1.2.2 Alarm signals

The renowned ethologist and Nobel-prize laureate Karl von Frisch wrote, 75 years ago, in his report "on the psychology of a fish shoal" about observations made during his summer holydays at lake 'Wolfgangsee' in Austria on the Eurasian minnows, *Phoxinus phoxinus* (von Frisch 1938). After cutting a small wound into the skin close to the caudal fin to label one member of 'his' tame minnow shoal and releasing it, he witnessed the whole shoal becoming visibly scared and hesitant. A similar phenomenon recurred another day, when one minnow got trapped and wounded under a feeding tube, provoking the flight of the entire shoal. To get to the bottom of it, he performed several tests and finally concluded that it was neither the sight nor a sound of the injured comrade that scared off the entire minnow shoal, but rather a chemical substance released from the wound. He called it 'Schreckstoff', which means 'scary stuff' and triggers an innate fright response in conspecifics (in Stensmyr and Maderspacher 2012). Since then, many researchers tackled the question of what 'Schreckstoff' is, until a first candidate, hypoxanthine-3-N-oxide was identified (Brown and others 2000; Pfeiffer and others 1985). The fright response seems to be common behaviour within the teleost superorder Ostaryophysi which have specific club cells in their epidermis that may be involved in the production of 'Schreckstoff' (Pfeiffer 1977). However, hypoxanthine-oxides do not elicit a (full) fright response in all Ostariophysan species and/or are not always present in skin extracts (Mathuru and others 2012), suggesting that other compounds have to be involved. Oligosaccharides from mucus and skin, chondroitin-sulfate fragments, have been recently shown to act as potent odorants in zebrafish and trigger full fright responses (Mathuru and others 2012). Yet, a controversial discussion remains on whether these alarm substances can be truly considered as pheromones (Magurran and others 1996). While the benefits to the receivers (predation avoidance) are obvious, the benefits to the sender are not. Clearly, even if the sender does not survive predator attack, the group as whole benefits from being warned. If the sender shares genes with other group members, kin selection may be a plausible explanation for the evolution of 'Schreckstoff'. Yet, shoal members are not necessarily close relatives (Bernhardt and others 2012). So it is open to debate, still, whether 'Schreckstoff' is only a by-product of the ruptured skin or a true chemical 'signal'.

Aside those alarm cues that require cell damage, there is also evidence for the existence of non-injury based odorants, so called 'disturbance cues' that may be released via the urine or gills by stressed or disturbed fishes prior to a predator attack (Wisenden and others 1995). Urea is a first candidate substance that was shown to function as an early warning signal to conspecifics in juvenile rainbow trout and convict cichlids (Brown and others 2012).

1.2.3 Chemical signals guiding migration

Many fishes, including lampreys, salmonids, river herrings and freshwater eels, perform long distance migrations over thousands of kilometers to reach their spawning areas, guided by chemical cues. The sea lamprey, *Petromyzon marinus* is an ancient jawless (Agnatha) fish that lives in freshwater streams for several years as a larva before it undergoes metamorphosis and travels to the sea where it starts hematophagous feeding. After a year of parasitic life, it travels back to the rivers, spawns and dies. Chemical cues, released by conspecifics, guide adults back to their spawning streams (Johnson and others 2009; Sorensen and others 2005) Three sulphated bile-acid like sterols, petromyzonol sulphate (PS), petromyzonamine disulphate (PADS) and petromyzosterol disulphate (PSDS), were identified as the attractants of larvae holding water and proposed to act as a migratory pheromone for adult sea lampreys (Sorensen and others 2005). In addition, the sex pheromone 3-keto-petromyzonol sulphate released by earlier arriving spermiated lamprey males attracts and induces upstream movements in ovulated females (Johnson and others 2009; Li and others 2002). Field studies have shown that the synthetic analogue of the male pheromone is powerful enough to direct ovulated females away from the natural pheromone source and summon them into traps (Johnson and others 2009). This discovery may find practice in an environmental-friendly attempt to control invasive sea lamprey in the Great Lakes of America, and provides a good example for (a) possible application(s) of fish pheromone research.

1.2.4 Reproductive pheromones

Reproductive pheromones are probably the best studied fish pheromones. Because most fishes are external fertilizers, they must coordinate reproduction to ensure that sperm and eggs are released at the same time and place; sex pheromones help this synchronization. Most reproductive pheromones that have been identified or suggested in teleosts are hormonal pheromones. Hormonal pheromones are thought to have evolved by 'chemical spying' of the receiver on 'leaking' (e.g. through the gills by passive diffusion from the bloodstream to the outside) or excreted (e.g. urine) reproductive hormones from the opposite sex (Sorensen and Stacey 1999). In early evolutionary stages, only the receiver benefitted from detecting the hormones released by the conspecific sender. However, if there was a selective advantage to the sender, with time, this may have turned into a bilateral benefit and specialization of the sender for the production and release of hormonal pheromones (Sorensen and Stacey 1999).

Use of hormone-derived sex pheromones is best understood in the goldfish (*Carassius auratus*). Females release first a pre-ovulatory primer pheromone and, later, a post-ovulatory releaser pheromone to stimulate the males' endocrine system and reproductive behaviour, respectively.

This non-territorial species has a polygamous, scramble-competition mating style and spawning occurs when temperature and day-length rise in spring. Maturation of the eggs leads to an increased release of maturation-inducing steroid 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) and its metabolite 17,20β-P sulphate to the water, especially during the afternoon/evening (Sorensen and Stacey 1999). The male olfactory system detects both steroids with extremely high sensitivity, and this stimulates gonadotropin release from the pituitary which, in turn, activates testicular sperm and seminal fluid production (Sorensen and others 1995). After ovulation late at night, 17,20β-P and 17,20β-P-S release reduces and, instead, females produce increasing amounts of hormonal F-type prostaglandins PGF2α and 15keto-PGF2α, which in turn stimulate and synchronize female sexual behaviour. The PGFs are released *via* the gills and *via* urine pulses which increase in frequency as soon as females enter suitable spawning areas (rich vegetation; Sorensen and Stacey 1999). These PGFs are as well detected by the male olfactory organ with high sensitivity and receptor sites distinct from the 17,20β-P receptors (Sorensen and others 1988; Sorensen and others 1995). They attract males to spawning sites and stimulate spawning behaviour early next morning (Appelt and Sorensen 2007). Many other cyprinids (e.g. common and crucian carp and zebrafish; Bjerselius and Olsén 1993; Bjerselius and others 1995b; Friedrich and Korsching 1998; Lim and Sorensen 2011) and salmonids (e.g. Atlantic salmon, Artic char, lake whitefish and brown trout; Laberge and Hara 2003a; Moore and Scott 1992; Moore and Waring 1996; Sveinsson and Hara 2000) also show high olfactory sensitivity or attraction to 17,20β-P (-metabolites) and/or prostaglandins, suggesting a wide distribution of these hormones as sex pheromones in teleosts.

In contest competition-based mating systems that are strongly driven by female choice, nest-guarding males are specialized in the production and release of hormone-derived sex pheromones to attract females. The first evidence emerged over 30 years ago, from a study reporting that testicular etiocholanolone 3α-glucuronate from black goby males (*Gobius jozo*) attracts ripe females (Colombo and others 1980). Some years later, a similar observation was made in African catfish (*Clarias gariepinus*) males, where the most potent testicular odorant was found to be 3α,17α-dihydroxy-5β-pregnan-20-one 3α-glucuronate. Androstanes and pregnanes with 5β,3α configuration are potent odorants as well for the round goby (Neogobius melanostomus; Murphy and others 2001) and recent studies have shown that round goby males release several conjugated forms of these steroids via their urine (Katare and others 2011), probably to attract females (Tierney and others 2012). Similar to many terrestrial mammals, urine seems to be a common vehicle for pheromones in fishes. Increased frequency of urine pulses in the presence of pre-ovulatory females was observed in Mozambique tilapia males (Barata and others 2008); more specific information is given in section 2.4 as well as chapter II and III of this thesis.

1.2.5 Chemical signals in social organisation

In complex animal societies, individuals interact via cocktails of chemical signals to recognize group, kin, individuals, age, sex or social position and a multitude of examples exists from insects (e.g. ants, termites, wasps, bees) and mammals (e.g. primates, rodents, gregarious ungulates, canine packs; Wyatt 2003e). Many teleost fishes form large social groups (shoals) and chemosensory-based recognition has been demonstrated in several behavioural studies (e.g. for cyprinids, salmonids and cichlids) although almost nothing is known about the nature of the chemical signals involved. Frequently, social organization involves both pheromones and individual signature mixtures. For the scientist, differentiation between the two may not always be easy because pheromones occur against a background of other odorants, pheromone and signature compounds may overlap, and one pheromone may mediate different functions (Wyatt 2010). Yet pheromones always elicit an innate and stereotyped response (although some conditionality is possible). Signature mixtures, on the other hand, are variable subsets of molecules; they are always learnt by other animals, allowing them to distinguish individuals or colonies and without necessarily any obvious benefit to the sender (Wyatt 2010). Thus, processes such as individual- or offspring-recognition are mediated by learnt signature mixtures rather than 'hard-wired', whereas information on social status or territoriality may, indeed, be conveyed by pheromones. Identifying the chemical code of social organization cues in fishes therefore provides an enormous challenge, and one may struggle in extracting, whether a 'real' pheromones is involved or not.

However, excellent models for such studies are the cichlids, as they show an advanced social structuring and a wide range of interactions, also in the laboratory. Olfactory cues play roles in a multitude of contexts, including species recognition (Plenderleith and others 2005), parent-offspring recognition (in Nelissen 1991), mate recognition (Reebs 1994), "self-recognition" (Thünken and others 2009) and social rank recognition (Barata and others 2007; Maruska and Fernald 2012). Females of the Lake Malawi cichlid *Maylandia emmiltos* (before *Pseudotropheus emmiltos)* prefer odours from conspecific males to those from closely related (sympatric) heterospecific males (Plenderleith and others 2005). African jewelfish parents (*Hemichromis bimaculatus)* choose holding water of their own fry over plain water or holding water of heterospecific fry (Kühme 1963). The African cichlid *Pelvicachromis taentiatus* differentiates between its own odour, and those of familiar and unfamiliar conspecifics, suggesting the learning of 'signatures' (Thünken and others 2009). Male tilapia (*Oreochromis mossambicus)* increase their urination frequency during aggressive disputes with rival males, and the olfactory potency of male urine increases with ascending social rank of the donor, suggesting the presence of a dominance pheromone (Barata and others 2008; Barata and others 2007). The work included in this thesis follows directly from the latter observations; thus, the next section will outline the model species of the current work and review its social behaviour and chemical communication.

2. The Mozambique Tilapia

2.1 Geographic occurrence and phylogenetic position

The Mozambique tilapia (*Oreochromis mossambicus*) was first described by the German naturalist Wilhelm Karl Hartwich Peters in 1852 in his work "Naturwissenschaftliche Reise nach Mossambik, Band 4 Flussfische" covering the specimens of river fishes he collected during his expedition to the river Zambezi and coastal region of Mozambique from 1842 to 1848. Native to South-East Africa, this cichlid occurs in Malawi, Mozambique, Swaziland, Zambia, Zimbabwe and the East coast of South Africa (Cambray and Swartz 2007) thriving in the slower flowing river sectors or, during the dry season, in backwaters, floodplains, pools and swamps. Due to its high salinity tolerance it also inhabits estuaries and lagoons but is not found in the open sea (Lowe-McConnell 1991).

With 1,658 currently described species (Fishbase 2013a), cichlids truly are the most diverse family in the order Perciformes (perch-like) which, in turn, comprises one third of extant teleosts. Many aspects of cichlid phylogeny are still under debate and many questions remain unanswered. According to their geographic separation, cichlids are divided into four phylogenetically distinct groups; the first split of the Indian and Madagascan cichlids dates back more than 200 million years (Myr) when the supercontinent Pangaea started to split, followed by the Neotropical cichlids which separated from the Africans probably 130 Myr ago with the geological divergence of the Gondwana supercontinent. (Streelman and others 1998). The African assemblage is a monophyletic group (except the basal Heterochromines) comprising more than 80 % of all extant cichlids (Stiassny 1991). African cichlids attract a lot of attention from evolutionary biologists because of their explosive speciation and radiation in the East African Lakes (Malawi, Victoria and Tanganyika) at an astonishing (in evolutionary terms) rate, i.e. in Lake Victoria in less than 12 400 years (Mayer and others 1998). Each lake has its own unique cichlid flock with an extremely high level of endemicity (99%; Ribbink 1991). The maternal mouth-brooding genus *Oreochromis*, however, clusters together with other West- and Pan African river cichlids and separates from the East African clades (Schwarzer and others 2009; Streelman and others 1998). One characteristic of *Oreochromis* is that it exhibits allopatric speciation; usually only one or two species occur per river (Lowe-McConnell 1991).

2.2 Economic value and environmental impact

Commercial production of tilapias has increased drastically over the last two decades. Tilapias currently hold the second position of the world's most important aquaculture fishes, after carp and before salmon. More than 100 countries around the world farm tilapia, with Egypt, Philippines, Thailand and Indonesia among the top five and China as the by far largest producer (Rakocy 2005). The three dominant species are the Nile tilapia (*Oreochromis niloticus*), the Mozambique tilapia (*O. mossambicus*) and the blue tilapia (*O. aureus*), whereby the Nile tilapia nowadays accounts for about 80 % of the total production. Breeders willingly hybridize *O. niloticus* males with *O. mossambicus* females to achieve 100 % male progeny which yield higher harvest and marketability than mixed sex populations. All Oreochromines feed very low in the food web and are easily reared on a vegetarian diet (Lowe-McConnell 1991). This presents a huge advantage over many cultured species, such as salmon, tuna, bass or seabream, that mostly receive feed based on fishmeal, thereby doing little to reduce the problem of overfishing and depletion of wild stocks. Moreover, tilapia have low requirements in terms of water quality, are relatively stress- and disease-resistant and readily reproduce in captivity. Their hardiness and adaptability to a wide range of culture systems in both fresh- and seawater, opened or closed setups, ponds, floating cages, raceways or tanks truly makes them a frontrunner among aquaculture fishes (El-Sayed 2006).

However, tilapia aquaculture currently in practice has negative aspects, too. Poor management of culture facilities lead to their escape or release, and the Mozambique tilapia in particular has become a highly successful invader of tropical and subtropical waters all around the world (database 2006). In many countries this species is becoming an increasing threat to native aquatic fauna, competing for habitat, nesting sites or food, and even preying on the fry and juveniles of other fishes (Canonico and others 2005; Morgan and others 2004).

Given its popularity as a food fish and its world-wide distribution, it therefore seems paradoxical that the IUCN red list currently classifies *O. mossambicus* as 'near threatened' in its natural habitat. But the Nile tilapia is invading parts of the river Zambezi and South Africa (Cambray and Swartz 2007), and hybridization - in addition to resource competition - is causing original *O. mossambicus* populations to decline in its native areas (Canonico and others 2005).

2.3 Aspects of the biology of the Mozambique tilapia

As all species within the *Oreochromis* genus, the Mozambique tilapia is a maternal mouth-brooder and arena spawner. It has a polygamous mating style; a male can fertilize the eggs of several females successively and females may visit several males and divide their eggs during one spawn (Neil 1966). In the spawning season, usually from October to February (southern hemisphere spring/summer), the males aggregate in so-called 'leks', become aggressive, occupy territories where they dig nests with their mouth into the substrate and feed very little (Bruton and Boltt 1975). Ovarian gametogenesis takes about 14 days and the entire mouth brooding cycle a minimum of 36 days, thus a female can spawn every 5-7 weeks. When pre-ovulatory, females pass by a 'lek' and choose (a) male(s) to mate with, then take the fertilized eggs in their mouths and retreat to sheltered water to brood them. After 20-22 days, they often congregate in shallow but well protected areas with rich vegetation or under logs to release their fry at 9-10 mm length (Bruton and Boltt 1975; Neil 1966). Juveniles stay together in large shoals, protecting themselves from predators. Adults that are momentarily not reproducing also shoal in mixed sex groups away from the male arenas (Neil 1966). In wild populations, sexual maturity is usually reached between 12 and 24 months (Arthington and Milton 1986; James and Bruton 1992); however, under unfavourable, stunting or captive conditions, *O. mossambicus* can become reproductively active early, at just 2-3 months (Fishbase 2013b). They may live for 10-11 years, and males can grow up to 40 cm and one kg (Fishbase 2013b).

The Mozambique tilapia is a diurnal omnivorous fish; fry and juveniles take up detritus, diatoms and other microalgae, while adults may additionally feed on macrophytes, insects and planktonic crustaceans. Large individuals feed on other small fishes, including their own species, too (Bruton and Boltt 1975; Lowe-McConnell 1991). Natural predators are mainly piscivorous birds, e.g. herons, kites, gulls and the African fish eagle, or larger fish. Juvenile tilapia, for instance, are preyed upon by the African toothed catfish *Clarias gariepinus* (Bruton and Boltt 1975).

2.4 Tilapia as a model to study chemical communication

The Mozambique tilapia provides an ideal teleost model to explore a wide variety of research questions experimentally at the molecular, physiological and ecological/behavioural levels, including chemical communication. As mentioned earlier, these modest animals are fairly easy to maintain, grow and reproduce in captivity. Their pronounced sexual dimorphism makes it easy to distinguish adult females from males, the latter being generally larger, exhibiting a conspicuous breeding colouration, wider lips, a wider mouth and higher anal and (pointed) dorsal fins (Oliveira and Almada 1995). The social behaviour of *O. mossambicus* in captivity is well-described (examples include Oliveira and Almada 1996; Oliveira and Almada 1998a; Turner 1986); it was found to be in many aspects highly similar to the behaviour of wild fish (Neil 1966). Males exhibit a comprehensive repertoire of behaviour- and colour changes, depending on the social condition. The neutral colour when hovering or swimming in the water column is an inconspicuous light grey. During aggressive disputes, males change to darker shades of grey and the winner of a contest usually adopts a dark-grey, almost black coloration (Oliveira 1995). Courting males are deeply black with only the lower parts of opercula and jaw in contrasting white and edges of the dorsal and caudal fin in red. Colour changes can occur rapidly, within seconds or minutes. Relatively linear social hierarchies that can remain stable for at least several days are established by males in captivity (Oliveira and Almada 1996; Oliveira and Almada 1998a). Depending on the availability of space and nesting sites, and the number of males in tank, there may be only one or several alpha (i.e. dominant) males. The males of the highest rank occupy a small territory wherein they dig a nest in the sand (Figure 2 A) that they defend aggressively (Oliveira and Almada 1998a; Oliveira and Almada 1998b). When a (pre-ovulatory) female is nearby they will invite and guide her into their nest, where they express shivering movements of the whole body, in a nearly perpendicular posture (head declined) with flattened fins (Oliveira 1995). In the presence of pre-ovulatory females and during courtship, males drastically increase their urination frequency (Barata and others 2008) to stimulate the females' reproductive system (Huertas and others 2014) and allure them to spawn (Barata and others submitted manuscript).

Other individuals entering the nest during this period are rapidly pursued and chased away. If the intruder does not flee, but challenges the resident, the two rivals might engage in frontal or lateral displays or even higher levels of aggression, such as circling fights (Figure 2 C) and mouth-to-mouth attacks. Frontal displays (Figure 2 B) are characterized by the mouth wide open, the opercula expanded and fins erected (Oliveira 1995), often accompanied by alternate advancing and retreating (Turner 1986). Lateral displays constitute a collateral position towards the rival with the jaw and opercula expanded, a dark grey colouration and often develop into tail beating

and large-amplitude swings of the entire body. Frequently, a conflict is resolved at the display stage and one male retreats or takes on a submissive posture (i.e. maintaining immobile in the water column with a light grey colouration, fins not erected and the head inclined.). In 1975, Bruton and Bolt wrote in their study on the a wild *O. mossambicus* population in south Africa, "A characteristic of this behaviour, which consists of broadside displays, chasing and jaw-locking, is that the aggressive conflicts take the form of symbolic displays, rather than actual fighting". Only 8 % or so of conflicts escalate to high aggression and mouth-to mouth fights that are energetically expensive (Ros and others 2006) and have a high risk of injuries. During these 'symbolic displays' males release urine in pulses with drastically increasing frequency (Barata and others 2007). High-frequency urination ends when the conflict is either settled or the opponents proceed to escalated fighting. Whilst submissive to its opponent, a male also stops urinating. Dominant males can store more urine in their urinary bladder than males of lower hierarchical status and the olfactory potency of male urine is positively correlated with the donor's social rank (Barata and others 2008; Barata and others 2007). The aggressiveness of receiver males is reduced during exposure to dominant male urine but amplified during exposure to urine from subordinates (Barata and others submitted manuscript). This reveals that the urine of male tilapia is a vehicle of pheromones that affect both intra- and intersexual interactions on the physiological and/or behavioural level. Since unveiling the chemical identity of male tilapia urine signals is one major goal of this thesis, the next section of this chapter comprises a conceptual introduction into the methodology of chemical signal identification, with emphasis on the particular techniques used in this study.

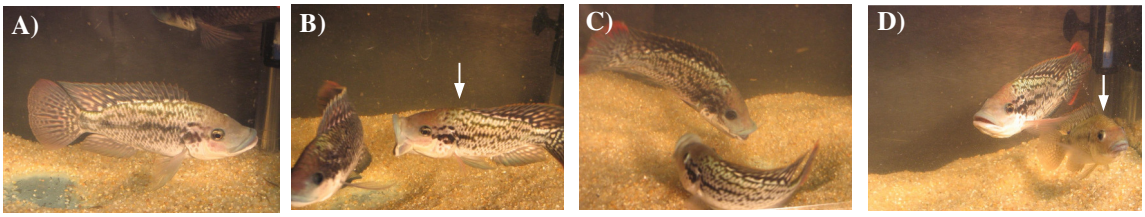

Figure 2 | Photographs of captive Mozambique tilapia males in different social contexts: **A)** territorial male above nest; **B)** frontal display (arrow); **C)** circling fight; **D)** male with female (arrow); pictures by Dr P Hubbard and Dr O Almeida.

3. Identification of chemical signals – An introduction to methodology

3.1 Bioassays

The key to identifying a pheromone is the bioassay; a repeatable, standardised and reliable way of measuring the response to a given chemical stimulus (reviewed in Wyatt 2003b). The design usually follows the observation of a behaviour or developmental shift that is mediated by a chemical signal and, thus, varies greatly depending on the study species and observed behavioural or physiological response. Setting up a functional bioassay and obtaining a clear effect under artificial laboratory conditions can be challenging; conversely, measuring an effect of a candidate compound in a lab experiment does not necessarily mean it will function also in the wild, so results must be interpreted with caution. Laboratory settings can deprive animals of some signals (and often achieve it deliberately), and overstimulate with others (noise, artificial lighting). In natural contexts animals frequently respond to multi-modal signals, e.g. including visual and/or auditory next to chemical and multiple environmental factors influence the response. In addition, pheromones naturally occur in a blend of other chemicals and some compounds can theoretically substitute for each other or, in contrast, a pheromone may only be functional if the correct ratio and concentration of all the other compounds is present (Wyatt 2003b). The bioassays used in this particular study are briefly summarized in the following subsections.

3.1.1 Electro-olfactogram (EOG) recordings

Using the olfactory organ of the animal as a detector gives valuable insight on its sensitivity to a chemical compound at the sensory level. This study used underwater electro-olfactogram (EOG) recordings (Figure 3 A) to assess the olfactory activity of biological (urine) samples, its fractions and synthetic chemicals. The EOG is a negative electrical potential recorded *in vivo* from above the olfactory epithelium, measured as a direct current (DC) voltage signal (Scott and Scott-Johnson 2002). Most probably, it originates from the sum of generator potentials of olfactory receptor neurons (ORNs) responding to a stimulus. The resulting negative amplitude is likely due to positive charges (e.g. Ca^{2+}) entering the cell during depolarization at the distal end of the receptor cell (but not because of the action potential generated in the axon; Scott and Scott-Johnson 2002). EOG responses are characterised by a rapid 'raising' phase (depolarization) and a slower decline (repolarization, Figure 3 B). In freshwater fishes, large EOG amplitudes can be obtained, for example, tilapia may respond with up to 25-30 mV to a social stimulus such as conspecific urine. Another advantage is that the EOG requires only little surgery (usually only slight enlargement of the nostril), allowing recovery of the fish after the experiment. Further, it is

relatively straight forward, is quickly performed and electrode position is not crucial, i.e. the relative amplitude is usually unaffected by the position (although it may influence the absolute amplitude).

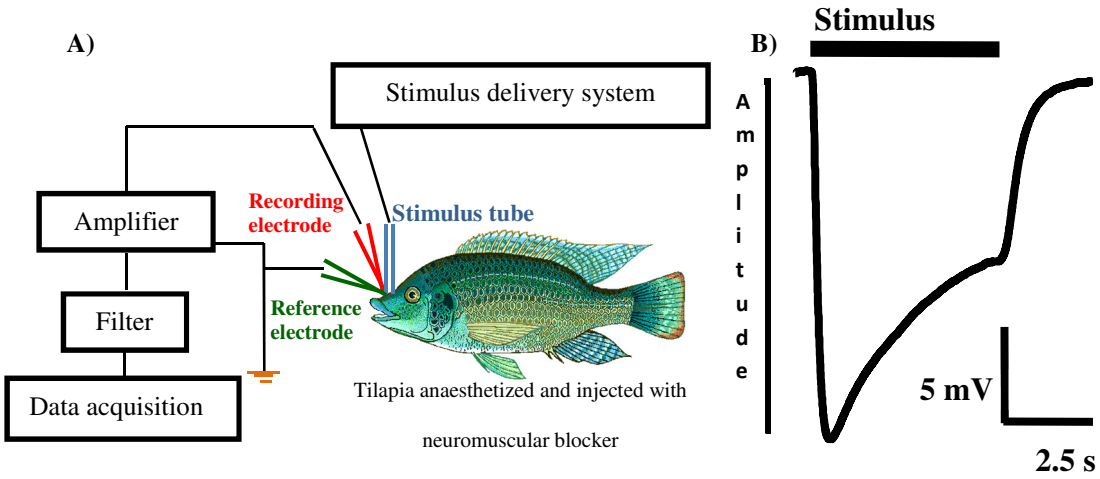

Figure 3 | Electro-olfactogram recording: **A)** simplified scheme of the EOG set-up. During the experiment, the fish is kept wet in a purpose-built chamber and the gills are constantly perfused with aerated water containing the anaesthetic; the stimulus tube irrigates the nostril with either water or stimulus-containing water; the recoding electrode is placed adjacent to the olfactory epithelium and the reference electrode in the water above the head and connected to earth; the signal is filtered and amplified and then digitalized and analysed with appropriate software; **B)** typical EOG from the olfactory epithelium of *Oreochromis mossambicus* in response to tilapia male urine at 1:10,000 v/v dilution.

3.1.2 Behavioural assays

Most studies investigating the role of chemical cues and pheromones in fish behaviour have used preference-, choice-, or attraction tests. For instance, the lamprey male pheromone was identified using a typical flow-through Y maze choice paradigm, in which the chemical stimulus was introduced at the distal end of one arm to test whether the fish is attracted to the stimulus, swimming towards it. (Li and others 2002; Li and others 2003). Yet, these assays cannot provide sufficient insights into how chemical signals may modify or mediate more complex social interactions, such as conflicts between male competitors. In this study, to address whether urinary pheromones mediate male-male aggression and what are the putative compounds involved, either staged dyadic encounters between rivals (resident-intruder paradigm; chapter II) or, confrontations of a male with its own mirror image (which he attacks as if it were a rival; chapter V), were manipulated and aggressiveness evaluated.

3.1.3 Physiological assays

The identification of primer pheromones requires bioassays that measure physiological effects. The difficulty is presented by the time lag between stimulus application and physiological response, and the often narrow time frame in which the latter is detectable. In male goldfish, for example, enlarged milt volumes can be stripped from 17,20β-P stimulated males only after over-night exposure (Stacey and Sorensen 1986). Since primer pheromones usually cause modifications in the endocrine system (e.g. Stacey and others 1989; Wyatt 2003a), measuring changes of hormonal concentrations in body-fluids or, more recently, hormonal release rates to holding water can be a good physiological bioassay. The latter, non-invasive approach allows repeated (i.e. time series) measurements on the same fish, increasing the chances that the endocrine response is 'captured' and avoiding interference from handling stress (Scott and Ellis 2007; Scott and others 2008). Through specific sequential extraction procedures, it is possible to measure the release of free, glucuronidated and sulfated steroids differentially. However, in most cases, focusing only on released free steroids is adequate (Scott and Ellis 2007), as those correspond best to the concentration of active steroids circulating in the blood at the moment of sampling. If the target is one or few specific known hormone(s), immunoassays using antibodies that bind to a certain hormone with high specificity and sensitivity are usually applied to assess the concentration in a given sample volume. Available methods are enzyme linked immunosorbent assays (ELISAs) or, more traditionally, radioimmunoassays (RIAs). In chapter III of this thesis, RIAs were used to measure the release of free 17,20β-P to the water by female tilapia.

3.2 Bioassay-guided fractionation and chemical identification

Biological samples are usually complex mixtures of compounds and pheromones occur against a background of many other molecules. Thus, the components in a crude sample first have to be separated from each other to target the ones with bioactivity, a process referred to as bioassay-guided fractionation. Once the bioactive chemicals are isolated and purified, they can then be identified by spectroscopic methods. This study used solid phase extraction (SPE) and high-performance liquid chromatography (HPLC) to separate the chemical substances of tilapia male urine into different fractions, which were tested for biological activity by EOG recordings. Electrospray ionisation mass spectrometry (ESI-MS), tandem mass spectrometry (MS^n) and nuclear magnetic resonance spectroscopy (NMR) unveiled the chemical identity of the active components. Figure 4 exemplifies the approach and the following subsections provide a brief introduction into the above mentioned techniques.

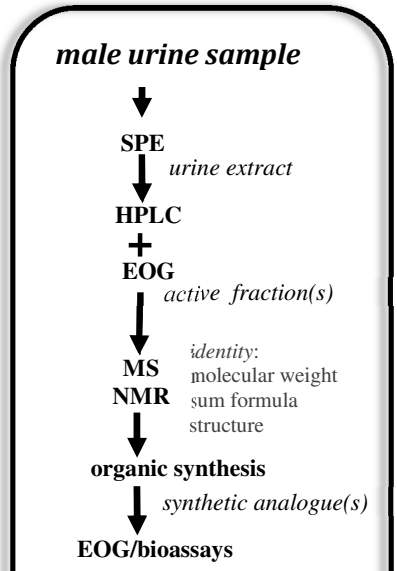

Figure 4 | Workflow used in this study to identify the pheromone in tilapia male urine. After a pheromone candidate has been identified, its biological activity should be confirmed with synthetic analogues.

3.2.1 Solid-phase extraction (SPE)

Solid-phase extraction is a sample preparation tool, useful to concentrate and purify the compounds in a sample. It can be used as a first step of separating different compounds and selectively remove undesired molecules and/or inorganic ions (Simpson and Wells 1998). The SPE principle is similar to that of liquid column chromatography. The liquid sample is passed through a short column (cartridge) containing a bed of solid particles (stationary phase). Depending on the affinity of the sample compounds to the stationary phase, they are either retained, or, pass through the cartridge bed together with the liquid phase. In the next step, the compounds remaining in the stationary phase can be removed (eluted) and collected by rinsing the column with an appropriate solvent. (Arsenault 2012). In this study, male urine samples were prepared using reverse-phase C18-SPE cartridges. C18 cartridges have a non-polar stationary phase, consisting of silica bonded to octadecyl hydrocarbon (C18) chains and are suitable for aqueous samples. Separation is based on hydrophobicity so that the compounds with moderate to low polarity are retained. Those compounds can then be eluted with an organic solvent (e.g.

methanol) that disrupts the hydrophobic interactions between analyte and stationary phase (Simpson and Wells 1998).

3.2.2 High-performance liquid chromatography (HPLC)

HPLC is a chromatography method used to separate solutes in a liquid on the basis of their physico-chemical properties. It can be used in a solely preparative fashion, i.e. to disunite compounds in a sample into different fractions and/or analytically, to identify and quantify the individual compounds. The separation process is achieved by injecting the liquid sample into a solvent stream (mobile phase) which is continuously pumped at high pressure through a column packed with small particles (McMaster 2007). The small particle size, typically 5 µm average diameter, allows for higher resolution as compared to traditional column liquid chromatography, but high pressure is needed. The higher the affinity of a component for the stationary phase the longer it will stick to it before leaving (eluting) from the column. The differential retention time of compounds on the column is the basis of HPLC separation. Typical HPLC separations are based either on the analytes' polarities (e.g. normal phase, reverse phase or HILIC), electrical charges (e.g. ion-exchange) or molecular sizes (size exclusion) but a huge variety of columns exists, giving HPLC its versatility. This study used reverse phase C18 silica (see chapter III) columns, wherein polar compounds elute first and non- polar compounds last.

The mobile phase strongly influences retention times and separation of analytes. The mobile phase can be a mixture of solvents for which the composition may be changed (gradient) during the run to achieve better separation. In gradients, the elution strength is increased with time to elute the more retained compounds, which is especially useful for samples containing compounds of very different polarities (Waters 2014). In this study, water and methanol (or acetonitrile) were used as solvents and percentage of the latter increased during separation. Another factor strongly influencing separation and retention time is the pH, especially for ionisable (acidic or basic) compounds. Co-existing protonated and deprotonated forms of ionisable compounds do not separate well on a hydrophobic stationary phase (e.g. C18) column. But buffers in the mobile phase can be used to adjust pH (Dong 2006) in a way the molecules are either mostly protonated or deprotonated. In chapter III of this study, ionization of weakly acid compounds was supressed by lowering the pH of the mobile phase with formic acid, leading to higher retention times and sharper peaks.

The eluting compounds can be detected by an appropriate detector that monitors a given property of the eluting molecules. The resulting signals (peaks) in the chromatogram, can be used to control the collection of purified material into fractions and/or to quantify known compounds by calculating the peak areas and comparing them with those obtained from known standards. A variety of HPLC detectors exist but not all detectors 'see' every component separated by the

column. (McMaster 2007). Here, separation and fractionation of male urine was monitored on a universal, evaporative light scattering detector (ELSD) that detects a wide range of non-volatile compounds, including carbohydrates, lipids, steroids and polymers and has the advantage of not being limited to compounds containing UV-absorptive chromophores (Varian 2008).

3.2.3. Mass spectrometry (MS)

Mass spectrometry is an analytical technique that allows determination of the mass of molecules or atoms in a sample. John Fenn and colleagues described MS as follows: "Mass spectrometry consists in 'weighing' individual molecules by transforming them into ions *in vacuo* and then measuring the response of their trajectories to electric and magnetic fields or both" (Fenn and others 1989). A mass spectrometer consists of three main components: **1)** the ion source to produce electrically charged molecules (ions) or charged fragments of molecules; **2)** the analyzer that allows the ions to make their 'flights' in a controlled way, i.e. it separates and sorts the ions under vacuum in either an electrical or magnetic field that changes speed and direction of the ions. The deflection depends on the ions' mass to charge ratio (m/z), according to which the ions are separated; **3)** the detector, the final end of the trajectories, which measures the m/z (Watson and Sparkman 2007a).

There are several different ways to produce ions, and the chosen technique depends also on the sample types and target molecules. For liquid biological samples containing large(r) non-volatile molecules, either matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI; used in chapter III) is suitable. In ESI-MS the sample is sprayed under a strong positive or negative electric field, typically +/- 4000 V, and the resulting spray droplets are evaporated using a desolvation gas, usually nitrogen. As the molecules are brought into the gas phase, they adopt a negative (positive electric field applied) or positive (negative electric field applied) charge (Watson and Sparkman 2007b). The ions may be formed by addition of a hydrogen cation, i.e. proton $[M+H]^+$, or, by loss of a proton $[M-H]^-$. However, other cations, such as sodium $[M+Na]^+$ or potassium adducts $[M+K]^+$ may also be formed, something that must be taken into consideration when deducing the molecular mass of a compound.

The deliberate production of fragment ions from a precursor ion is a process referred to as tandem mass spectrometry (MS/MS) and can be useful to reveal some structural features of the molecule which may help in identifying it (Watson and Sparkman 2007c). It could be thought of as the production of a secondary mass spectrum from the primary mass spectrum to reveal new information. A precursor ion is selected and fragmented into new product ions. Multiple stages of mass analysis can be performed, i.e. MS to the n (MS^n); in chapter III of this study MS^2 to MS^4 studies were carried out on male tilapia urine samples.

Mass spectrometers are frequently coupled to gas chromatography (GC-MS) or liquid chromatography (LC-MS) systems, allowing separation of the molecules in a complex sample and identification of their molecular masses at same time; in chapter III, mass spectrometers were coupled to HPLC or UPLC (ultra-performance liquid chromatography) systems to analyse male tilapia urine.

3.2.4. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy is an analytical technique that allows elucidation of the molecular structure of unknown compounds and can provide information about the connectivity of atoms and the properties of molecules. In an NMR spectrometer, the sample is subjected to a strong static magnetic field. NMR relies on the phenomenon that many atomic nuclei (with an odd number of protons and/or neutrons) have an intrinsic magnetic moment and thus a non-zero spin. The most commonly studied nuclei ^{1}H and ^{13}C, for instance, have a spin = ½. If these nuclei are subjected to a magnetic field, two different energy levels, i.e. high (-½) and low (+½) are generated, but most nuclei will stay in low energy state (they will align with the magnetic field; Diehl 2008). In the spectrometer, transitions between these energy levels are achieved by employing radio frequency pulses on the nuclei. This excites the nuclei and causes them to resonate at a frequency characteristic for the nucleus/isotope and the strength of the magnetic field and gives rise to the NMR peaks. But superimposed on this basic resonance frequency is an effect from the local atomic environment as the electrons around the nucleus shield it from the magnetic field. This causes slight frequency shifts, which are also referred to as chemical shifts (in ppm) and provide information on the bonding and arrangements in a molecule (Butler 2003; Diehl 2008). Different functional groups can be distinguished by their chemical shift, for example, in a ^{1}H spectrum, a methyl group (CH_3) has usually a chemical shift around 0.9-1 ppm, whereas alcohols (CH-OH) have a shift around 3.4-4 ppm. But also identical functional groups with different neighboring substituents will still give distinguishable signals. For example, the shape of the peaks, i.e. their splitting, informs on the number of protons present on neighboring substituents. The signal intensity corresponds to the (relative) number of protons that are responsible for this specific signal.

However, to unveil the identity of an unknown, more complex molecule, a ^{1}H spectrum alone may sometimes not be informative enough. Yet, ^{13}C spectra as well as two dimensional spectra (data are plotted in a space defined by two frequency axes) provide further insights into the molecular arrangements and three dimensional structure(s) of the compound(s) in a given sample, allowing full structure elucidation. In chapter III of this thesis, ^{1}H, ^{13}C and several different types of 2D-NMR spectra were recorded to elucidate the structure of the compounds present in the most active urine fraction of tilapia males.

Chapter II

Muscular hypertrophy of urinary bladders in dominant tilapia facilitates the control of aggression through urinary signals

The chief objective of this chapter is to establish whether the ability of dominant males to store and release large urine volumes is linked to physiological (urine production) and/or morphological (bladder, kidney) differences between males of different social status and/or between sexes. Moreover, it is investigated whether male-male aggression is modulated by urination.

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Muscular hypertrophy of urinary bladders in dominant tilapia facilitates the control of aggression through urinary signals

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Abstract

The urination pattern of the Mozambique tilapia (*Oreochromis mossambicus*) depends on social context, and the olfactory potency of urine released depends on social rank (males) and reproductive status (females). This strongly suggests that urine mediates chemical communication in this species. The current study tested, firstly, whether urine production rate depends on sex or social status and, secondly, whether differences in urination pattern and volume of urine stored are associated with variation in the morphology of the urinary bladder. Finally, the effect of urination during aggressive male-male interactions was assessed. Urine production in catheterized fish depended neither on sex nor social status (males). Nevertheless, males had larger kidneys than females. Dominant males had heavier urinary bladders than subordinate males or females, mainly due to enlarged muscle fibres, thicker urothelium and a thicker smooth muscle layer. In male pairs wherein urination was prevented by temporary constriction of the genital papillae, social interactions escalated to aggression (mouth-to-mouth fighting) more rapidly and frequently than in control pairs. This was accompanied by elevated plasma testosterone and 11-ketotestosterone levels. In control encounters, the male that initiated the aggressive behaviour was usually the winner of the subsequent fight; this did not happen when the males could not urinate. These results suggest that the larger, more muscular bladder of dominant males is an adaptation, facilitating higher urination frequency, post-renal modulation and storage of larger urine volumes

for longer. It is likely that urinary pheromones modulate aggression in male-male encounters by providing information on the social rank and/or motivation of the emitter; males are unlikely to invest in costly highly aggressive fights if they judge their opponent to be more dominant. Thus, a morphological explanation for the differing urination patterns of dominant and subordinant males, and females, has been provided, and a possible function for this behaviour in male-male interactions is suggested.

Keywords | social dominance**,** chemical communication**,** urine signals**,** urinary bladder, muscle, aggression**,** *Oreochromis mossambicus*

Running title | Urine, urination and aggression in tilapia

Introduction

In many terrestrial mammals, the release of urinary signals which are detected by conspecifics plays an essential role in the communication of social rank. The best studied model species in this respect, the male house mouse (*Mus musculus*), excretes a complex mixture of proteins and volatile pheromones in its urine to mark territorial boundaries and to signal sex and social status (Brennan and Zufall 2006; Humphries and others 1999; Hurst and Beynon 2004). Dominant males release more urine than subordinate males, and castration reduces urine output (Drickamer 1995). The urination pattern is variable and dependent on hormonal and social status; when in a new environment, intact males urinate sooner and release a larger number of smaller drops than castrated males (Mucignat-Caretta and others 2004). These variations in urine release are accompanied by structural differences in the urinary bladder; bladder weight is higher in intact males and testosterone-treated castrated males than castrated males. The difference in bladder weight is mainly due to thicker muscle layers (Mucignat-Caretta and others 2004).

Unlike terrestrial animals, those living in freshwater are continually exposed to a hypotonic environment; to maintain their hydro-mineral balance, excess water entering by osmosis must be filtered in the kidney and released as dilute urine. However, urine may be stored for some time prior to discharge. Hence, many aquatic animals emit urinary signals which transmit information on their social or physiological status (Appelt and Sorensen 2007; Barata and others 2007). Crayfish males, for example, release more urine during agonistic interactions, and the eventual winner of a fight releases more urine than the loser (Breithaupt and Eger 2002). Physically blocking urine release leads to a significant increase in fight duration between size-matched males (Schneider and others 2001) and anosmic crayfish do not decrease fight duration, in repeated pairings, as control crayfish do. This implies that these animals recognise the social rank of their opponent *via* urinary signals, and that these signals are important for establishing a dominance hierarchy (Breithaupt 2011). Similar observations were made on lobsters *(Homarus americanus)*; larger urine volumes could be collected from catheterized lobster when they were disturbed by the presence of an unfamiliar conspecific than from lobsters that were simply disturbed by a rapidly moving plate, or left undisturbed (Breithaupt and others 1999).

Freshwater teleosts also use urine as a vehicle for chemical communication. Goldfish (*Carassius auratus*) females release hormone-based conjugated steroids in their urine to prime males and signal fertility (Appelt and Sorensen 2007; Stacey and Sorensen 2002). African cichlids alter their urination pattern depending on social context (Barata and others 2007; Maruska and Fernald 2012). In the case of the Mozambique tilapia (*Oreochromis mossambicus*), urinary signals are released by males to demonstrate their social dominance to rival males as well as receptive females (Barata and others 2008; Barata and others 2007). In their natural habitat, males

aggregate in spawning areas or `leks` and receptive females enter these areas to choose a male to mate with. After spawning in the territory (a pit in the substrate), the female picks up the fertilized eggs in her mouth and broods them elsewhere separate from the males (Bruton and Boltt 1975). In captivity, *O. mossambicus* males establish relatively linear hierarchies in which dominant individuals adopt a characteristic black colouration and aggressively defend a small territory. Although these hierarchies are not necessarily stable, there are fewer reversals of position between higher-ranked than lower-ranked males (Oliveira and Almada 1996). Females preferentially mate with the most dominant males (Turner 1986). Although the role of urine has not been followed in nature, in captivity the tactical release of urinary pulses seems to play an important role in mediating aggressive interactions between males. Dominant males store more urine in their urinary bladder than subordinates and increase urination frequency during aggressive disputes as well as courtship behaviour (Barata and others 2008; Barata and others 2007). In contrast, a male never releases urine when submissive to its opponent. When no loser or winner emerges during symmetrical aggression (biting or circling each-other or mouth-to-mouth fighting), high urination frequency occurs immediately before, or soon after, the start of aggressive displays. When one male becomes submissive after engaging in symmetrical aggression, the dominant male stops urinating. Females, in contrast, release urine pulses at a higher frequency than males (Almeida and others 2005), but whether they also change urination rate according to social context is as yet unclear. The olfactory potency of male urine, assessed by the electro-olfactogram, is positively correlated with the social rank of the donor. A sterol-like urinary odorant has been proposed to act as a pheromonal signal of dominance (Barata and others 2008).

The underlying mechanisms behind differences in urination pattern between dominant and subordinate *O. mossambicus* males are as yet unknown. Subordinate males may produce less urine, for example, as a result of lower glomerular filtration rate, or dominant and subordinate males may produce urine at the same rate but subordinates are unable to store as large volumes as dominant males due to differences in bladder morphology, or both. The chemical information conveyed through increased urination by dominant males is likely to include a putative pheromone that advertises dominance and may modulate aggression between rivals thereby contributing to the stability of social hierarchy. Increased urine release associated with visual and/or auditory displays from dominant males conveys multimodal sensory information that could provide accurate and reliable information to receivers about the social status of the sender (e.g., Amorim and others 2003; Barata and others 2007; Maruska and Fernald 2012; Ward and Mehner 2010).

The aims of the present study were therefore to determine in *O. mossambicus*: **1)** whether urine production depends on social status or sex; **2)** if bladder morphology varies between social

status or sex and relates to observed differential urine volume and urination frequency; and **3)** if aggression between rival males is modified by urination.

Materials and methods

Ethical statement

Fish care and experimentation complied with the guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals under a 'group-1' license issued by the veterinary directorate "Direcção Geral de Veterinária" of the Ministry of Agriculture, Rural Development and Fisheries of Portugal.

Selection of dominant and subordinate males

Sexually mature Mozambique tilapia were raised in captivity from a brood-stock maintained at the University of Algarve (Faro, Portugal). Social groups of five males and five females (26 groups in total) of similar standard length (SL in mm) and body weight (BW in g; coefficient of variation of BW less than 5 %) were maintained for nine days in plastic tanks (93 x 55 x 50 cm; ca. 200 l) containing re-circulated and bio-filtered freshwater at 27 °C and sand substrate. The photoperiod was 12L : 12D and fish were fed once a day after behavioural observations with commercial cichlid food pellets. Spawning occurred spontaneously but eggs were removed from the female's mouth to stimulate the initiation of a new ovulatory cycle. Males were tagged with coloured plastic labels (T-Bar anchor FD94, Floy Tag Inc., Seattle, WA, USA) attached to the muscle near the dorsal fin. Systematic focal observations of each male started on the fourth day after formation of each social group and were carried out over five consecutive days at noon. The frequency of submissive displays during agonistic interactions or absence of dark coloration without social interaction and dominant behaviours such as aggression (biting, chasing, lateral displays, circling or mouth-to-mouth fights), nest digging, courtship towards females or dark coloration without social interaction was recorded over five min for each male. A dominance index (DI) ranging from zero to one was calculated for every male each day as the sum of all dominant behaviours and subsequent division by the sum of all dominant and subordinate behaviours (Barata and others 2007). Accordingly, after five days of observation the mean DI was assessed for every male. Males with a DI ≤ 0.16 were selected as subordinates while males with a DI ≥ 0.8 were chosen as dominants. After daily observation, urine was collected by gently squeezing the abdomen immediately above and anterior to the urogenital papilla and collecting the resultant stream of urine in a plastic tube. Urine volumes were measured gravimetrically to explore eventual correlations with urinary bladder weights.

Assessment of urine production

Urine production was measured by urinary cannulation (Summerfelt and Smith 1990; Wood and Patrick 1994) whereby the urine is siphoned as soon as it enters the bladder from the mesonephric ducts without remaining in the bladder. The cannula was a soft polyethylene (T10-PE) tube of small length (5 to 6 mm; O.D. = 0.60 mm, ID = 0.28 mm) with four additional small side-openings (to reduce likelihood of blockage), which was glued to a T50-PE tube (O.D. = 0.97 mm, I.D. = 0.58 mm) of ca. 1 m length. The end of the T50-PE tube was bent at a 45° angle to facilitate entire insertion of the T10-PE tube and initial length of the T50-PE tube into the urogenital papilla. During the procedure, fish were anesthetized in water containing 200 $mg.l^{-1}$ 3-aminobenzoic acid ethyl ester (MS-222, Sigma-Aldrich, St. Louis, MO, USA) and placed in a V-shaped sponge with their gills irrigated with aerated water containing the anaesthetic. The cannula was carefully inserted through the urogenital papilla and secured in place with three ligatures, one just anterior to, and two to the side of the anal fin. After the catheter was well secured without leaks, the area around the first two ligatures was thoroughly wiped to remove mucus and a thin film of tissue cement (3 M Vetbond) was applied preventing the catheter from slipping out. In preliminary tests, correct urinary cannulation was verified by dorsal intramuscular injection of 100 µl phenol red (100 $mg.kg^{-1}$ in 0.9 % NaCl), which readily appeared in the urine flowing through the cannula; correct placement of the cannula in the bladder was further confirmed by dissection of the fishes and careful observation under a stereo microscope. After cannulation, each fish was placed in individual plastic aquaria (11.4 x 25.8 x 38 cm) with ca. 2.4 l aerated biofiltered freshwater (27 °C) flowing continuously (ca. 20 $ml.min^{-1}$). The cannula was led out of each aquarium and connected to one of two holes in the lid of a 1.5 ml Eppendorf tube hanging below the aquarium bottom. Urine production were measured 24 h after cannulation every hour over 5 h in dominant (mean ± SD; $N = 5$; SL = 130 ± 19 mm; BW = 65 ± 24 g) and subordinate males ($N = 6$; SL = 129 ± 8 mm; BW = 60 ± 10 g) and non-ovulatory females ($N = 6$; SL = 112 ± 6 mm; BW = 41 ± 7 g) and expressed as ml per hour per kg fish.

Morphometric analyses

Dominant (mean ± SD; $N = 11$; SL = 172 ± 11 mm; BW = 162 ± 35 g) and subordinate (N = 13; SL = 174 ± 15 mm; BW = 162 ± 40 g) males and females ($N = 10$; SL = 181 ± 11 mm; BW = 201 ± 38 g) of similar standard length and body weight (ANOVA, $F_{2,29} = 1.223$, $P = 0.309$ and $F_{2,29} = 3.155$, $P = 0.058$) from social groups were sacrificed with a lethal overdose of anaesthetic (MS-222; 1 $g.l^{-1}$). Empty urinary bladder-, liver- (HSI), kidney- (KSI) and gonad- (GSI) somatic indices (%) were determined for each fish, as the organ weight relative to the fish's total body weight. For histological analysis a section of urinary bladder was cut and fixed in Bouin's fixative solution. Tissue samples were sagitally oriented and embedded in paraffin, serially sectioned (7 µm thickness) and stained with Masson's trichrome. For the morphometric analysis, the widest

serial sagittal histological section through the urinary bladder body of each specimen was chosen and digitalized using a camera (Leica DC Image) coupled to a light microscope (Leitz Dialux 20). The measurements were carried out through interactive image analysis software (Sigma Scan Pro 5). To calculate the thickness of the urothelium, four regions of the urothelium not detached from the underlying connective tissue were chosen and digitalized at x25 magnification. Because the inner surface of the bladder is highly folded in its empty state, five measurements of the thickness of the urothelium were taken randomly in each region. The average urothelial thickness per fish (total of 20 measurements) was considered for data analysis. The relative area of the detrusor muscle layer in the wall of the urinary bladder was calculated as the ratio between the area measured for each tissue and the total area of the histological section on digitalized images captured at x10 magnification. The cross-sectional area of the muscle fibres was measured only in fibres wherein the nucleus was completely visible. Measurements were obtained from 20 cross-sectioned muscle fibres per specimen on digitalized images of the widest histological section of the urinary bladder captured at x40 magnification.

Effect of urination during male-male aggressive interactions

Reproductively-active males (N = 22, mean ± SD; SL = 130 ± 12 mm; BW = 71 ± 23 g) were used to study the effect of urination on aggressiveness. Before experiments and in-between trials, each male was housed in residence tanks together with four or five females but without male-competitors. The experimental aquarium (79 cm x 35 cm x 45 cm) for encounter experiments was supplied with sand substrate and divided at the centre by a removable opaque partition covered with plastic material. Application of a common food dye colorant to one compartment confirmed that the opaque plate prevented diffusion between one half and the other. Male pairs of similar size (difference in SL = 0.8 ± 0.6 mm; BW = 2.4 ± 1.6 g) were removed from their residence tanks at a time, anaesthetised with MS-222 (200 $mg.l^{-1}$) and their urogenital papillae tied using surgical silk. For the control experiments, males were treated similarly but surgery only simulated by manipulating the papillae with forceps without actually tying them. Fish were allowed to recover from anaesthesia and then introduced into the experimental aquarium (one fish per compartment); after 30 min, the central partition was removed and males were allowed to interact for 45 minutes. Encounters were symmetrical in that both males were either tied or controls. Subsequently, all males were returned to their residence tanks, after removal of surgical silk when appropriate. Pilot experiments using dye-injected males (see Miranda and others 2005) showed that the treatment was effective in preventing urination, but caused no obvious signs of discomfort. Each pair of males was used twice, with a one week interval; once with untied urogenital papillae (control) and once with tied urogenital papillae whereby they were unable to urinate. The order of the two treatments was alternated among male pairs. The interactions of each male pair were video recorded and behaviours analysed using the software “The Observer”

(version 5, Noldus Technology, Wageningen, The Netherlands). The behaviour repertoire was divided into levels of aggression: frontal and lateral displays; chasing; circling fight; biting and mouth-to-mouth fighting. Numbers of aggressive behaviours as well as latencies were counted for each individual and level. The hierarchical stability (Gonçalves-de-Freitas and others 2008) in both treatments was calculated considering the sum of all agonistic behaviours from the pair of males (A + B) in all levels as the following: [given attacks by A/(given attacks by A + received attacks from B)] – [given attacks by B/(given attacks by B + received attacks from A)]. Values closer to one indicate hierarchical stability and more aggressive asymmetry; contrarily, values closer to zero indicate instability and more aggressive symmetry. For each male the fight outcome in respect to their fighting initiative was categorized as follows: winning, losing, unresolved. Urine was collected from all males by gently squeezing their abdomens after each interaction period and urine volumes measured. Plasma samples for subsequent measurement of testosterone and 11-ketotestosterone levels were collected only after the second experiment (N = 6 for each control and tied males) to minimize stress factors for the fish between trials and stored at -20 °C until analysis.

Quantification of androgen levels

Testosterone (T) and 11-ketotestosterone (11-KT) were extracted from plasma with diethylether, the solvent evaporated and the residue re-suspended in 0.5 M phosphate buffer. Steroids were analysed by radioimmunoassay as previously described: T (Scott and others 1984) and 11-KT (Kime and Manning 1982). Extraction efficiency was 90 % for both steroids, with intra-assay and inter-assay co-efficients of variation of 7.5 % and 12.4 % (T), and 9.1 % and 9.2 % (11-KT), respectively (Condeça and Canário 1999).

Statistical analysis

One-Way ANOVA was used to compare relative urinary bladder- and kidney weights, muscle cell diameter, urothelial thickness in the urinary bladder wall and primary urine production between dominant and subordinate males, and females. Datasets were normally distributed and of equal variance (F-test). Square root transformation was used to normalise muscle cell diameter data. When the ANOVA was significant the Holm-Sidak post-hoc was used to identify which groups differ. Kruskal-Wallis ANOVA on Ranks followed by Dunn's method was used to compare muscle layer areas in the urinary bladder between sex and social status. Two Way Repeated Measures (TW-RM) ANOVA followed by the least square mean (LSD) test for planned comparisons was used to compare response latencies and number of aggressive behaviours among tied versus normally urinating males and different aggression levels; the Dunn-Sidak method was used to obtain exact probabilities where more than two comparisons between means were lower than 0.05. The Wilcoxon Signed Rank Test was used to compare hierarchical stability between pairs of

tied and control males in the behavioural experiment. Student's *t*-test was applied to compare androgen levels in those treatments. Chi-squared (χ^2) tests were applied on the distribution of fighting outcomes in urogenital papillae tied males and control males. Spearman's rank correlation coefficient was calculated to measure statistical dependence between the urinary bladder weight and the total urine volumes stored and between morphological parameters of the urinary bladder (i.e., relative bladder weight, muscle layer area, muscle fibre diameter). All data are shown as mean ± SEM and statistical significance was established at $P < 0.05$.

Results

Urine production and morphometric analyses

Dominant males stored significantly more urine in their bladder than subordinate males (5.3 ± 0.7 ml versus 1.5 ± 0.5 ml; unpaired *t*-test, $N = 24$, $t = 4.654$, $P < 0.001$). In contrast, urine production was variable between individuals and not statistically different among groups: 3.74 ± 1.66 $ml.h^{-1}.kg^{-1}$ in dominant males, 2.73 ± 1.42 $ml.h^{-1}.kg^{-1}$ in subordinate males and 6.53 ± 1.50 $ml.h^{-1}.kg^{-1}$ in females (Figure 1). Therefore, the larger urine volumes stored in dominant male bladders are unlikely due to higher urine production.

Relative urinary bladder (empty) weight was significantly larger in dominant (mean ± SEM. 0.097 ± 0.009 %) than in subordinate (0.061 ± 0.004 %) males and both larger than females (0.028 ± 0.008 %; Figure 2A). Relative kidney weight was similar in males of different social status (dominant 0.302 ± 0.021 %; subordinate 0.275 ± 0.012 %; Figure 2B) and both were larger in males than females (0.133 ± 0.01 %). Also, the GSI did not significantly differ between dominant (0.559 ± 0.045 %) and subordinate (0.698 ± 0.085 %) males (unpaired *t*-test, $N = 22$, $t = -1.199$, $P = 0.243$). No differences were found in relative liver weights, neither between sexes (females 4.181 ± 0.800 %) nor social status of males (dominant 3.017 ± 0.397 %; subordinates 2.732 ± 0.327 %; $N = 32$, One-Way ANOVA, $F_{2,29} = 2.264$, $P = 0.122$).

Histological examination of cross-sections of the urinary bladder showed differences in urothelial thickness, relative area of the detrusor muscle layer (M; Figure 3A, C, E) and muscle fibre diameter among females, dominant and subordinate males (Figure 3B, D, F). The urothelium was highly folded in all individuals, regardless of sex and social status. Urothelial thickness was significantly different between the three groups ($P < 0.001$; Figure 4A); thickest in dominant males (61.6 ± 3.7 µm), thinner in subordinate males (52.0 ± 2.4 µm) and thinnest in females (38.6 ± 2.2 µm). Dominant males (43.1 ± 2.1 %) had a thicker detrusor muscle layer than subordinates (30.7 ± 1.1 %; $P = 0.004$; Figure 4B). Dominant males also had the largest muscle fibres (58.2 ± 3.0 µm), followed by subordinate males (38.9 ± 0.9 µm) and females (17.1 ± 0.6 µm; $P < 0.001$; Figure 4C).

In males, relative urinary bladder weights correlated positively with the total urine volumes stored ($N = 22$, $r = 0.65$, $P < 0.005$; Figure 4D). Further, urinary bladder weights correlated positively with the detrusor muscle area ($N = 25$, $r = 0.477$, $P = 0.0161$), muscle fibre diameter ($N = 25$, $r = 0.664$, $P < 0.001$) and urothelium thickness ($N = 18$, $r = 0.501$, $P = 0.0338$) measured in the urinary bladder wall of dominant and subordinate males and females. A strong positive correlation was also found between the area occupied by the detrusor muscular layer and the muscle fibre diameter in subordinate and dominant males ($N = 17$, $r = 0.860$; $P < 0.001$). However, when female data were included in this analysis, no statistically significant correlation

was present (N = 25, r = 0.344, P = 0.091; Figure 4E). Urothelial thickness of males and females correlated strongly with the muscle fibre diameter (N = 18, r = 0.872, P < 0.001) but not at all with the detrusor muscle area (N = 18, r = -0.0299, P = 0.902).

Effect of urination during male-male aggressive interactions

Both control and urogenital papillae tied males showed a lower number of low aggressive behaviours such as displays and chasing than highly aggressive behaviours, with no significant differences between the two types of males (Figure 5A). However, at higher levels of aggression, tied males engaged in significantly more circling and mouth-to-mouth fighting (mean ± SEM; 108 ± 35, P = 0.003 and 103 ± 35, P = 0.007) than control males (72 ± 22 and 71 ± 22). Moreover, tied males engaged more quickly in circling (P = 0.003) and mouth-to-mouth (P = 0.013) fighting than control males (Figure 5B). The behavioural sequence always followed the same order from the lowest to the highest level of aggressiveness and latencies to the first display shown by any of the rivals were significantly shorter than latencies until circling (P = 0.036) or mouth-to mouth fights (P = 0.035). Only one pair out of eleven in both tied and control dyads did not escalate to circling- and mouth-fighting. In addition, the fight duration, measured as time between start of circling fights until the end of all fighting events (emergence of a clear winner and loser), tended to be lower in control males than in tied males but did not reach statistical significance (paired t-test, t = 2.194, P = 0.053). Submissive behaviours were observed only at low rates, and the latency until one of the rivals showed submission or fleeing was high and similar to that of high aggression. There was neither a statistical difference in number of submissive events (7 ± 3 and 5 ± 3) nor in latency (15.35 ± 5.08 min and 14.26 ± 4.86 min) between tied and control males. Preventing rival males from urination had a clear negative effect on their hierarchical stability (Figure 5C, P < 0.001). Males that could urinate normally showed a more asymmetric aggression pattern, whereas pairs of tied males were symmetric in their aggressiveness. For each male, the outcome in respect to their fighting initiative was categorized as follows: winning, losing, unsolved. In eight out of eleven fights, males that initiated the fight also won the fight when they could urinate normally; only one male lost a fight and two fights were unresolved (χ^2 = 7.818; P = 0.02). In contrast, when tied males initiated a fight, they were winners only in three out of eleven cases, lost four fights and four fights remained unresolved (χ^2 = 0.182; P = 0.913). As expected tied males had much more urine (0.87 ± 0.17 ml) stored in their urinary bladder than control males (0.29 ± 0.09 ml; paired t-test, t = -4.298 P < 0.001). Blood plasma levels of the two androgens T and 11-KT after the aggressive encounter were significantly higher in males with tied urogenital papillae as compared to control males (Figure 6).

Discussion

The present study shows that urinary bladder morphology in the tilapia depends on both sex and social status, and helps to explain the different urination patterns and urine storage capacities in dominant and subordinate males, and females. Dominant males had a thicker detrusor muscle layer than subordinates, but not necessarily females. In addition, the muscle fibre diameter and the urothelium thickness were also larger in dominant males. These histological differences might explain the higher bladder weights of dominant males. Structural differences in the urinary bladder of dominants, subordinates and females are most likely related to variations in urination behaviour. Females urinate at higher frequency than males (Almeida and others 2005), whereas dominant males store more urine in their bladder, for longer, than subordinates or females (Barata and others 2008). This allows males to release large volumes of urine under in specific social contexts such as in the presence of pre-ovulatory females (Barata and others 2008) or intruder males (Barata and others 2007).

The thicker muscle layer and larger diameter of muscle fibres in dominants may be necessary to resist higher internal pressure on the urinary bladder wall. Thicker detrusor muscle and enlarged muscle fibres are likely to improve the mechanical properties of the bladder by increasing the contractile force needed for context-specific urine release. The urothelium was highly folded and thicker in all bladders allowing them to accommodate large urine volume changes. A thicker urothelium in dominants can also support increased urination frequency at certain times which are expected to cause greater tensile forces upon the bladder surface. It has been suggested recently that epithelium thickness, in general, is closely related to the internal and external forces acting on it and might also be important to infer interfacial (net surface) tensions (Chen and Brodland 2009). Our results are similar to previous studies on male mice, where increased muscular mass and urinary bladder weight were observed in intact males as compared to castrated males or females. These differences could be related to a distinct urination pattern and a higher volume of urine retained in the bladder after voiding in intact males (Mucignat-Caretta and others 2004). Testosterone-treated castrated male and female mice developed larger bladders and could retain larger quantities of urine in their bladder as compared to controls; thus T induces morphological modifications of the urinary tract necessary to support the dominant male urination pattern in mice (Mucignat-Caretta and others 2004). The effect of T supplementation on bladder morphology and urine storage capacity on female and subordinate male tilapia was not investigated in this study. However, androgen (T and 11-KT) and progestogen (17α, 20α-dihydroxy-4-prengen-3-one (17, 20α-P) and 17α, 20β-dihydroxy-4-prengen-3-one (17, 20β-P)) levels in the urine of dominant tilapia males have been shown to be higher than in subordinates (Oliveira and others 1996). Thus, it is likely that the morphological changes in the urinary bladder

and urine storage capacity in dominant tilapia males are androgen dependent. Although the current study showed no difference in the GSI of dominant and subordinate males, other studies ((Oliveira and Almada 1999) showed that GSI - plus a number of other external morphometric parameters (fin length, jaw size etc.) - were positively correlated with dominance index.

Although dominants had larger urinary bladders with larger urine volumes stored, the primary urine production rate was independent of sex and social status. However, cannulated animals were isolated 24 h before and during experiment and possible influences of such short isolation on urine production rates are unknown. Nevertheless, our results are in agreement with rainbow trout in which the glomerular filtration rates are also independent of hierarchical status, although urine volume released over time was significantly lower in dominant fish (Sloman and others 2004). Although not tested in the present study, it is also possible that morphology and physiology of the urinary system may change with ovarian cycle in females. Nevertheless, kidneys were larger in male tilapia than females. Adult male freshwater sculpins (*Cottus hangiongensis*) have hypertrophied kidneys during the spawning period, equipped with a high number of secretory granules whereas female kidneys change little (Goto and others 1979). During the spawning season, the kidneys of male sticklebacks also grow and produce mucus that aids nest building (Ikeda 1933) and treatment of goldfish and masu salmon males with 17β-estradiol and/or 17α-methyl-T contributes to an increase in kidney weight (Yamazaki and Watanabe 1979; Yambe and Yamazaki 2006). Thus, although urine production is unaffected, the kidneys and bladders of male fish clearly have some function related to reproduction. In the case of the tilapia, this may involve the active secretion of putative pheromones into the urine and/or subsequent post-renal modification during its longer storage in the bladder. Either could explain the higher olfactory potency of dominant male urine.

Male tilapia unable to urinate during dual encounters engaged significantly more frequently, and more rapidly, in highly aggressive behaviours and escalating fights than control males. Also previous investigations on crayfish males have shown that blockage of urine release increases fighting intensity (Schneider and others 2001). Although the current experimental design necessarily precluded the fleeing of one male before aggression escalated, the results suggest that the release of urinary signals reduces male-male aggression and escalation of conflicts. Escalating battles are energetically costly for *O. mossambicus* males (Ros and others 2006); therefore, any method of conflict resolution which avoids this would save energy and be beneficial to both rivals. This assumes that males are **(a)** able to predict the outcome of a fight based on the chemical signals that they perceive in their opponent's urine and that **(b)** the production costs of a putative dominance pheromone are energetically favourable over escalating fights. Indeed, the majority of fight initiators also won the conflict under control conditions, whereas in tied males the outcome was not dependent on the instigator. This strongly suggests that males can predict fighting outcomes based on the olfactory information present in their rival's urine. Such odorants

could amplify information about the aggressive motivation of the sender and its dominance status and/or health condition. Once a male has gained a high hierarchical status in a social group, maintaining dominance may result partially from its ability to respond to challengers by urine release.

Plasma T and 11-KT levels were higher in tied males as compared to normal males after the encounter. Higher androgen levels during periods of hierarchical instability when social interactions are more frequent and more intense have been reported previously in teleosts (Cardwell and Liley 1991; Hirschenhauser and others 2004). In fact, hierarchical stability values calculated in our study were lower in tied than in control dual encounters. However, *O. mossambicus* males facing their own mirror image - by definition without an eventual winner or loser - show no hormonal responses (urine measurements), despite escalating aggression (Oliveira and others 2005). In the current study, males unable to urinate seemed unable to predict the outcome of the fight, whereas males able to urinate or - more importantly - able to smell their rival's urine, could. A male facing its mirror image only has visual information from his 'rival'. Though tied males cannot predict their opponent's aggressive motivation based on urinary signals, they are experiencing the physical strength of the rival and eventually injuries. This is not given for males fighting only their mirror image. The differences in hormonal responses in these two experimental paradigms may therefore depend on the relative importance of olfactory, visual and physical input.

In conclusion, the morphology of the urinary system in *O. mossambicus* is dependent on sex and the social rank of males, whereas urine production rate is not. Morphological differences in the urinary bladder between dominant and subordinate males, and females, may facilitate different urination behaviours - and release of urinary odorants - in different social contexts. The larger, more muscular bladder of dominant males is an adaptation, allowing higher urination frequency and post-renal modulation of large urine volumes. Urinary odorants - putative pheromones - are likely acting as a signal of dominance, thus contributing to hierarchical stability and conflict resolution.

Acknowledgements

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Figures and figure legends

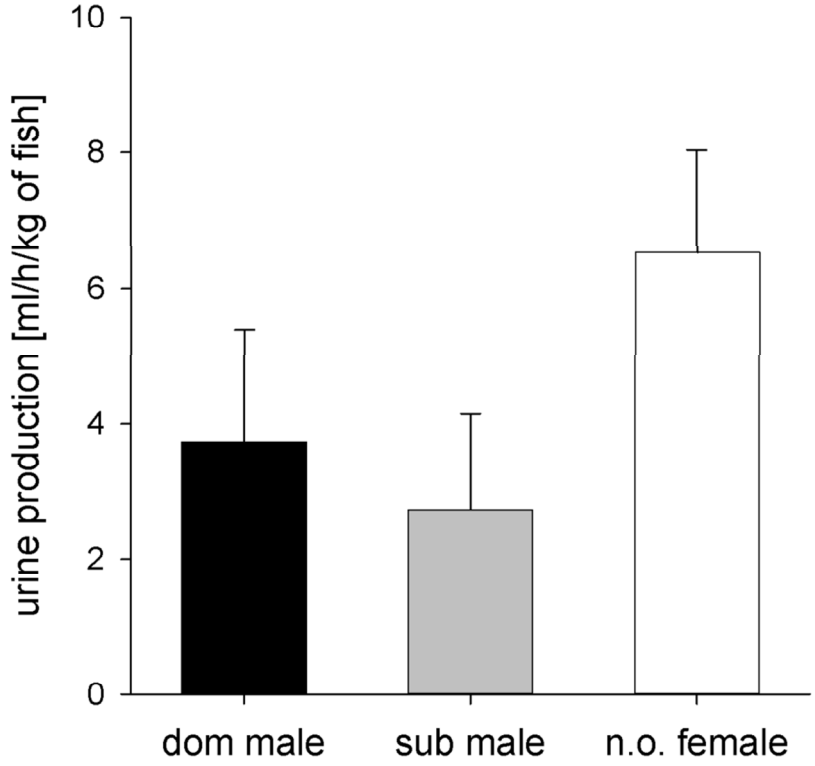

Figure 1 | Comparison of urine production rates (mean + SEM; $ml.h^{-1}.kg^{-1}$) in cannulated dominant (black bar; N = 5) and subordinate (grey bar; N = 6) males and non-ovulatory (n.o.) females (white bar; N = 6); ANOVA, $F_{2,14} = 1.764$, $P = 0.207$, NS.

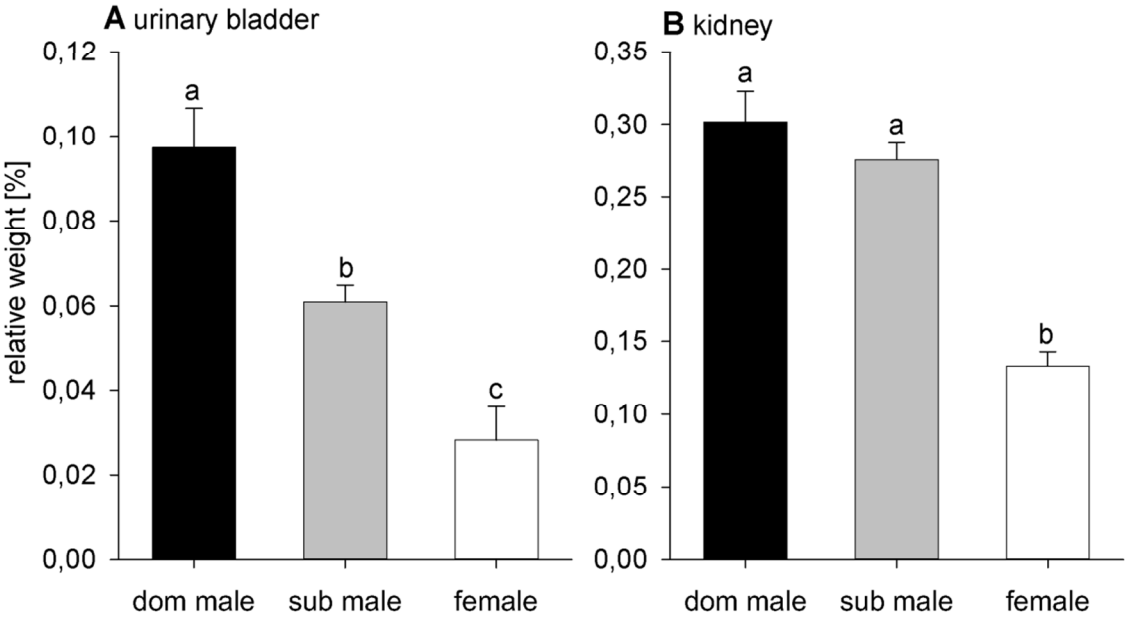

Figure 2 | Comparison of relative urinary bladder and kidney weights. Organ-somatic indices (mean + SEM; %) of dominant (black bars), subordinate (grey bars) males and females (white bars). (**A**) Relative urinary bladder weight of dominant males (N = 9) was significantly larger than of subordinate males (N = 13) and females (N = 8); ANOVA, $F_{2,27}$ = 22.63, P < 0.001, followed by Holm-Sidak method. **(B)** Relative kidney weight was larger in dominant (N = 11) and subordinate (N = 13) males than in females (N = 8); ANOVA, $F_{2,29}$ = 28.3, P < 0.001, followed by Holm-Sidak method. Different letters over the bars indicate significant differences.

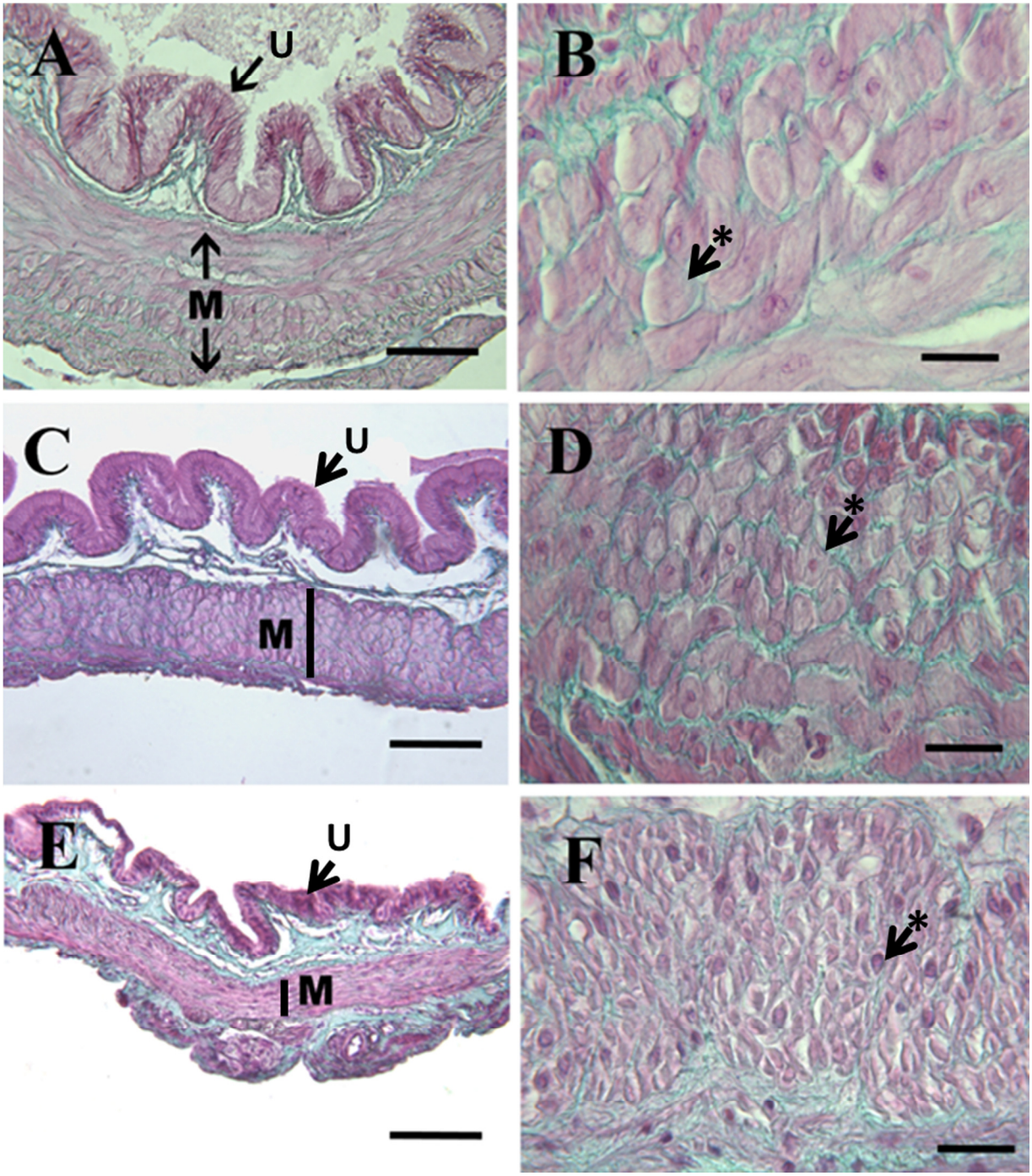

Figure 3 | Histological sections of the urinary bladder from *O. mossambicus* stained with Masson's trichrome. Images **A**, **C** and **E** show the detrusor muscular layer **(M)** in the wall of the urinary bladder of dominant males, subordinate males and females. Note that the urothelium **(U)** is highly folded, regardless of sex or social rank. Scale bar in **A**, **C**, and **E** = 50 µm. Muscle fibers **(*)** are larger in dominant (**B**) compared to subordinate males (**D**) and females (**F**). Scale bar in **B**, **D** and **F** = 10 µm.

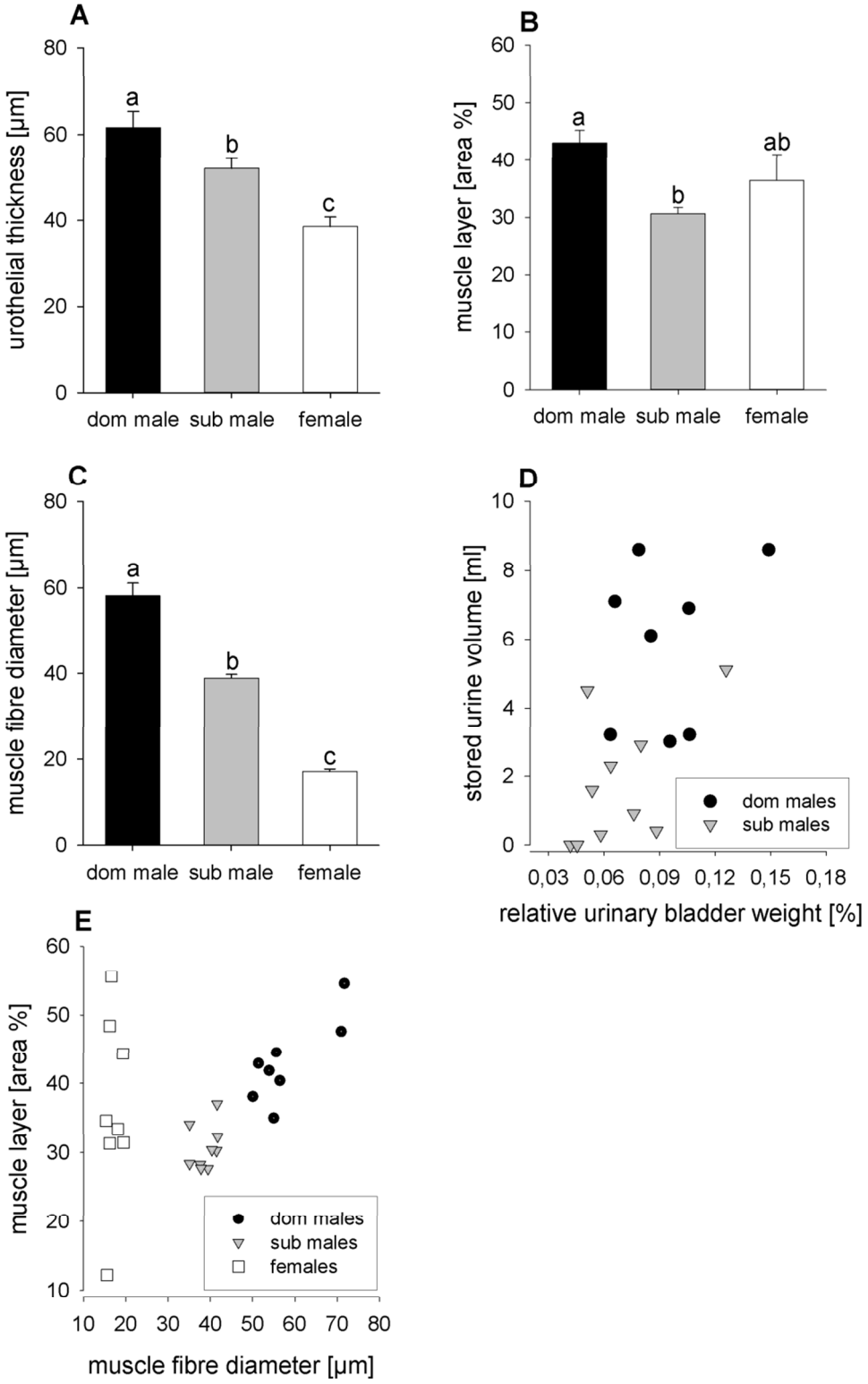

Figure 4 | Morphological differences in the urinary bladder of *O. mossambicus.* (**A**) *y*-axis: urothelial thickness (mean + SEM; µm) was largest in dominant (N = 10), followed by subordiante males (N = 10) and smallest in females (N = 10); ANOVA, $F_{2,25} = 14.69$, $P < 0.001$, followed by Holm-Sidak method. (**B**) *y*-axis: detrusor muscular layer area (mean + SEM; %) of

dominant males (N = 8) was larger than in subordiante males (N = 9) with females (N = 8) between the two; Kruskal-Wallis ANOVA on Ranks, H = 10.94; df = 2; P = 0.004, followed by Dunn's Method. **(C)** y-axis: muscle fibre diameter (mean + SEM; µm) was largest in dominant (N = 8), followed by subordiante males (N = 9) and smallest in females (N = 8); ANOVA, $F_{2,22}$ = 204.49, P < 0.001, followed by Holm-Sidak method. Different letters over bars indicate significant differences. **(D)** Positive correlation between stored urine volume (ml) collected during 5 consecutive days of behavioural observation from subordinate and dominant males and their relative urinary bladder weights; Spearman correlation, N = 22, r = 0.65, P< 0.005. **(E)** Positive correlation between muscular layer area (%) and muscle fibre diameter in the urinary bladder wall of subordinate and dominant males; Spearman correlation, N = 17, r = 0.86; P < 0.001; but not when females were included into the analysis; N = 25, r = 0.344, P = 0.09.

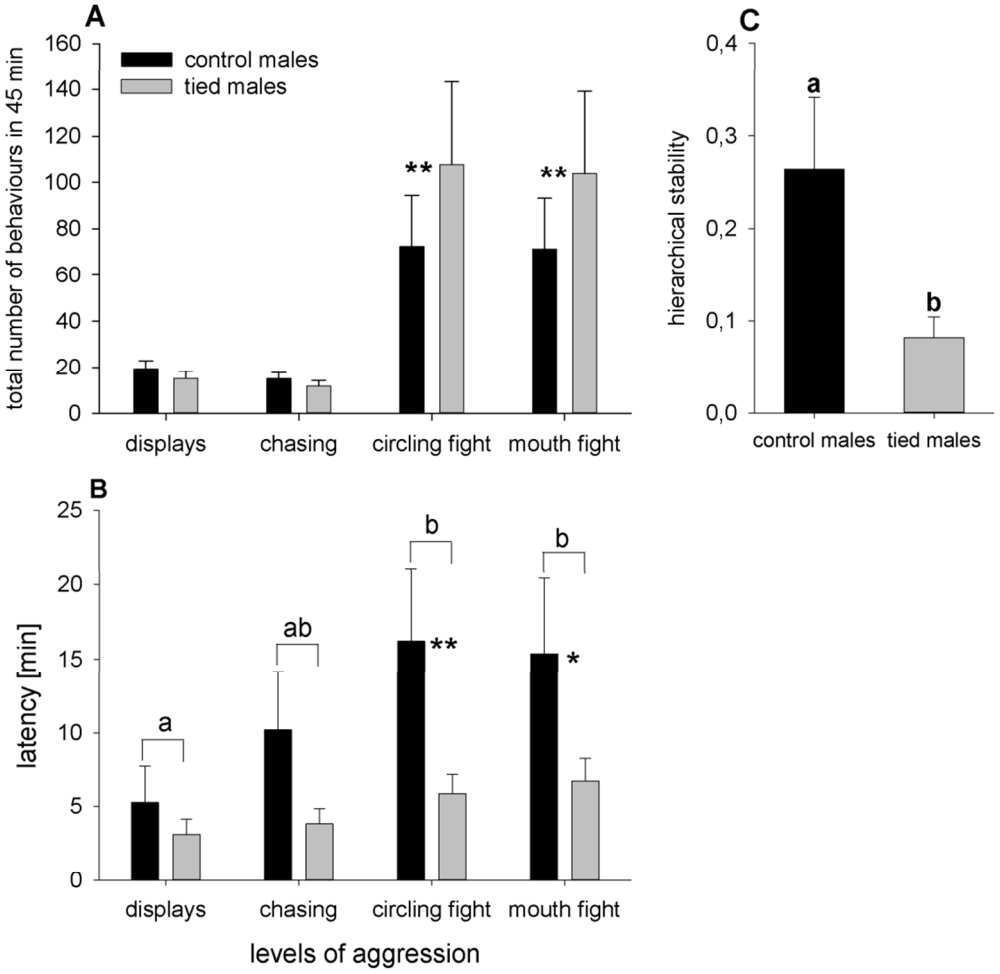

Figure 5 | Levels of observed aggressive behaviours from pairs of control males versus the same pairs of males with tied urogenital papillae during 45 min. **(A)** Total number of agonistic behaviours given by each male pair (mean + SEM). Tied males engaged in significantly more highly aggressive behaviours (circling and mouth fight) than males that could urinate normally, as indicated by asterisks above bars (** $P < 0.01$); TW RM ANOVA, $F_{1,10} = 2.71$, $P = 0.13$ (treatment); $F_{3,30} = 6.36$, $P = 0.002$ (level of aggression) and $F_{3,30} = 4.59$, $P= 0.009$ (treatment x level of aggression), followed by LSD test. **(B)** Latency (mean + SEM; min) to the first aggressive behaviour given by one of the two rivals. Latencies to highly aggressive behaviours in tied males were shorter than in untied males (* $P < 0.05$; ** $P < 0.01$; differences between treatments); highly aggressive behaviours (circling and mouth fight) appeared significantly later in time than lower aggression (displays), as indicated by the different letters above bars (differences among behaviours); TW RM ANOVA, $F_{1,10} = 4.87$, $P = 0.052$ (treatment); $F_{3,30} = 5.99$, $P = 0.003$ (level of aggression) and $F_{3,30} = 1.61$, $P = 0.21$ (treatment x level of aggression), followed by LSD test. **(C)** Hierarchical stability within each male pair (mean + SEM) expressed by the subtracted ratios of attacks from each male to the sum of attacks given by both individuals. Control males were

hierarchically more stable than tied males; Wilcoxon Signed Rank Test, $N = 11$, $W = -66.0$, $P < 0.001$.

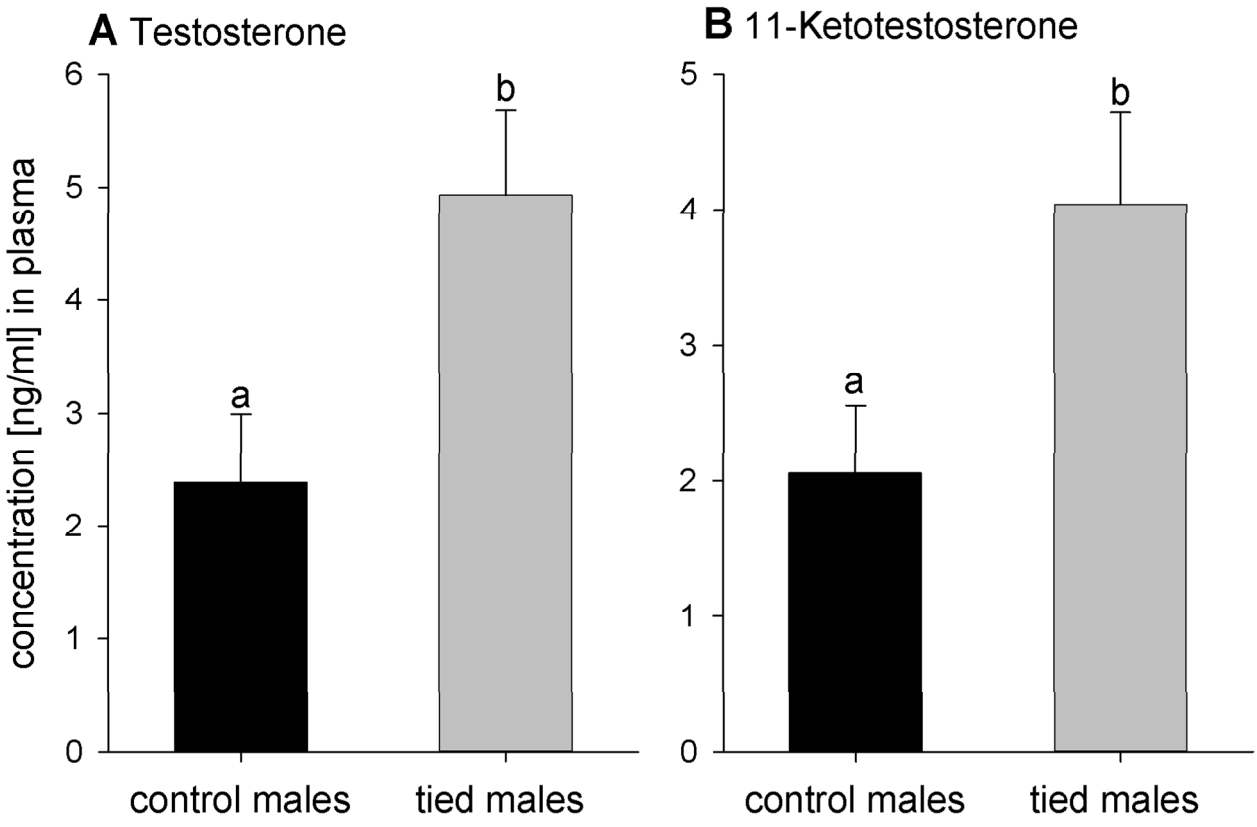

Figure 6 | Testosterone (A) and 11-ketotestosterone (B) plasma levels (mean + SEM. $ng.ml^{-1}$) in control and urogenital papilla tied males after the encounter period of 45 min. Urogenital papillae tied males had significantly higher levels of T and 11-KT in their plasma than control males; unpaired *t*-test, $N = 12$, $t = 2.623$, $P = 0.025$ and $t = 2.345$, $P = 0.041$.

Chapter III

Identity of a tilapia pheromone released by dominant males that primes females for reproduction

The chief objectives of this chapter were to isolate the compound(s) present in the olfactory most active male urine fraction, elucidate the chemical structure, to verify the olfactory activity of the identified compound(s) with synthetic analogues and to unveil the pheromonal function of the identified compounds.

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REPORT

Identity of a tilapia pheromone released by dominant males that primes females for reproduction

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Abstract

Knowledge of the chemical identity and role of urinary pheromones in fish is scarce, yet necessary to understand the integration of multiple senses in adaptive responses and the evolution of chemical communication. In nature, Mozambique tilapia (*Oreochromis mossambicus*) females mate preferentially with dominant territorial males which they visit in aggregations or leks (Bruton and Boltt 1975). Dominant males have thicker urinary bladder muscular walls than subordinates or females and store large volumes of urine which they release at increased frequency in the presence of subordinate males or pre-ovulatory, but not post-spawned, females (Barata and others 2008; Barata and others 2007; Keller-Costa and others 2012). Females exposed to dominant male urine augment the release of the oocyte maturation-inducing steroid 17α,20β-dihydroxypregn-4-en-3-one (17,20β-P; Huertas and others 2014) and spawn in the vicinity of males artificially scented with urine from dominant males (Barata and others submitted manuscript). Here we isolate and identify a male Mozambique tilapia urinary sex pheromone as two epimeric (20α- and 20β-) pregnanetriol 3-glucuronates. The 20β-epimer is 10-20 times more abundant than the 20α-epimer and its concentration increases with dominance. We show that both males and females have high olfactory sensitivity to the two steroids which cross adapt upon stimulation. Females exposed to both steroids show a rapid, 10-fold increase in production of 17,20β-P. Thus, the identified urinary steroids prime the female endocrine system to accelerate oocyte maturation, which likely promotes spawning synchrony. Tilapias are both important as a

source of food worldwide and an invasive species with devastating impact on local freshwater fauna (Canonico and others 2005; Morgan and others 2004). Knowing the chemical cues that mediate reproduction may provide a tool towards population control (Corkum and Belanger 2007; Madliger 2012; Sorensen and Stacey 2004).

Key words | pheromone, chemical communication, chemical identification, pregnanetriol 3-glucuronate, fish, tilapia, *Oreochromis mossambicus*

Running title | Identification of the tilapia male sex pheromone

Results and discussion

Bioassay guided fractionation of male urine samples

The Mozambique tilapia olfactory response to male urine and C18-SPE solid phase extracts of urine is positively correlated to social status (dominance index, DI) of the donor (Spearman correlation; urine: $r_s = 0.537$, $P = 0.0258$; urine extract: $r_s = 0.591$, $P = 0.0124$). The dominance index DI of the donor males was assessed by daily observing the donor males dominant (aggressive displays, circling or mouth-to-mouth fights, courtship towards females, nest digging, dark colour) and subordinate (submissive displays, fleeing, light grey colour) behaviours (Barata and others 2008; Keller-Costa and others 2012). Urine samples were subsequently collected from dominant (DI≥0.8), intermediate (0.16<DI<0.8) and subordinate males (DI≥0.16). Bioassay-guided fractionation of dominant male urine extracts by high performance liquid chromatography (HPLC) with light scattering detection (ELSD), revealed one fraction (fraction A) with particularly strong olfactory potency (Figure 1a (Barata and others 2008)). Fraction A did not absorb ultraviolet radiation indicating absence of chromophores. Fraction A was also present but at lower intensity (Figure 1a) in urine from intermediate and subordinate individuals. Furthermore, peak area of fraction A taken from different individuals correlated positively with the amplitude of the olfactory responses of the recipients (Figure 1b; Spearman correlation, $r_s = 0.939$, $P < 0.001$) and with the donor DI (Spearman correlation, $r_s = 0.743$, $P < 0.001$).

Identification, structure elucidation and quantification

Ultra-performance liquid chromatography coupled to high-resolution mass spectrometry (UPLC-HRMS) of fraction A revealed the presence of two compounds showing near retention times of R_t 12.78 min and R_t 12.82 min (extended data (ED), Figure 1a) and molecular ions at *m/z* 511.2908 and 511.2912, in negative ionization polarity (ED Figure 1b and c), indicating a neutral mass of the compounds of 512 Dalton. The indicated molecular formula $C_{27}H_{44}O_9$ (Δppm 1.292) fits both compounds. Detailed ESI-MS^n studies in the positive polarity (ED Figure 2) further revealed that these compounds contain a hexose acid moiety attached to a steroid backbone (aglycone). The consecutive losses of water observed after serial fragmentations indicated the presence of at least three oxygen atoms bound to the aglycone unit.

The proton-nuclear magnetic resonance (^{1}H NMR) spectrum confirmed the presence of two closely related compounds, i.e. stereoisomers (ED Figure 4a), as suggested by UPLC-HRMS. Comparison of the ^{1}H NMR spectrum of the HPLC fraction A with the spectra of the unconjugated progestogen standards 5β-pregnane-3,17α,20β-triol (**2**; ED Figure 6) and 5β-pregnane-3,17α,20α-triol (**3**; ED Figure 6) preliminarily identified the aglycone moiety of the urinary steroids as 5β-pregnane-3,17,20-triols. The combined use of ^{1}H-^{1}H and ^{1}H-^{13}C shift-correlated 2D NMR allowed full structural and stereochemical elucidation of the two steroid

conjugates as the sodium salts of epimeric 5β-pregnane-3α,17α,20α-triol-3α-glucuronate (**14**, Figure 2a) and 5β-pregnane-3α,17α,20β-triol-3α-glucuronate (**10**, Figure 2b). To confirm their chemical structure and test for bioactivity, both isomers were synthesized from the precursor 5β-pregnane-3α,17-diol-20-one (**1**, ED Figure 6) via a slightly modified previously described route (Cooley and others 1980) (ED Figures 6, 7 and 8). Retention times on LC-MS (Figure 2c) and all the spectral data for the synthetic and natural compounds were identical (ED Figure 4 and ED Figure 5).

Quantification by LC-MS of **14** and **10** in individual urine samples (mean ± SEM) unveiled, respectively, 2.1 ± 1.3 and 25.2 ± 8.6 µM in subordinate (N = 6) and 12.8 ± 3.8 and 229.5 ± 64.4 µM in dominant (N = 7) males. Furthermore, as with urine, urine extract and fraction A, the dominance index was positively correlated to the urinary concentration (ED Figure 9) of **10** (Spearman correlation, r_s = 0.790, P < 0.0001, N = 19) and **14** (Spearman correlation, r_s = 0.550, P = 0.0145, N = 19).

Olfactory sensitivity to the identified and synthesized compounds

Stimulation of the olfactory epithelium with increasing concentrations (10^{-11} M to 10^{-5} M) of the two synthetic pregnanetriol 3-glucuronates (Figure 3c) produced sigmoidal concentration-response curves in males (Figure 3a) and females (Figure 3b). The thresholds of detection were near 10^{-9} M and plateaus were reached at about 10^{-6} M. This distinct shape of the curves suggests the presence of a relatively specific receptor mechanism for detection of these steroids in tilapia. Interestingly, for both males and females compound **10** had a significantly lower (mean ± SEM) apparent EC_{50} (27.55 ± 7.76 nM) and maximum response I_{max} (1.8 ± 0.1) than compound **14** (88.18 ± 11.68 nM and 1.99 ± 0.12; two-way repeated measures ANOVA followed by Holm Sidak test; P < 0.001 and P = 0.019). The lower apparent EC_{50} value indicates a higher affinity of **10** than **14** for the olfactory receptor(s). The similar apparent Hill-coefficients of about 1 for both isomers (**14** 1.1±0.1 and **10** 1.2±0.1) are consistent with a 1:1 binding ratio to the receptor(s), with no cooperativity. There was no olfactory response to the aglycones **2** and **3** even at 10^{-6} M. D-glucuronic acid sodium salt alone or a mixture of the aglycone steroids and D-glucuronic acid sodium salt at 10^{-6} M was not detected, neither were sulphated or C-17- or C-20-glucuronidated androgens or progestogens. Similar olfactory sensitivity was only found to structurally related C-3α-glucuronidated steroids (e.g. 3α,17α-dihydroxy-5β-pregnan-20-one 3-glucuronate or etiocholane-3α-ol-17-one 3-glucuronate), which demonstrates that C-3α glucuronidation is essential for the olfactory response to the two isomers and underpins the specificity of the underlying olfactory receptor mechanism.

Whether the two compounds share common olfactory receptor mechanisms was tested by EOG cross-adaptation tests (Caprio and Byrd 1984) in which the sensory epithelium is first adapted to one isomer and then tested with the second isomer. In compound **10** adapted olfactory

epithelium (Figure 3d), the response to compound **14** was not significantly different from the self-adapted control, indicating a shared receptor mechanism. In contrast, exposure to the bile acid taurochenodeoxycholic acid (TCD) in **10** or **14** adapted olfactory epithelium failed to reduce the response below 80 % of the unadapted TCD alone, indicating independent receptor mechanism(s). Reciprocal adaptation of **10** to **14** (Figure 3e) strongly reduced the response, unlike TCD, which confirms the presence of a common olfactory receptor mechanism for the two isomers. The difference in response of **10** compared to the self-adapted control may reflect the higher affinity of **10** for the olfactory receptor as measured by the lower apparent EC_{50}. These results indicate that **10** and **14** act through a common olfactory receptor distinct from that of TCD.

Can females use this sensory information to assess the social status of the male? As the urine is released to the water it is diluted allowing for differential perception. For example, a 1/10,000 v/v dilution of dominant male urine evokes strong olfactory (Frade and others 2002) and endocrine responses in females (Huertas and others 2014). Such a dilution would contain 23 nM of compound **10,** which is close to the EC_{50} value and lies on the steepest (i.e. linear) part of the EOG concentration response curve, while dilution of subordinate male urine would bring it to near or below detection limit. Furthermore, dilution of subordinate male urine would bring compound **14** necessarily to below the limit of detection. The effect of urine dilution together with the dynamics of urine release by dominant males (Barata and others 2008) suggests a possible mechanism for females to distinguish dominant from subordinate males by sensing pregnanetriol 3α-glucuronate concentration.

Biological function of the identified compounds

Having established that both male and female tilapia are sensitive to **10** and **14** we tested whether the two synthetic steroids are sufficient to emulate the priming effect of male urine on maturation-inducing steroid production by females (Huertas and others 2014). Pre- and post-spawned females were exposed separately to five different test stimuli: **i**) dominant male urine (1:10,000 v/v in tank water), **ii**) the corresponding C18-SPE extract (i.e. the steroids containing urine fraction), **iii**) the aqueous flow-through of C18-SPE, **iv**) 50 nM of a 4:1 mixture of the two isomers **10** and **14**, and **v**) methanol control. Before stimulation, females in the five trials (N = 8) released 17,20β-P to the water at similar rates (mean ± SEM; 135.6 ± 11.1 ng kg^{-1} h^{-1}; Figure 4). One hour after applying the stimulus female tilapia exposed to dominant male urine showed a highly significant increase (nearly 10-fold), in the release rate of 17,20β-P (1282.4 ± 247.7 ng kg^{-1} h^{-1}; Figure 4), in agreement with previous observations (Huertas and others 2014). Similar increases in 17,20β-P release rates were also measured in females stimulated with the urine extract (1324 ± 250.5 ng kg^{-1} h^{-1}) and with the synthetic steroid mix (1970± 434.3 ng. kg^{-1} h^{-1}). In contrast, no significant change in 17,20β-P release rates was observed when the stimulus was the aqueous C18-SPE flow-through or methanol control. The slightly larger release rate of 17,20β-P after stimulation with the

synthetic steroids as compared to raw urine or urine extract (Figure 4) may be related to the higher concentration of **14** and **10** in the synthetic steroid mix (96.1 μM and 413.1 μM) than in the dominant male urine pool (17.7 μM and 263 μM). These results demonstrate that the two identified pregnanetriol 3-glucuronates are responsible for the observed priming effect of urine from dominant tilapia males. Steroidal pheromone mediated priming also exists in goldfish, in which females release the maturation-inducing steroid 17,20β-P (primarily via the gills) and its sulphate (primarily via the urine) to stimulate gonadotropin secretion, milt production and sperm motility in males (Defraipont and Sorensen 1993; Dulka and others 1987; Stacey and others 1989). Although 17,20β-P and 17,20α-P (including their sulphate and glucuronide conjugates), which are produced in the testis (Martins and others 2009; Scott and others 2010) are present at higher concentrations in the urine of dominant Mozambique tilapia males than in subordinates (Oliveira and others 1996), the species lacks olfactory sensitivity to any of these steroids (Frade and others 2002). We suggest that the urinary pregnanetriol 3-glucuronates **10** and **14** from dominant tilapia males are honest signals, carrying information to the female about the male's reproductive performance, i.e. sperm-quality. Also, the two pregnanetriol 3-glucuronates **10** and **14** may have a releaser (i.e. behavioural) effect on female mate choice and spawning decision (Barata and others submitted manuscript), thus increasing the probability of reproductive success. Given that **10** is correlated with DI, is present at much higher concentrations and has higher olfactory potency than **14**, we predict that **10** is the main biologically active component. However, the relative contributions of both steroids remain to be investigated.

Although reproductive pheromones in teleosts have been the focus of several studies during the past two decades, only in goldfish (*Carassius auratus*) and the masu salmon (*Oncorhynchus masou*) (Yambe and others 2006) their identity is known and their biological functions defined. Yet in both of the aforementioned species, females are the signalling sex and mating strategies and compounds released are different from the Mozambique tilapia. The current study presents not only the first chemical identification of a cichlid sex pheromone, but also the first sex pheromone from a teleost with a mating system in which territorial males signal to females, and that is strongly driven by female mate choice. Fish are the largest group of vertebrates and can use diverse signals for communication; visual cues, sound, electrical fields and chemicals. The chemical identification of a sex pheromone in male tilapia urine that primes the female reproductive system and possibly promoting spawning synchrony will stimulate further research into chemical communication and behaviour in particular and how different sensory information is integrated. This should shed light on the role of chemical communication on inter- and intra-sexual selection, and how the diversity of urinary pheromone signalling in freshwater fishes has shaped reproductive strategies, social structure and evolution.

Materials and methods

Experimental animals and urine collection

Fish care and experimentation complied with the guidelines of the European Union Council (86/609/EU) and Portuguese 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.

Sexually mature Mozambique tilapia were raised in captivity from a brood-stock maintained at the University of Algarve (Faro, Portugal). Males and females were kept together in large 500 l stock tanks until used for experiments. Social groups were created in 200 l tanks with five males and five females of similar standard length (SL in mm) and body weight (BW in g; coefficient of variation of BW less than 5 %) as previously described (Keller-Costa and others 2012). Males were colour tagged (T-Bar anchor FD94, Floy Tag Inc., Seattle, WA, USA) and systematic focal observation of their behaviour carried out daily (Keller-Costa and others 2012). An average dominance index (DI) for each male was calculated from the behavioural analysis of five consecutive daily observations, ranging from zero to one (Barata and others 2007; Keller-Costa and others 2012). Subordinate males had a DI < 0.2 and dominant males a DI ≥ 0.8, the others were intermediates. After each daily observation urine was collected from each male by gently squeezing the area immediately above and anterior to the urogenital papilla and stored at -20 °C until further analysis.

Urine extraction and fractionation

Urine samples (pooled over five observation days) from dominant (mean ± SD; *N* = 6; BW = 150 ± 31 g; SL = 168 ± 11.7 mm), intermediate (*N* = 5; BW = 156 ± 26.9 g; SL = 171 ± 12.3 mm) and subordinate (*N* = 6; 150 ± 42.5g; SL = 170 ± 13.5 mm) males were extracted using solid-phase cartridges (C18, 500 mg, Isolute®, Biotage) and eluted with methanol. Aliquots of each extract (500 µl) were supplemented with 5 µl 4 mM chenodeoxycholic acid (CDC; ≥ 98%, Sigma®; the internal standard), dried under nitrogen gas, reconstituted in 55 µl methanol/water [60/40 v/v, containing 0.001% formic acid (FA)] and injected into a HPLC system (Smartline KNAUER, Berlin, Germany) with a C-18 column (3.9 mm x 300 mm; 4 µm particle size; Nova-Pak, Waters). HPLC conditions were as follows: mobile phase was water (MilliQ) and methanol (HPLC-grade), both containing 0.001% FA; 0-4 min isocratic at 15 % methanol, 5-91 min linear gradient from 15 % to 100 % methanol, 91-96 min isocratic at 100 % methanol; flow-rate: 0.7 ml min^{-1}. The column eluate was first routed to a diode array UV detector (Smartline 2600, KNAUER, Germany), then split off and half routed to an evaporative light scattering detector (Varian 380-LC ELSD, Polymer Laboratories) and the other half collected by an Advantec CHF100SA

fraction collector into 30 fractions, each 3 min. ELSD conditions were as follows: nitrogen carrier gas (≥ 98 % purity, 1.6 SLM), 65 °C nebulisation temperature, 110 °C evaporation temperature. Data were visualized and analysed using the Data-Apex Clarity™ Software. Peak areas were normalized to the peak area of the internal standard CDC. Negative blank fractions, generated by injecting only 55 µl of 60/40 v/v methanol/water (0.001 % FA) under similar conditions, were used to control for uncontaminated and unbiased experimental conditions. All HPLC fractions from each male were kept at -20 °C until assessment of olfactory potency.

Identification/Structure elucidation

A large urine pool (~30 ml), collected from various dominant donor males held in social groups (described above) provided the material for subsequent chemical analyses. MS^n studies were performed on a Bruker Daltonics HCT *ultra* mass spectrometer (Bruker Daltonics, Bremen, Germany). The ionisation was made by electrospray (ESI) in the negative and positive polarities. Typical spray and ion optics conditions were as follows: capillary voltage, 3.5 kV; drying gas (nitrogen), 300 ºC at 5 $l.min^{-1}$; nebulizer gas pressure, 20 psi; capillary exit voltage, 130 V; skimmer voltage, 40 V. The LC-MS system was an Agilent Technologies 1200 Series LC coupled to the above described mass spectrometer. Under LC operation the spray and ion optics conditions were the following: negative ionization polarity; capillary voltage, 3.5 kV; drying gas (nitrogen), 330 ºC at 10 $l.min^{-1}$; nebulizer gas pressure, 50 psi; capillary exit voltage, 130 V; skimmer voltage, 40 V. A Hamilton PRP-1 reversed phase LC column (15.0 cm length, 2.1 mm internal diameter, 5 µm average particle diameter), stabilised at 25 °C was used. The eluent system was ultra-pure water (**A**) and acetonitrile (**B**), both with 0.1 % FA. The gradient started with 20 % of **B**, followed by a linear increase up to 80 % in 20 min. In a second gradient step an increase up 100 % took place in 5 minutes. A final cleaning step using 100 % of **B** during 5 min was made after each run. The eluent was then allowed to recover the initial conditions (80 % of **A** and 20% of **B**) in 1 min and then stabilise for additional 6 min before the next run. High-resolution mass spectra were recorded on an UPLC-RLX 3000 system (Dionex) and an Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). UPLC was performed using an Acclaim C18 column (150 x 2.1 mm, 2.2 µm, Dionex, Germany) at a constant flow rate of 300 $\mu l.min^{-1}$ using ultra-pure water with 0.1% FA (solvent **A**) and acetonitrile with 0.1% FA (solvent **B**). Isocratic conditions of 50 % **A** and 50 % **B** were used. Full-scan mass spectra were generated in the range of *m/z* 94.00-1400.00, both under negative and positive ionization polarity and analysed with the Xcalibur Qual Browser software (Thermo Scientific).

NMR spectra were measured on a Bruker Avance NMR spectrometer (Bruker-Biospin, Karlsruhe, Germany), operating at 500.13 MHz for ^1H and 125.75 MHz for ^{13}C. An inverse triple channel cryoprobe (5 mm) was used. The spectra were recorded in methanol-d_4. Samples were measured in 5 mm tubes (600 µl methanol-d_4) or 2 mm capillaries (mass-limited samples; 85 µl

methanol-d_4). Chemical shifts are referenced to the ^{1}H- and ^{13}C NMR signals of methanol-d_4 (Gottlieb and others 1997).

In the high-field region of the ^{1}H NMR spectrum of the obtained mixture (HPLC fraction A), each of the two compounds displayed two singlets typical of angular methyl groups and a doublet of a methyl group (J ~ 6 Hz) attached to a methine carbon (ED Figure 4). As revealed by HMBC, the angular methyl signals are part of the steroid ring system. The combined use of 2D NMR (^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC) correlations, assigned the methyl group doublet to the steroid side chain and also identified the side chain as 2-hydroxyethyl unit attached to the hydroxylated C-17, in both compounds. Direct comparison of the ^{1}H NMR spectrum with pregnane standards (5β-pregnane-3α,17α,20β-triol (**2**) and 5β-pregnane-3α,17α,20α-triol (**3**)) allowed preliminary assignment of all ^{1}H resonances of the aglycone moiety, identifying the urinary steroid aglycons as 5β-pregnane-3,17,20-triols.

The doublet of an axial H-1' methine proton (δ 4.41, d, J = 7.8 Hz) characteristic of a proton at the anomeric centre of a β-hexopyranose and signals of four other methine protons, confirmed the hexose unit as already suggested by the MS^n experiments. The absence of hydroxymethylene group signals in the ^{1}H NMR spectrum together with doublet signals of axially oriented H-2'–H-5' ($^3J_{H\text{-}H}$ ~8-9 Hz) and HMBC correlations of H-5' with C-6' (δ 176.6) indicated a glucuronic acid unit. The glycosidation site at 3-OH of the steroid was established by downfield shifts of the H-3 and C-3 resonances observed in the spectra of the steroids as compared to 5β-pregnane-triol standards. H-3 was shifted from δ 3.54 to δ ~3.8 and C-3 from δ 72.5 to δ ~79. Finally, comparing ^{1}H NMR (ED Figure 4) and 2D NMR spectra (^{1}H-^{1}H COSY, ROESY, TOCSY, HSQC (ED Figure 5), HMBC and H2BC) of the mixture (HPLC fraction A) with the corresponding spectra of synthetic glucuronate standards allowed full structure elucidation and stereochemical assignment of the two steroid conjugates as the 20-epimers, sodium 5β-pregnane-3α,17α,20β-triol 3α-glucuronate (**10**) and sodium 5β-pregnane-3α,17α,20α-triol 3α-glucuronate (**14**) (see ED Figures 4 and 5 and Table 1).

Steroid synthesis

The steroidal glucuronates were prepared via a slightly modified route previously described (Cooley and others 1980) using 5β-pregnane-3α,17α-diol-20-one (**1**) (ED Figure **6**) (Sigma-Aldrich) as starting material. Following a standard protocol, reduction with $LiAlH_4$ in absolute THF gave, after hydrolysis with diluted H_2SO_4 and extraction with CH_2Cl_2, a chromatographically inseparable mixture of the 5β-pregnane-3α,17α,20-triols (**2**,**3**) (20β:20α=3:2 based on ^{1}H-NMR) in quantitative yield. The mixture was subjected to diol protection (Lebwart and Schneider 1969) followed by a deprotection step using Dowex 50Wx8 (Hun Park and others 1994), making use of the fact that one of the desired products **4** reacts very slowly. Separation of **4** (20β) by means of chromatography and re-protection of **3** gave the desired diol-protected compound **5** (20α). The

average overall yield of **4** and **5** was 84 %. For linking the glucuronic acid to the 20β-steroid (ED Figures 6 and 7), commercially available benzoyl-protected methyl glucuronate (**6**) was reacted with **4** in the presence of activated molecular sieve (4Å) and freshly prepared Ag_2O in absolute benzene at room temperature. After overnight stirring in darkness the pure product (**7**) was obtained after chromatography in 93 % yield. Subsequently, deprotection of **7** using Dowex 50x8 gave **8** in a yield of 38 %. De-benzylation of the glucuronate was accomplished with sodium methylate/sodium hydroxide to give the desired sodium 5β-pregnan-3α,17α,20β-triol 3α-glucuronate (**10**). The overall yield calculated from **1** was 11 % and from **4** 24 %, respectively. An analogous procedure was used to produce the 20α-configured steroidal glucuronate **14** (ED Figure **8**). After attachment of the protected glucuronate, the deprotection of the steroidal diol function was accomplished within 9 h to give **12** in a yield of 70 % calculated from **5**. Cleavage of the protection groups from the glucuronate proceeded in 42 % yield calculated from **12**. The total yield of sodium 5β-pregnan-3α,17α,20α-triol-3α-glucuronate (**14**) calculated from **1** was 7 % and from **5** 29 %, respectively.

Electro-olfactogram (EOG) recordings

Preparation of animals and recording of the EOG was carried out as previously (Frade and others 2002). The DC voltage signal was pre-amplified, then filtered (low-pass 50 Hz) and amplified (NL106, Digitimer Ltd: final gain x100 or x1000 depending on the potency of the stimulus), and recorded on a PC running Axoscope software (version 9.1, Axon Instruments, Inc., Foster City, CA, USA). The olfactory potency of urinary HPLC fractions from dominant (N = 6), intermediate (N = 5) and subordinate (N = 6) males from social groups was assessed on three adult males (mean ± SD; BW = 157.9 ± 19.1 g) and three females (BW = 110.3 ± 15.4 g) at a dilution of 1:10,000 in water (v/v). A screening of all 30 urinary HPLC fractions from selected dominant and subordinate males showed that one fraction (fraction A) contained most olfactory activity and was therefore selected for subsequent EOG recordings to explore in detail the relationship of olfactory potency with peak areas (concentrations). EOG data were log(x+1)-transformed and linear regression analysis was performed on pooled data from both sexes since EOG amplitudes of male and female responses were similar. Paired t-test was used to compare slopes and elevations of EOG responses to the peak area in HPLC fraction A and EOG responses to the whole C18-SPE urine extract. Data were normally distributed and of equal variance.

To investigate the olfactory sensitivity to the two synthesized steroids **10** and **14**, EOG concentration-response curves were generated. Ten females (mean ± SD: BW = 51.8 ± 36.3 g; SL = 131.7 ± 44.9 mm) and 14 males (BW = 35.1 ± 11.4 g; SL = 106.4 ± 11.5 mm) were exposed to increasing concentrations from 10^{-11} M to 10^{-5} M in $\log_{10}$ molar increments (in addition $5x10^{-8}$ M was tested) of 4 s odour pulses allowing ca. 1 min between exposures. Given the sigmoidal shape of these curves, apparent maximal olfactory response (I_{max}), apparent half-maximal effective

concentration (EC_{50}) and apparent Hill-coefficient values were calculated by fitting a sigmoidal regression curve using the Hill-equation [3 parameter: $y = \mathbf{a}x^{\mathbf{b}}/(\mathbf{c}^{\mathbf{b}} + x^{\mathbf{b}})$; $\mathbf{a} = \max(y) = I_{max}$; $\mathbf{b} = 1$ = Hill co-efficient; $\mathbf{c} = x50(x,y) = EC_{50}$] as mathematical model, in which y is the EOG response and x is the stimulus concentration. Two-way repeated measures analysis of variance (RM ANOVA) followed by the Holm-Sidak post-hoc method was used to compare EC_{50} values and I_{max} values of male and female responses to the two synthesized steroids. The steroids 5β-pregan-3α,17α,20α-triol (**3**), 5β-pregan-3α,17α,20β-triol (**2**) and the sodium salt monohydrate of D-glucuronic acid (all purchased from Sigma-Aldrich, Spain) were tested for olfactory potency in the same fish but found to elicit no olfactory response, even at concentrations as high as 10^{-6} M.

Cross-adaptation experiments were carried out to test for the presence of single or multiple receptor mechanisms (Caprio and Byrd 1984). Firstly, EOG responses to 4 s pulses of 10^{-6} M solutions of compounds **14** and **10** were recorded from males (N = 10; mean ± SD: BW = 38.7± 11.6 g SL = 109 ±12.7 mm). A 10^{-6} M solution of the adapting steroid was then used to perfuse the olfactory epithelium until voltage stabilised (about one minute). Test solutions (10^{-6} M test steroid in 10^{-6} M adapting steroid) were then administered as 4 s pulses, beginning with the adapting steroid (the self-adapted control at 2 x 10^{-6} M). The bile acid taurochenodeoxycholic acid [TCD; 3α,7α-dihydroxy-5β-cholan-24-oic acid N-(2-sulfoethyl)amide, Sigma-Aldrich] at 10^{-5} M was included as a negative control, since it was expected to act through different receptor mechanisms, and it has been shown to evoke in tilapia large EOG responses of similar magnitude as 10^{-6} M solutions of the above mentioned steroids (Huertas and others 2010). EOG responses to the test solutions during adaptation were converted to a percentage of the initial (unadapted) response (% R_I). For each cross-adaptation dataset, mean % R_I were compared using one-way RM ANOVA followed by the Holm-Sidak post-hoc test.

Hormone measurements

The basic methodology to analyse the endocrine response of females was as described (Huertas and others 2014). Groups of four tagged females and one male were kept together in 250 l tanks. A pre- or post-ovulatory (two days prior the predicted ovulation date or three days after the last ovulation) female was placed in a glass tank overnight and moved to an identical tank with clean de-chlorinated tap water (volume normalised to the fish weight 10 $g.l^{-1}$) the next morning. After 1 h, 1 l of water was collected and C18-SPE extracted (eluted with 5 ml methanol). This volume of water was replaced with clean water and one of the following stimuli was applied to the tank using a micropipette: a volume to give a final dilution of 1:10,000 of **i**) pooled urine of dominant male, **ii**) the corresponding C18-SPE male urine extract, **iii**) the corresponding C18-SPE aqueous flow-through, **iv**) a 4:1 mixture of the two synthesized steroids **10** (400 µM) and **14** (100 µM) or **v**) methanol control. After 1 h of stimulation, another litre of water was collected and C18-SPE extracted. At the end of the experiment, females were returned to their original group tank and

allowed to undergo another ovulatory cycle before the experiment was repeated using one of the above-mentioned but different stimulus. Thus, each stimulus was tested once in each female and eight females (mean ± SD; BW = 48.2 ± 13.3 g; SL = 112.8 ± 11.7 mm) were used as replicates. C18-SPE methanol extracts from all females were dried under nitrogen gas, re-suspended in radioimmunoassay buffer and assayed for 17,20β-dihydroxypregn-4-en-3-one (17,20β-P; Huertas and others 2014). Comparison of 17,20β-P release rates between groups and urine as control was done by Two-way RM ANOVA followed by the Holm-Sidak post-hoc test.

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Figures and figure legends

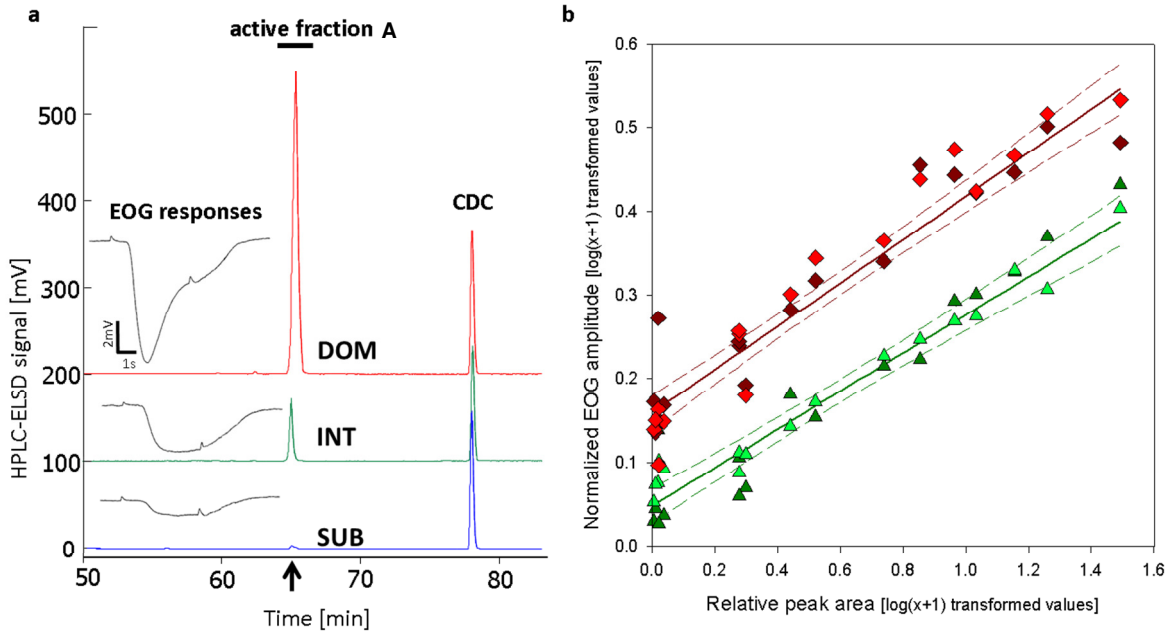


Figure 1 | Olfactory responses to the most active urine fraction (A) are concentration dependent. a) Details of representative C18-HPLC chromatograms of urine extracts from a dominant (DOM), intermediate (INT) and subordinate (SUB) tilapia male. The olfactory most active peak (fraction A) is indicated by an arrow; chenodeoxycholic acid (CDC) was added as internal standard. Typical electro-olfactogram responses to the active fraction at 1:10,000 v/v dilution in water are presented next to the peaks. **b)** Mean EOG responses (normalised to 10^{-5} M L-serine standard) of males (dark symbols; $N = 3$) and females (light symbols; $N = 3$) elicited by the C18-SPE urine extract (red diamonds) and the most active HPLC fraction A (green triangles) at 1:10,000 v/v dilution from tilapia males ($N = 17$) of different social rank. EOG responses are plotted over the HPLC peak areas (relative to 0.4 mM CDC standard). Linear regression analysis was performed on pooled data from both sexes (solid lines and 95 % confidence interval dashed lines). The C18-SPE urine extractt [red lines; $R^2 = 0.871 \pm 0.071$ (±SE); $P < 0.001$; log(EOG+1) = 0.159 (±0.011) + (0.26 (±0.215) x log(rPA+1))] and HPLC fraction A [green lines, $R^2 = 0.847 \pm 0.069$, $P < 0.001$; Log(EOG+1) = 0.0482 (±0.0105) + (0.228 (±0.0143) x Log(rPA+1))] had similar slopes but significantly different elevations (paired *t*-test; $P < 0.001$).

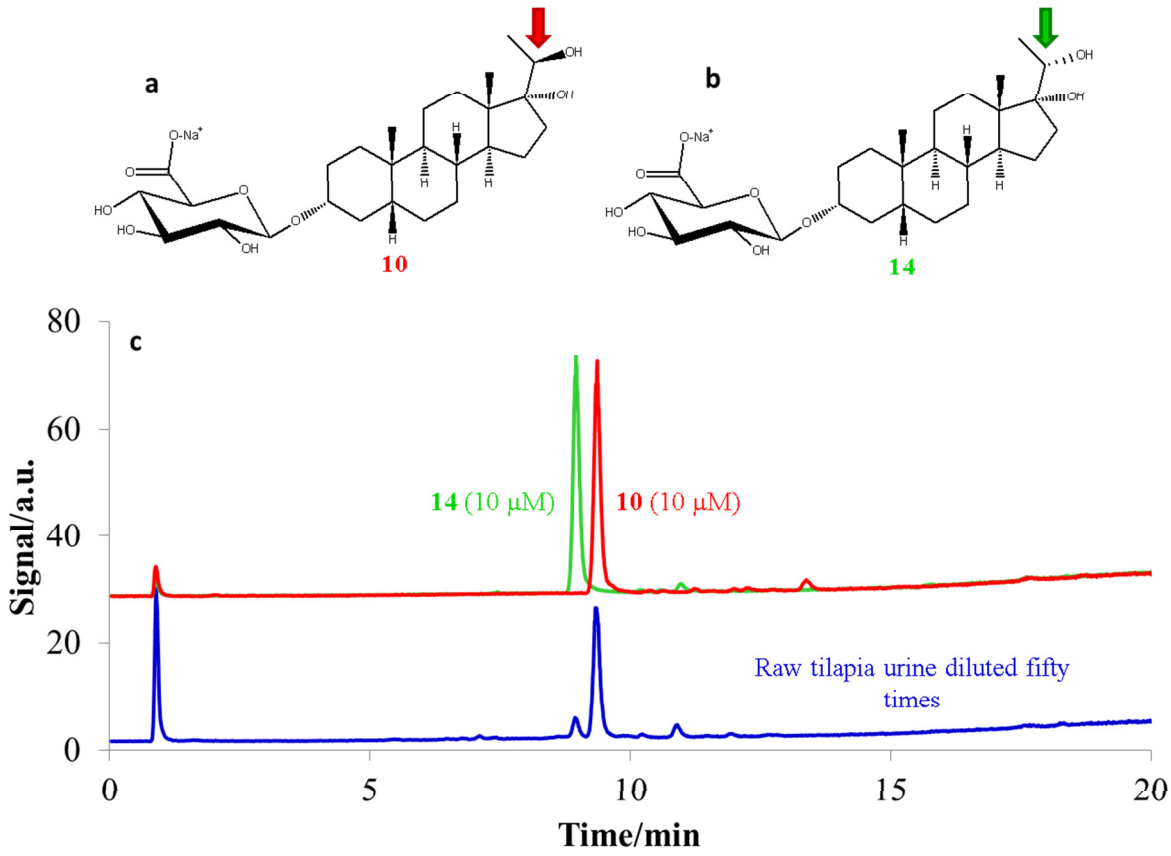


Figure 2 | Two isomers of 5β-pregnanetriol 3α-glucuronate are present in the most active male urine fraction. (a, b) Structures of the two principal components of Mozambique tilapia male urine extract: 5β-pregnan-3α,17α,20α-triol 3-glucuronate (**14**) and 5ß-pregnan-3α,17α,20β-triol 3-glucuronate (**10**). Arrows indicate the epimeric centre. **c**) LC-MS traces of raw Mozambique tilapia urine (blue) diluted fifty times; 5β-pregnane-3α,17α,20α-triol-3α-glucuronate (**14**, green) and 5β-pregnane-3α,17α,20β-triol-3α-glucuronate (**10**, red) at 10 µM in negative ionization polarity.

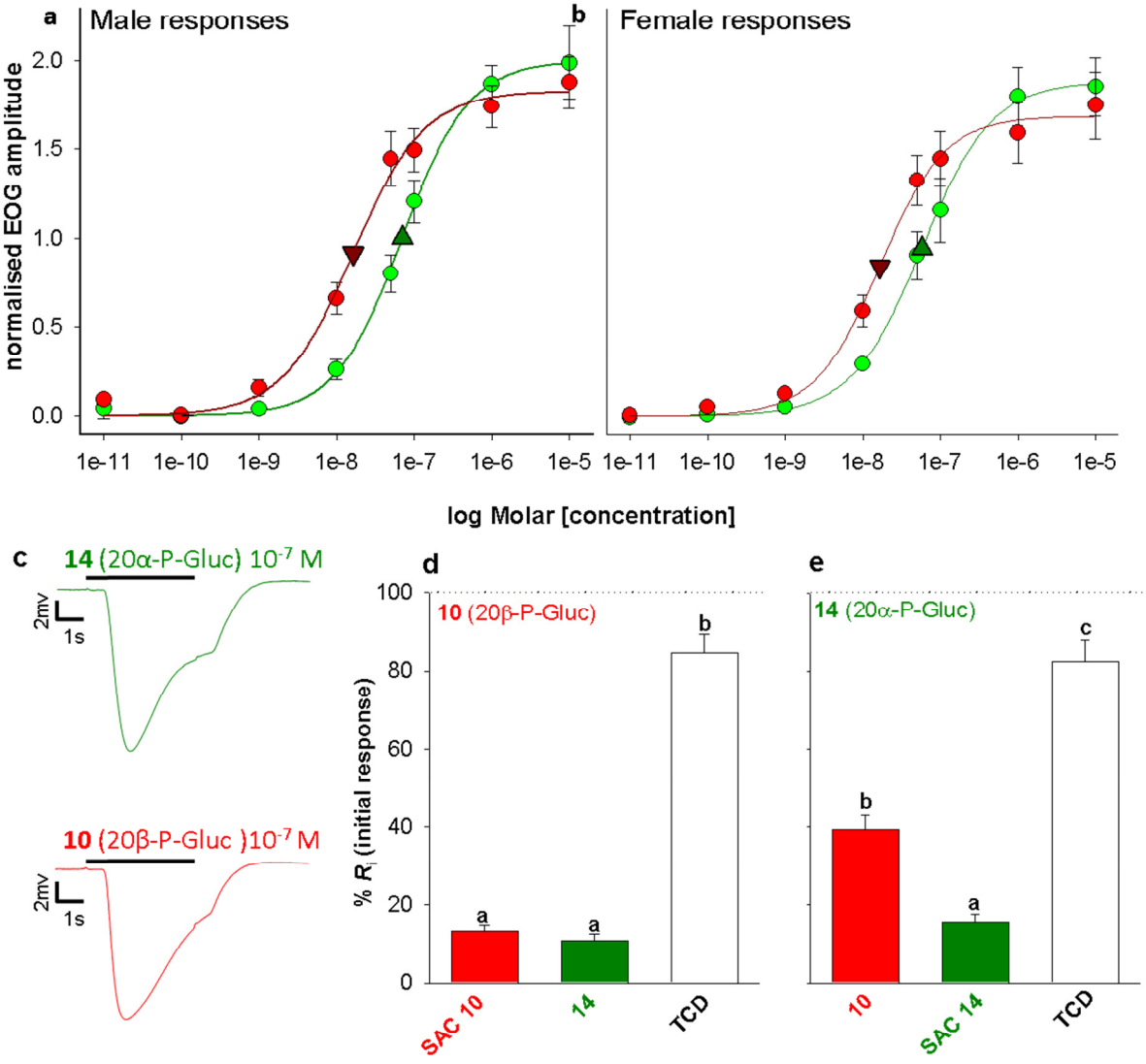


Figure 3 | Synthetic compounds 10 and 14 evoke olfactory responses in females and males. **a,b)** Normalised (to response amplitude of 10^{-5} M L-serine standard) EOG concentration-response curves (mean ± SEM) recorded from males (**a,** N = 14) and females (**b,** N = 10) for the two synthetic analogues **14** (green circles) and **10** (red circles). Triangles on the curves represent respective mean apparent EC_{50} values. **c**) Typical EOG responses to **14** and **10** at 10^{-7} M recorded from a tilapia male. **d,e)** EOG cross-adaptation results. Relative EOG response (mean + SEM) to 10^{-6} M of **14** (green, N =10), to 10^{-6} M of **10** (red, N = 10) steroids and 10^{-5} M TCD (empty, N = 6) expressed as percentage of the initial response (% R_I) to these compounds during 10^{-6} M adaptation to either **10 (d)** or **14 (e)**. SAC = self-adapted control. Different letters over the bars indicate significant differences: one-way RM ANOVA followed by the Holm-Sidak post-hoc test, $F_{2,14}$ = 223.3, $P < 0.001$ **(d)**, $F_{2,14}$ = 95.1, $P < 0.001$ **(e)**.

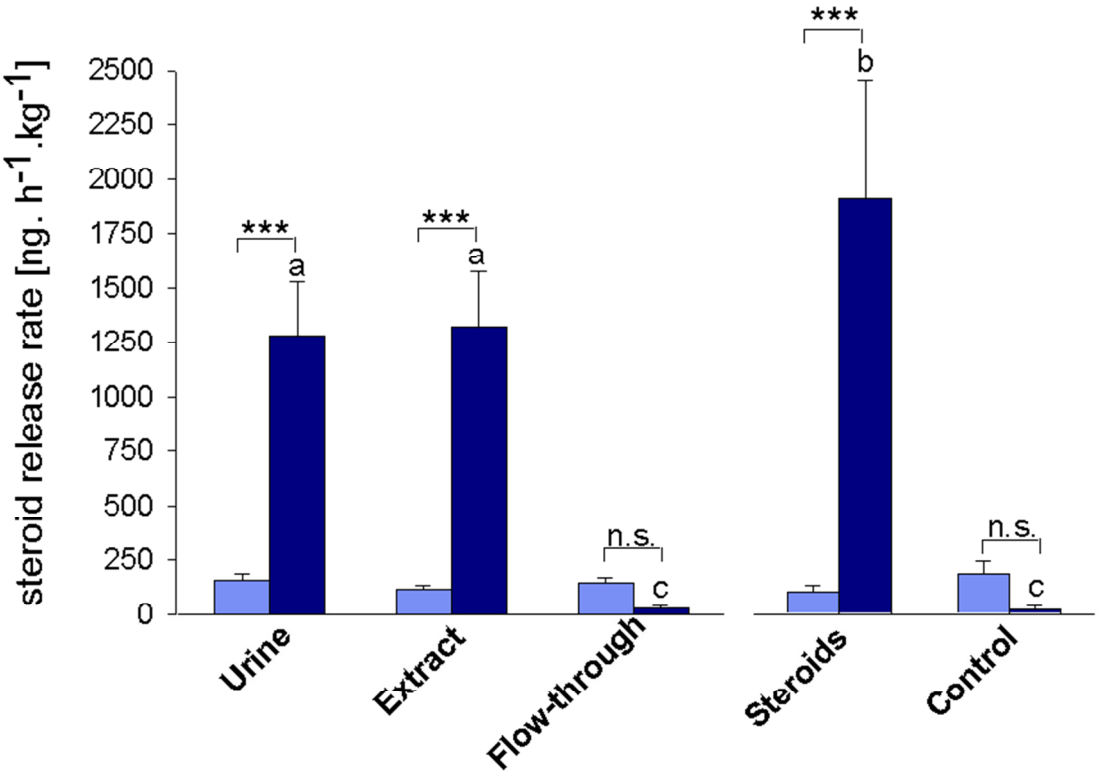


Figure 4 | Male urine and the synthetic steroid glucuronates increase the release of the oocyte maturation inducer 17,20β-P in females. Release rates (mean ± SEM) of 17,20β-dihydroxypregn-4-en-3-one (ng.kg^{-1}.h^{-1}) of eight female tilapia during 1 h before (light blue) and 1 h after (dark blue) exposure to the following stimuli, all diluted 1:10,000 v/v: conspecific dominant male urine; C18-SPE male urine extract; aqueous C18-SPE male urine flow-through; 4:1 mixture of synthetic steroid glucuronates **10** and **14** or methanol (control). All females had similar 17,20β-P release rates before any stimulus was added. Females significantly increased 17,20β-P release after stimulation with either urine, urine eluent or the synthetic steroid mixture (***$P < 0.001$), but not aqueous urine flow-through or the methanol control. Different letters above bars indicate significant differences in 17,20β-P release rates after stimulation, comparing the effect of raw male urine to the other stimuli; two-way RM ANOVA followed by the Holm-Sidak post-hoc test: $F = 14.222$, $P < 0.001$ (stimulus); $F = 41.104$, $P < 0.001$ (time); $F = 10.898$, $P < 0.001$ (interaction stimulus x time).

Extended data

Extended data Figure 1 | a) UPLC-ESI-MS of the active urine fraction A. HR-ESI-MS spectra of the peak at **b)** Rt 12.78 min and **c)** Rt 12.82 min.

Extended data Figure 2 | a) ESI- MS^2 and MS^3 fragmentation of the active urine fraction A and **b**) ESI-MS^4 fragmentation of the active urine fraction A.

Extended data Figure 3 | Structure of the steroidal glucuronates with numbering.

Extended data Figure 4 | a) ^{1}H NMR spectra of the synthetic references vs. active urine fraction A, **b**) Details of ^{1}H NMR spectra.

Extended data Figure 5 | Comparison HSQC spectra. Synthetic references vs active urine fraction A.

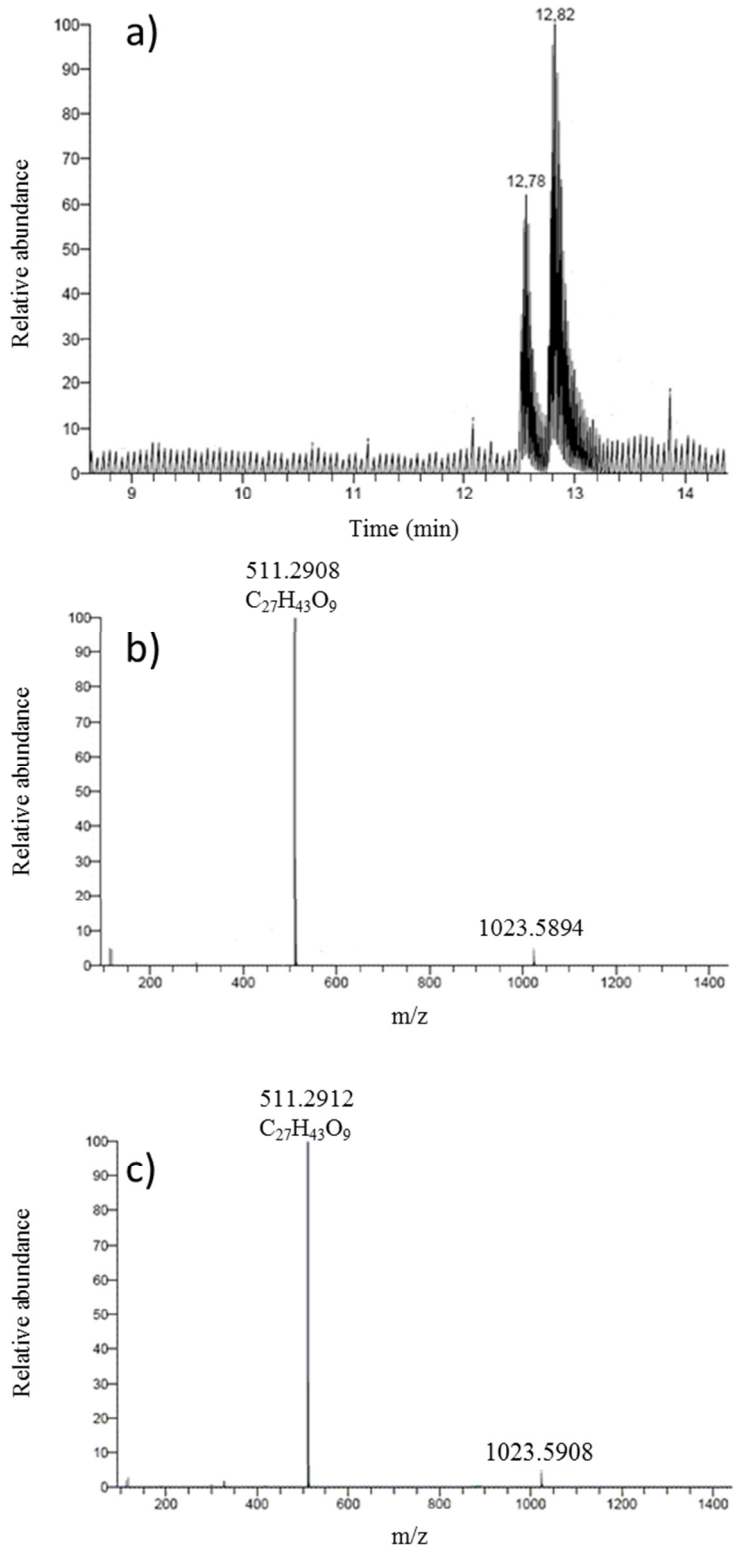
Extended data Figure 6 | Synthesis of the steroidal aglycones.

Extended data Figure 7 | Synthesis of the steroidal glucuronate **10**.

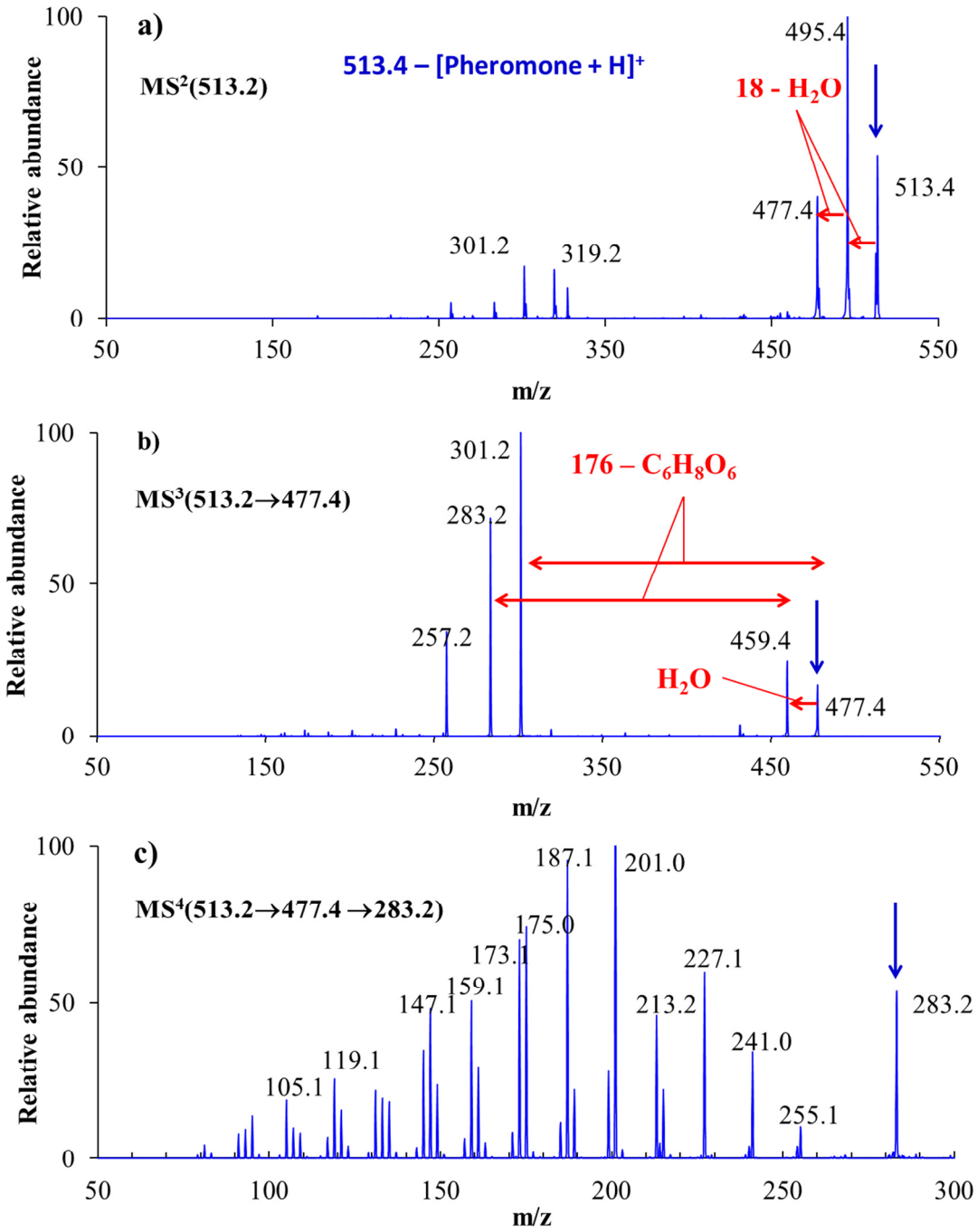
Extended data Figure 8 | Synthesis of the steroidal glucuronate **14**.

Extended data Figure 9 | Correlation between male social status (dominance index) and urinary concentration of the sex pheromone.

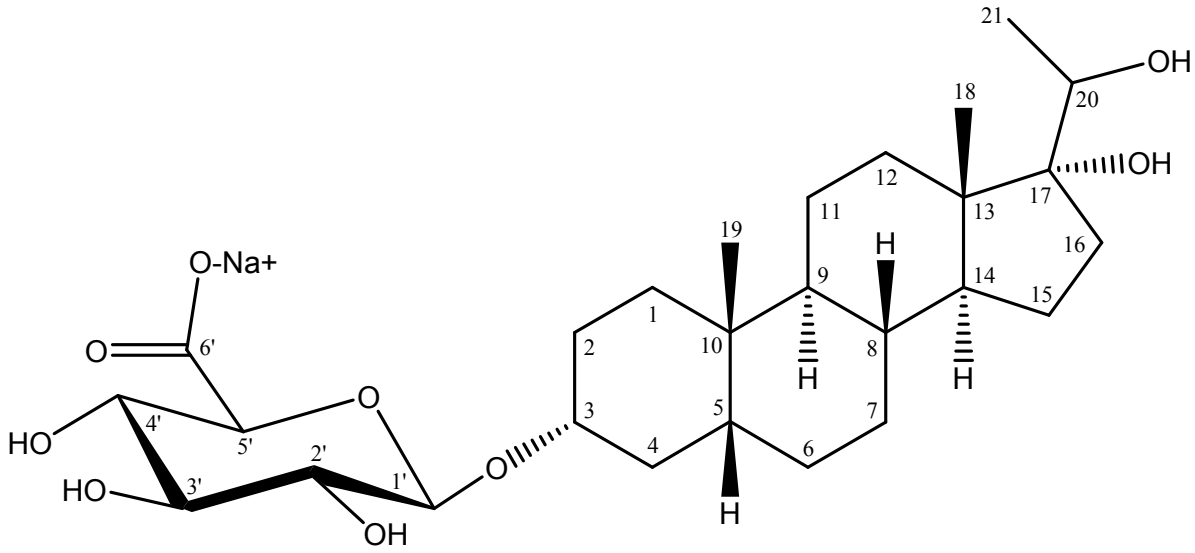
Extended data Table 1 | Chemical shifts of the synthetic steroidal glucuronates **10** and **14.**



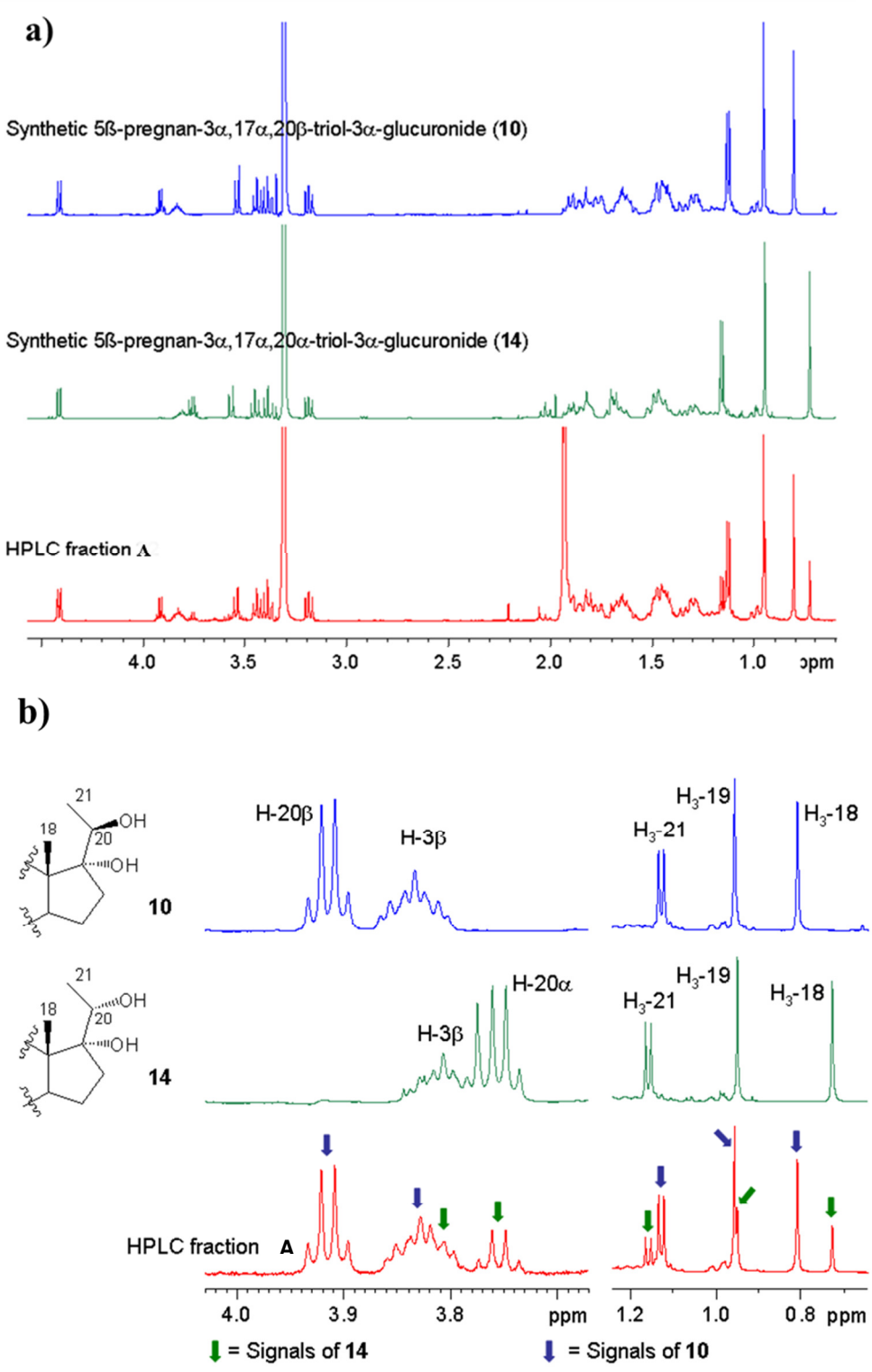
Extended data Figure 1 | a) UPLC-ESI-MS of the active urine fraction A. HR-ESI-MS spectra of the peak at **b)** Rt 12.78 min and **c)** Rt 12.82 min in the negative polarity.



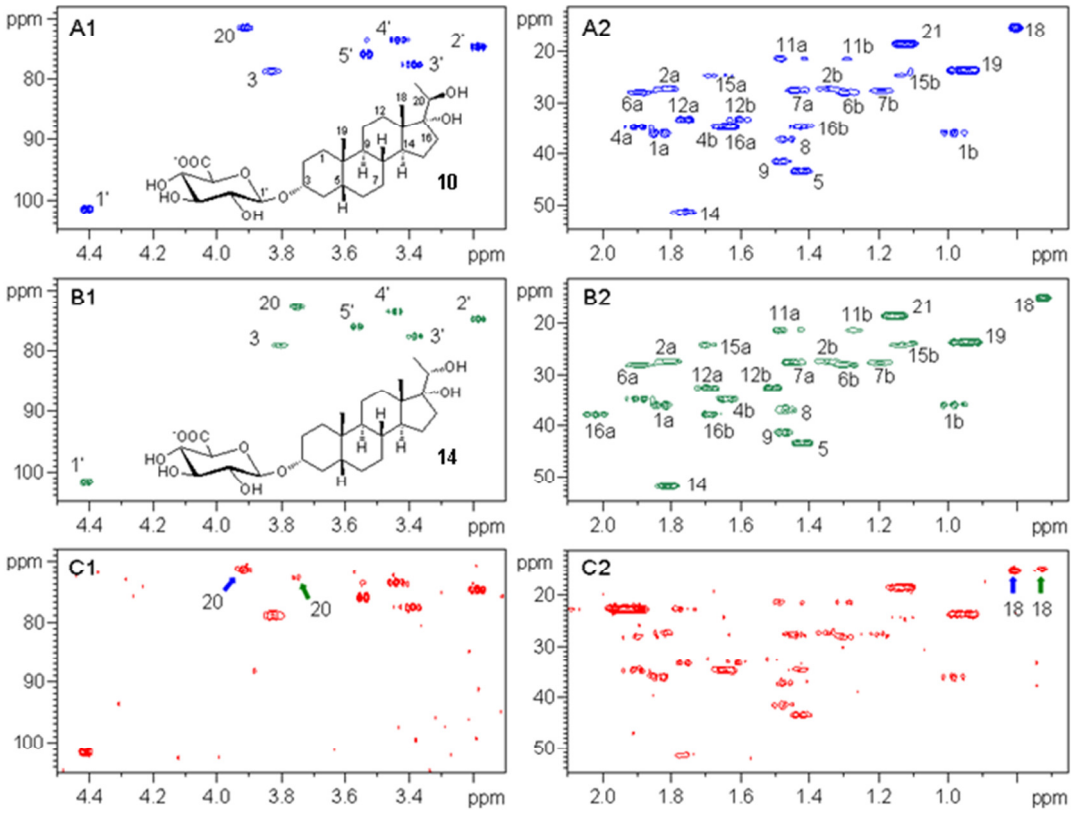
Extended data Figure 2 | ESI-MS fragmentation of compounds from the active urine fraction A in positive polarity. **a**) MS^2 (513.4), **b**) MS^3 (513→477.4), **c**) MS^4 (513→477.4→283.1). The vertical blue arrows indicate the fragmented ions. The neutral loss of 176 Da corresponds to a monodehydrated glucuronic acid residue. The remaining structure possesses at least three OH groups. Spectrum **c**) shows high number of peaks with mass differences of 14 Da, typical of steroids.



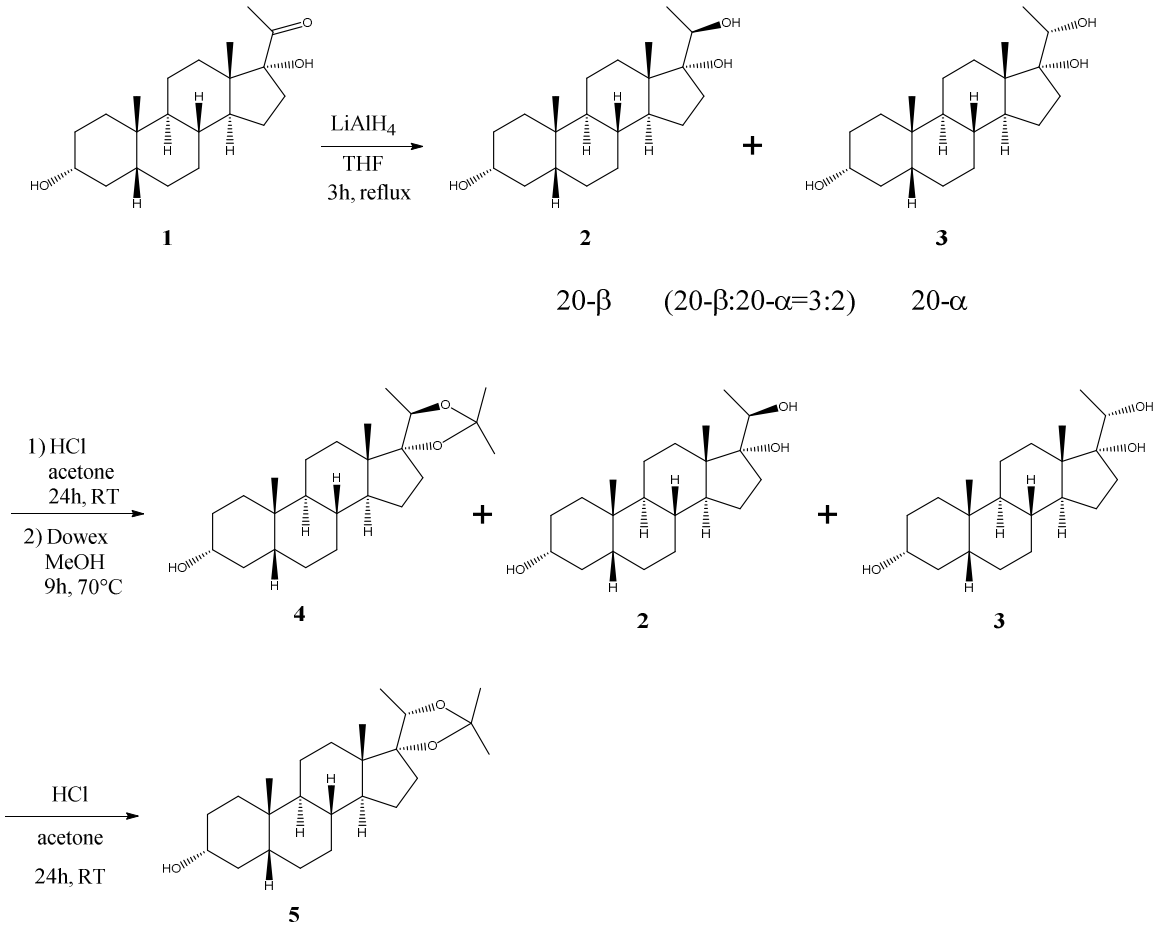
Extended data Figure 3 | Structure of the steroidal glucuronates **10** and **14** with numbering. Epimeric center at position 20.



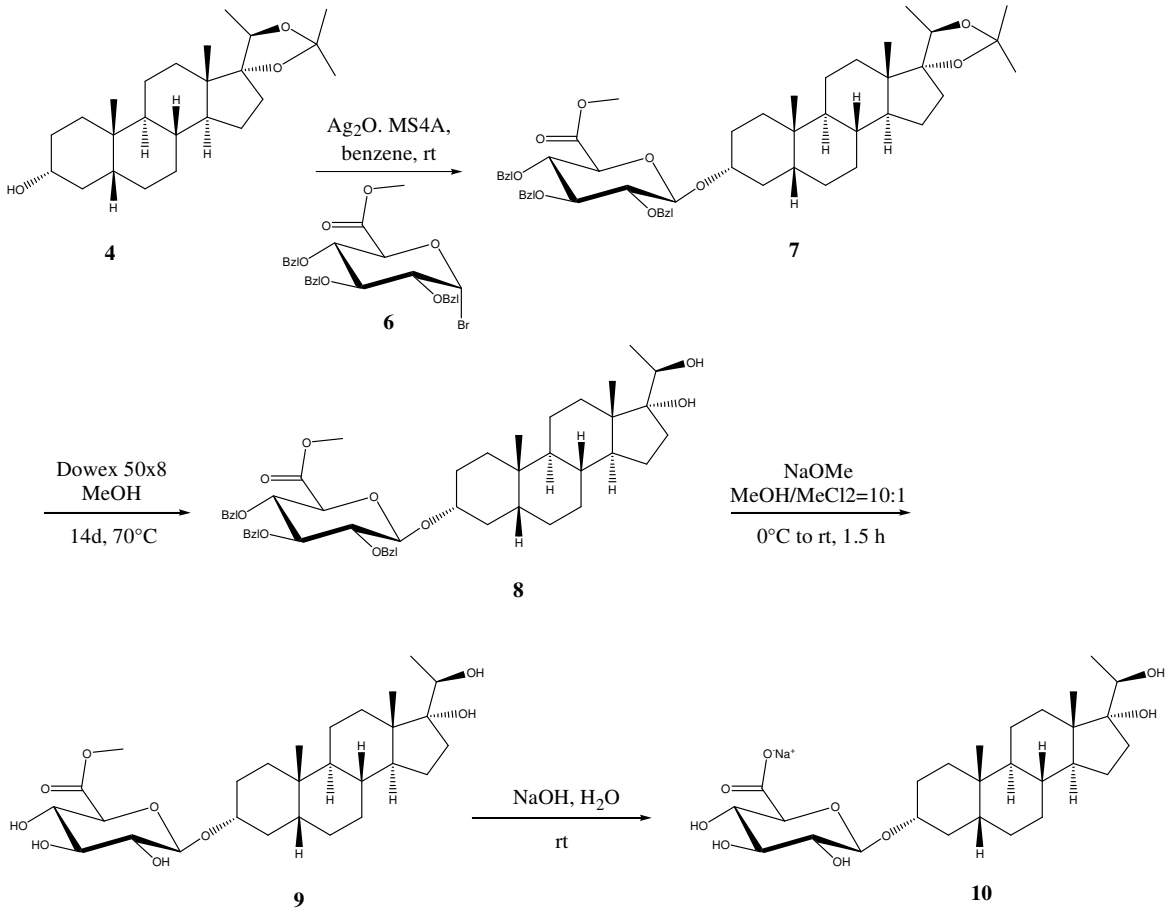
Extended data Figure 4 | a) ^{1}H NMR spectra (500 MHz, methanol-d_4) of synthetic reference compounds **10** and **14** and HPLC fraction A, containing sodium 5β-pregnan-3α,17α,20α-triol-3α-glucuronate and sodium 5β-pregnan-3α,17α,20β-triol-3α-glucuronate. For assignment of relevant signals, see partial spectra in ED Figure 5. **b**) Partial ^{1}H NMR spectra (500 MHz, methanol-d_4) of synthetic reference compounds **10** and **14** and HPLC fraction A.



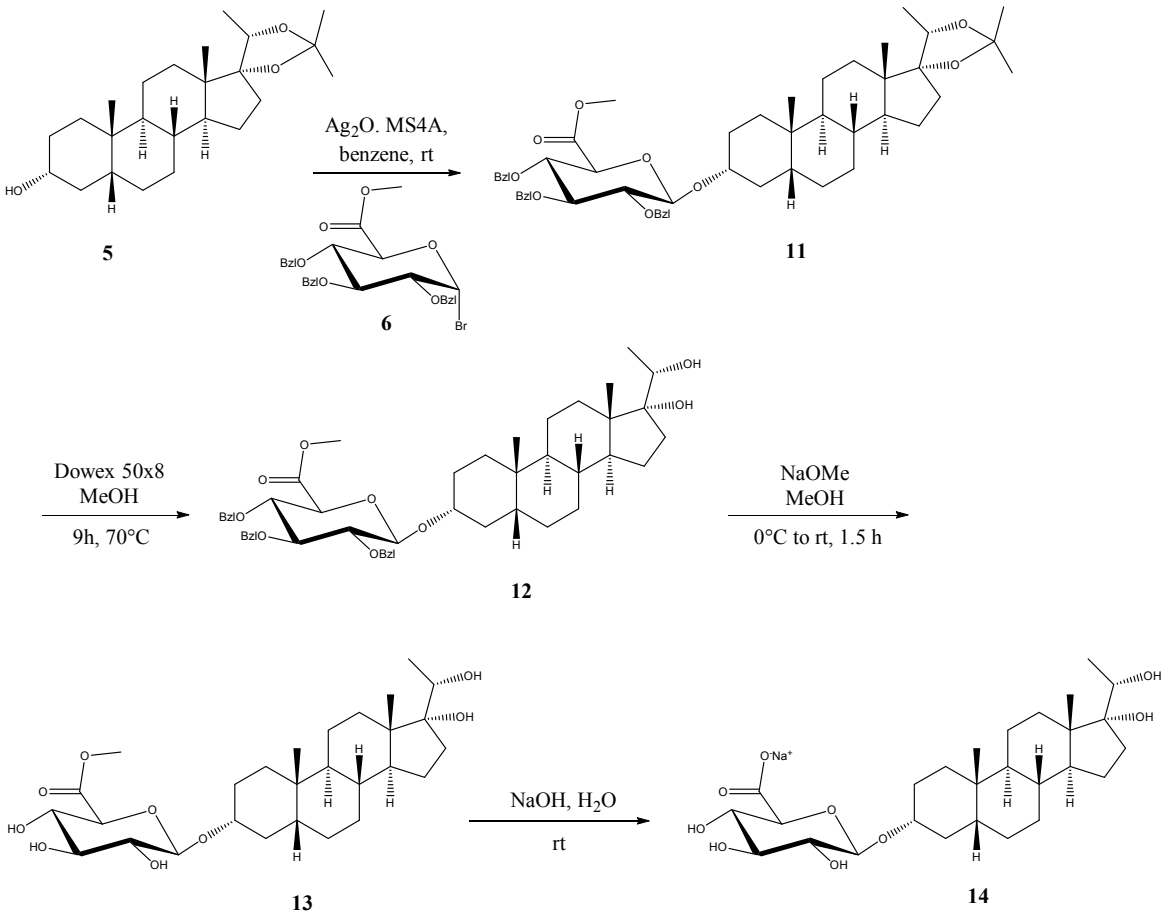
Extended data Figure 5 | Partial HSQC NMR spectra (500 MHz, methanol-d_4) of sodium 5β-pregnan-3α,17α,20α-triol-3α-glucuronate (**14**) and 5β-pregnan-3α,17α,20β-triol-3α-glucuronate (**10**). **A1**, **A2**: Synthetic **10**; **B1**, **B2**: Synthetic **14**; **C1**, **C2**: HPLC fraction A. Arrows (⬇⬇) in C1 and C2 indicate signals H/C-18 and H/C-20 which are relevant to distinguish the two isomers.



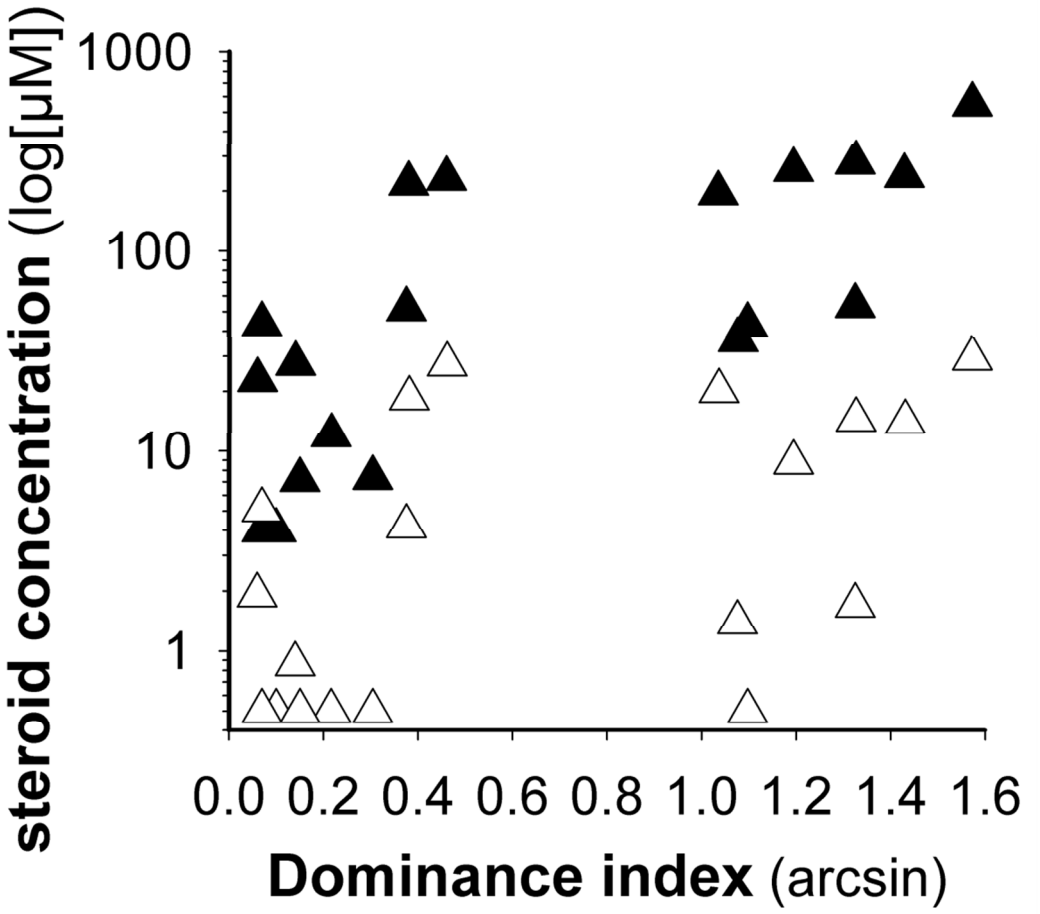
Extended data Figure 6 | Synthesis of the epimeric aglycons **2** (20β) and **3** (20α).



Extended data Figure 7 | Synthesis of 5β-pregnan-3α,17α,20β-triol-3α-glucuronate sodium salt (**10**).



Extended data Figure 8 | Synthesis of 5β-pregnan-3α,17α,20α-triol-3α-glucuronate sodium salt (**14**).



Extended data Figure 9 | Correlation between male social status (dominance index) and urinary concentration of the sex pheromone. Urinary concentrations in µM (common logarithm scale) of **14** (open triangles) and **10** (black triangles) as a function of the dominance indices of the donor males. The dominance index was positively correlated to the urinary concentration of **10** (Spearman correlation, $r_s = 0.790$, $P < 0.0001$, $N = 19$) and **14** (Spearman correlation, $r_s = 0.550$, $P = 0.0145$, $N = 19$). Note that for some males concentrations were below LC-MS detection limit of 1 µM for **14** (5 males) and 8 µM for **10** (2 males). Values assigned were 0.5 x detection limit.

Extended data Table 1 | ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of sodium synthetic 5β-pregnan-3α,17α,20α-triol-3α-glucuronate (**14**) and 5β-pregnan-3α,17α,20β-triol-3α-glucuronate (**10**) in methanol-d_4.

			14			**10**	
No.	**Type**	δ_C	δ_H	**mult., *J***	δ_c	δ_H	**mult., *J***
1	CH_2	36.5	1.84/0.98	m/m	36.5	1.84/0.98	m/m
2	CH_2	27.7	1.82/1.35	m/m	27.6	1.82/1.35	m/m
3	CH	79.4	3.81	m	79.2	3.84	m
4	CH_2	35.3	1.90/1.65	m/m	35.3	1.90/1.65	m/m
5	CH	43.8	1.42	m	43.8	1.42	m
6	CH_2	28.5	1.90/1.29	m/m	28.5	1.90/1.29	m/m
7	CH_2	27.9	1.45/1.19	m/m	28.0	1.45/1.19	m/m
8	CH	37.3	1.47	m	37.6	1.47	m
9	CH	41.7	1.48	m	41.8	1.48	m
10	C	36.0			36.0		
11	CH_2	21.7	1.45/1.28	m/m	21.8	1.45/1.28	m/m
12	CH_2	32.9	1.70/1.51	m/m	33.7	1.76/1.61	m/m
13	C	47.5			48.8		
14	CH	52.4	1.81	m	51.9	1.76	m
15	CH_2	24.6	1.67/1.16	m/m	25.1	1.67/1.12	m/m
16	CH_2	38.2	2.03/1.69	m/m	35.1	1.65/1.42	m/m
17	C	87.1			87.1		
18	CH_3	15.1	0.73	s	15.5	0.81	s
19	CH_3	24.0	0.95	s	24.1	0.96	s
20	CH	73.1	3.75	q, 6.4	71.7	3.92	q, 6.2
21	CH_3	18.9	1.16	d, 6.4	18.9	1.13	d, 6.2
1`	CH	102.0	4.41	d, 7.8	101.8	4.41	d, 7.8
2`	CH	75.1	3.19	dd, 7.8/9.0	75.1	3.19	dd, 7.8/9.0
3`	CH	78.0	3.39	dd, 9.0/9.0	78.0	3.39	dd, 9.0/9.0
4`	CH	73.9	3.45	dd, 9.0/9.5	74.0	3.44	dd, 9.0/9.4
5`	CH	76.4	3.57	d, 9.5	76.3	3.54	d, 9.4
6`	C	176.7			177.2	-	

Chapter IV

Olfactory sensitivity to steroid glucuronates in Mozambique tilapia suggests two distinct and specific receptor mechanisms for pheromone detection

This chapter gives more insights into the olfactory sensitivity and receptor specificity for the two steroid 3-glucuronides identified in chapter III, as well as for other steroids and prostaglandins.

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Olfactory sensitivity to steroid glucuronates in Mozambique tilapia suggests two distinct and specific receptor mechanisms for pheromone detection

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Abstract

Cichlids offer an exciting opportunity to understand vertebrate speciation; chemical communication could be one of the drivers of African cichlid radiation. Chemical signals mediate key aspects in the lives of vertebrates and often are species-specific. Dominant male Mozambique tilapia (*Oreochromis mossambicus* Peters 1852) release a sex pheromone, 5β-pregnan-3α,17α,20β-triol 3-glucuronate and its 20α-epimer, *via* their urine. The objective of this study was to assess sensitivity, specificity and versatility of the olfactory system of *O. mossambicus* to other steroids and their conjugates using the electro-olfactogram. *O. mossambicus* was sensitive to several 3-glucuronidated steroids, but did not respond to prostaglandins, unconjugated steroids or 17- or 20-conjugated steroids. Stimulation of the olfactory epithelium with increasing concentrations (10^{-12} M to 10^{-5} M) of 5β-pregnan-3α,17α,20β-triol 3-glucuronate, 5β-pregnan-3α,17α,20α-triol 3-glucuronate, 3α,17α-dihydroxy-5β-pregnan-20-one 3-glucuronate, etiocholanolone 3α-glucuronate and 17β-estradiol 3-glucuronate produced characteristic sigmoidal concentration-response curves. However, tilapia were most sensitive to 17β-estradiol 3-glucuronate, which also had the lowest apparent EC50 and maximal response amplitude. Cross-adaptation and binary mixture experiments suggested that 5β,3α-reduced pregnan- and androstan 3-glucuronates share a common olfactory receptor mechanism, whereas 17β-estradiol 3-glucuronate is detected *via* a distinct olfactory receptor. In conclusion, the Mozambique tilapia has evolved high olfactory sensitivity and specificity to 3-glucuronidated steroids through two distinct olfactory receptors; one detecting a male sex pheromone and a second detecting 17β-estradiol 3-glucuronate, a putative female-derived signal. However, *O. mossambicus* differs much in its olfactory perception from to the more recently derived East African cichlid *Astatotilapia burtoni*, suggesting that chemical communication could, indeed, be involved in speciation.

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Key words | chemical communication, pheromone, steroids, electro-olfactogram, olfactory receptor, cichlids

Running title | Olfaction of steroids in tilapia

Introduction

Sex steroids and their conjugates are potent odorants to several teleost fishes and are released into the water as sex pheromones, facilitating the location and choice of suitable mates or triggering endocrine changes in conspecifics that prompt gonadal maturation and improve fertility to enhance reproductive success (Stacey and others 2003; Stacey and Sorensen 2005). Pheromones may be composed of a single or multiple component(s) and are detected by olfactory receptors wherefrom the signal is transduced to specific brain areas that integrate the information and trigger the appropriate behavioural or endocrine response. A reliable method to study chemosensory perception in freshwater fishes and to explore whether different odorants are detected by separate or shared receptor mechanisms is recording of the electro-olfactogram (EOG) from the surface of the olfactory epithelium (for general review see Scott and Scott-Johnson 2002). In EOG cross-adaption tests, the response amplitude to one test odorant is measured prior to adaptation and then again during adaptation to a second odorant. If test and adapting odorant act through independent olfactory receptor mechanisms, the response to the test odorant during adaptation should be unaffected, i.e. not greatly reduced, compared to the signal measured prior to adaptation (Caprio and Byrd 1984; Cole and Stacey 2006; Sorensen and others 1995). In binary mixture tests, receptor mechanisms are separate if the EOG response to a mixture of two odorants is approximately the sum of responses to the individual odorants. Conversely, EOG responses to the mixture that are smaller or equivalent to twice the concentration of either odorant indicate a shared olfactory receptor mechanism (Cole and Stacey 2006). In goldfish *(Carassius auratu*s), for example, EOG recordings including cross-adaptation and binary mixture tests established that the pre- and postovulatory pheromones, released by females, are detected by conspecific males with high sensitivity through separate olfactory receptor mechanisms (Sorensen and others 1988; Sorensen and others 1995). The preovulatory pheromone includes free and sulphated 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P), acting via different receptors (Sorensen and others 1995). The postovulatory goldfish pheromone, on the other hand, consists of F-type prostaglandins, mainly PGF2α and 15K-PGF2α, which both too have distinct olfactory receptor sites (Sorensen and others 1988).

Within the Perciformes, the largest teleost order, studies on the perception and pheromonal function of hormonal steroids are scarce and derive from a few representatives of the Gobidae (Colombo and others 1980; Murphy and others 2001) and Cichlidae (Cole and Stacey 2006; Keller-Costa and others 2014). Cichlids are an extremely diverse taxon with currently 1,656 described species (Fishbase 2013a), mostly native to Africa, and adaptation of the sensory- and signaling systems to different environmental conditions has been suggested as an important driver in African cichlid radiation (Seehausen and others 2008). Focus so far has mainly been on the

evolution of colour polymorphism linked to light heterogeneity in the habitat (Seehausen and others 2008) alongside specialisation for particular trophic niches (Greenwood 1991). Divergent selection on chemical communication systems may, however, constitute an additional speciation factor. But knowledge of the identity, perception and functions of chemical signals across cichlids is limited, with the exception of two maternal mouth-brooders, *Astatotilapia burtoni* and *Oreochromis mossambicus* (Mozambique tilapia). *A. burtoni* has olfactory sensitivity to a variety of hormonal steroid conjugates (Robison et al 1998) with five distinct olfactory receptors, classified according to the type and position of the conjugate in the steroid (Cole and Stacey 2006). Unfortunately, it is not yet known whether *A. burtoni* synthesizes or releases any of these steroid conjugates and, if so, what their pheromonal function may be. *O. mossambicus* males establish dominance hierarchies in aggregations or 'leks' (Bruton and Boltt 1975) and use urine signals to mediate aggression between males and attract and prime females to spawn (Barata and others submitted manuscript; Keller-Costa and others 2014). Dominant male urine contains high concentrations of 5β-pregane-3α,17α,20β-triol 3-glucuronate (20β-P-3-G) and of its alpha-epimer (20α-P-3-G) which stimulate the endocrine system of females (Keller-Costa and others 2014). Both steroids evoke large olfactory responses mediated by a common receptor (Keller-Costa and others 2014). In contrast, steroids known to be present in blood plasma of *O. mossambicus* males, including 11-ketotestosterone, 17,20β-P and their glucuronate and sulphate conjugates, are not sensed by the olfactory epithelium (Frade and others 2002). Yet, it is not known whether prostaglandins or other steroid types, including steroids structurally related to the urinary pregnanetriol 3-glucuronates, are detected and, if so, how many different receptor sites are involved. But such insights are necessary to assess the olfactory steroid receptor diversity in African cichlids and therefore address the hypothesis of chemical signal diversification as a putative driver for African cichlid radiation.

Thus, the objectives of this study were, firstly, to assess olfactory sensitivity of *O. mossambicus* to steroids, and secondly, to establish, by cross-adaptation and binary mixture tests, whether steroid odorants act via shared or independent olfactory receptors; and thirdly, to compare these results to findings in *A. burtoni* (Cole and Stacey 2006), a more recently derived African cichlid.

Materials and methods

Fish

Fish care and experimentation complied with the guidelines of the European Union Council (86/609/EU) and Portuguese 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. Sexually mature Mozambique tilapia were raised in captivity from a brood-stock maintained at the University of Algarve (Faro, Portugal). Males and females were kept together in large 500 l stock tanks with sandy bottom, aerated freshwater at 27 ºC under a 12L : 12D photoperiod and fed daily with commercial cichlid feed (Sparos Lda., Portugal).

Odorants

The odorants tested in this study are given in table 1. Test odorants (steroids, prostaglandins, bile acid, L-serine) were purchased from Steraloids Inc. (Newport, RI, USA) or Sigma-Aldrich (Spain). The male tilapia sex pheromone components 5β-pregnan-3α,17α,20β-triol 3-glucuronate and 5β-pregnan-3α,17α,20α-triol 3-glucuronate were synthesized from the precursor 3α,17-dihydroxy-5β-pregnan-20-one as described previously (Keller-Costa and others 2014). All steroids, prostaglandins and the bile acid were dissolved in ethanol or methanol at 10^{-3} M (stock solution) and stored at -20 ºC until use. Stock solutions were diluted to the appropriate dilution in charcoal-filtered tap-water immediately prior to use in EOG recording (see below). A solution of 10^{-5} M L-serine to normalise EOG responses was similarly prepared from 10^{-3} M aliquots stored at -20 ºC.

Electro-olfactogram (EOG) recording

The method for EOG recording in tilapia has been described in detail (Frade and others 2002). Briefly, tilapia were anaesthetized with $NaHCO_3$-buffered MS222 (3-aminobenzoic acid ethyl ester, Sigma-Aldrich) in water (200 $mg.l^{-1}$) and immobilized with 3$mg.kg^{-1}$ gallamine triethiodide (Sigma-Aldrich). They were then maintained in a purpose-built padded 'fish-box', with 100 $mg.l^{-1}$ MS222 in aerated water pumped over the gills, within a Faraday cage. The olfactory rosette was exposed by cutting away a bit of skin and bone around the nostril and a glass tube with a constant flow of freshwater (4-6 $ml.min^{-1}$) was placed close to the raphe. Stimulus solutions were introduced into this flow by a computer-controlled solenoid valve. Borosilicate glass micropipettes filled with 4 % agar in 0.9 % NaCl were placed near the centre of the rosette (recording electrode) and lightly in contact with the skin of the head nearby (reference electrode). The DC voltage signal was amplified (either Neurolog NL102, Digitimer Ltd, Welwyn Garden

City, UK or Grass AC/DC strain gauge CP122; Astro-Med, West Warwick, RI, USA) and digitized (Digidata 1322A, Axon Instruments, Inc., now Molecular Devices, Sunnyvale, CA, USA). To determine which steroids and prostaglandins *O. mossambicus* detects, 3-6 mature fish were exposed to 4 s pulses of increasing concentrations (from 10^{-9} to 10^{-6} M). Compounds that did not evoke olfactory responses, or, inconsistent responses at high concentrations only, were excluded from further concentration-response, cross-adaptation and binary mixture studies (Table 1). Consistent responses were obtained from the bile acid TCD and all tested 3-glucuronidated steroids and EOG concentration-response curves generated. Mature female (*N* = 10-14; mean ± SD: BW = 44.1 ± 34.7 g; SL = 120.3 ± 44.6 mm) and male (*N* = 8-14; BW = 35.1 ± 11.4 g; SL = 106.4 ± 11.5 mm) recipients were exposed to increasing concentrations from 10^{-12} M to 10^{-5} M in $\log_{10}$ molar increments (plus 5 x 10^{-8} M) of 4 s odour pulses allowing at least 1 min between exposures. Given the sigmoidal shape of these curves, apparent maximal olfactory response (I_{max}), apparent half-maximal effective concentration (EC_{50}) and apparent Hill-coefficient values were calculated by fitting a sigmoidal regression curve using the Hill-equation [3 parameter: $y = \mathbf{a}x^{\mathbf{b}}/(\mathbf{c}^{\mathbf{b}} + x^{\mathbf{b}})$; **a** = max(y) = I_{max}; **b** = 1 = Hill co-efficient; **c** = x50(x,y) = EC_{50}] as mathematical model, in which **y** is the EOG response and **x** is the $\log_{10}$ stimulus concentration. Two-way (TW) ANOVAs followed by the Holm-Sidak post-hoc method for multiple pairwise comparisons were used to look for statistical differences within I_{max} and EC_{50} values.

EOG cross-adaptation tests

Cross-adaptation studies including 20α-P-3-G, 20β-P-3-G, 20one-P-3-G and ETIO-3-G were performed at saturating concentration because response magnitudes were similar at 10^{-6} M, whereas considerable variation existed from 10^{-9} M to 10^{-7} M concentration (linear part of the sigmoidal curves). Firstly, EOG responses to 4 s pulses of 10^{-6} M solutions of the steroids were recorded from mature males (*N* = 6-12; mean ± SD: BW = 46.9 ± 18.1 g; SL = 116.1 ± 16.4 mm). A 10^{-6} M solution of the adapting steroid was then used to perfuse the olfactory epithelium until voltage stabilised (about one minute). Then, a blank was recorded (10^{-6} M adapting steroid in 10^{-6} M adapting steroid). Test solutions (10^{-6} M test steroid in 10^{-6} M adapting steroid) were then administered as 4 s pulses, beginning with the adapting steroid (the self-adapted control (SAC) at 2 x 10^{-6} M). The bile acid TCD at 10^{-5} M was included as a negative control; it is a potent odorant for tilapia (Huertas *et al.*, 2010), is steroidal in nature, but likely acts via a different olfactory receptor mechanism. Initial EOG responses to the steroids before cross-adaption were blank-subtracted using the response to blank water; same water used to dilute stimuli. EOG responses to the test solutions during adaptation were blank-subtracted using the adapted response to the 1 x 10^{-6} M adapting steroid blank. EOG responses to the test solutions during adaptation were then converted to a percentage of the initial (unadapted) response (% R_I).

Cross-adaptation tests involving E2-3-G were performed separately on males (N = 5-7; five to seven BW = 73.3 ± 19.5 g; SL = 135.4 ± 12 mm) at 10^{-8} M concentrations because response amplitudes of 20α-P-3-G and 20β-P-3-G were roughly comparable to E2-3-G, at 10^{-8} M, whereas enormous differences existed at 10^{-6} M.

For each cross-adaptation dataset, mean % R_I were compared using Kruskal-Wallis-ANOVAs on ranks followed by the Dunn's post-hoc method with multiple comparisons versus the self-adapted control (SAC).

EOG binary mixture tests

Odorants at 10^{-6} M (10^{-5} M for TCD) were tested at the same concentrations used in cross-adaptation tests on six to fourteen mature males (mean ± SD: BW = 49.5 ± 22.9 g; SL = 117.3 ± 19.3 mm). Tests involving E2-3-G were performed at 10^{-8} M on nine mature tilapia males (BW = 68.7 ± 20.6 g; SL = 132.3 ± 13.6 mm). First, fish were exposed consecutively to steroid A (response $\boldsymbol{R_A}$) and B ($\boldsymbol{R_B}$) at 10^{-x} M, then to steroids A ($\boldsymbol{R_{2A}}$) and B ($\boldsymbol{R_{2B}}$) at twice the concentration (2x 10^{-x} M) and finally to a mixture of A and B (each at 10^{-x} M) to induce response R_{A+B}. The independent component index $\boldsymbol{I_{CI}}$ **(1)** and the mixture discrimination index $\boldsymbol{I_{MD}}$ **(2)** were generated as reported earlier (Kang & Caprio, 1991, Li & Sorenson, 1997, Cole & Stacey 2006).

$$\boldsymbol{I_{CI}} = \frac{\mathbf{RA+RB}}{\mathbf{(RA + RB)}} \quad \mathbf{(1)}$$

$$\boldsymbol{I_{MD}} = \frac{\mathbf{RA+B}}{0.5\ \mathbf{(R2A + R2B)}} \quad \mathbf{(2)}$$

The $\boldsymbol{I_{CI}}$ is predicted to be around 1 in case of independent receptor mechanisms and below 1 (about 0.5) in case of a shared receptor mechanism. The $\boldsymbol{I_{MD}}$ is predicted to be 1 in case of a shared receptor and >1 if there is receptor independence. Kruskal-Wallis-ANOVAs on ranks followed by the Dunn`s post-hoc method with multiple comparisons versus a control group (20α-P-3-G / 20β-P-3-G mix) were used to compare the binary mixture results.

Results

Detected steroids and EOG concentration response tests

Mozambique tilapia responded consistently to 3-glucuronidated steroids (Figure 1 and 6), but did not give EOG responses to representatives of 17-, or 20-glucuronidated or sulphated steroids. Neither did they respond to any of the unconjugated steroids, E2-3-S or prostaglandins, even at concentrations as high as 1 µM (Table 1, Figure 6). ETIO-3-S and E2-3,17-diG induced small EOG responses, yet only at high concentrations of 10^{-7} M and 10^{-6} M. However, the latter responses were not consistent and were not pursued further.

Sigmoidal concentration response curves were obtained from all 3-glucuronidated steroids (Table 1) and no differences were found between the responses of male and female recipients (Figure 1). The detection threshold was lower for E2-3-G (10 pM), followed by 20one-P-3-G (100 pM) and for the majority was around 1 nM. EOG response magnitudes of 20α-P-3-G, 20β-P-3-G, 20one-P-3-G and ETIO-3-G increased rapidly before reaching an apparent maximum around 1 µM, which suggests saturation of the olfactory receptors (Figure 1). For E2-3-G, both the EOG amplitude and saturation (1nM) was much lower. Accordingly, the E2-3-G apparent half-maximal effective concentration EC_{50} (mean ± SEM; ♂ 0.07 ± 0.02 nM; ♀ 0.14 ± 0.08 nM) and apparent maximal olfactory response I_{max} (♂ 0.38 ± 0.05; ♀ 0.34 ± 0.04) were significantly lower than apparent EC_{50} and I_{max} values of all the other 3-glucuronidated steroids (Figure 2 A and B). As for the other steroids, 20α-P-3-G (♂ 89.62 ± 16.15 nM; ♀ 86.3 ± 18.74 nM) and ETIO-3-G (♂ 54.38 ± 18.4 nM; ♀ 40.74 ± 10.92 nM) had similar and highest apparent EC_{50} values, followed by 20β-P-3-G (♂ 25.72 ± 9.73 nM; ♀ 30.12 ± 12.92 nM) and 20one-P-3-G (♂ 4.78 ± 1.01 nM; ♀ 3.67 ± 0.71 nM; Figure 2 A). Apparent I_{max} of 20α-P-3-G, 20β-P-3-G and ETIO-3-G were similar and nearly twice the response to 10^{-5} M L-serine (Figure 2 B). Apparent I_{max} values of male (but not female) responses to 20one-P-3-G were lower than to 20α-P-3-G. The apparent Hill-coefficients were close to 1 for all steroids, suggesting a simple 1:1 binding ratio to the olfactory receptors, with no cooperativity.

The concentration-response curve for TCD showed a rapid increase of EOG amplitudes at supra-threshold (around 10 nM) concentrations, without reaching an apparent maximum up to 10 µM (Figure 1); TCD was used as a 'negative control' in cross-adaptation and binary mixture tests.

EOG cross-adaptation tests

To assess whether the continuous perfusion and sequential exposure with steroids during cross-adaptation did not desensitize the olfactory epithelium, responses to the unadapted steroids were again recorded after cross-adaptation tests finished (after a 10 minute wash-out) and compared to the initial unadapted responses. No reduction of EOG responses was observed for any of the tested steroids, regardless the concentration. However, significant increases (mean %) in mean EOG response amplitudes were noted for some steroids, i.e. 20β-P-3-G (~27 %) and 20one-P-3-G (~ 26 %) at 10^{-6} M and 20α-P-3-G (22 %) at 10^{-8} M concentrations; paired *t*-tests, $P = 0.045$, $P = 0.021$ and $P = 0.046$, respectively. Increasing EOG response magnitudes over time are a commonly observed phenomenon in fish; since responses to test steroids during adaptation were compared only to the initial responses recorded before cross-adaptation, the increase noted here for some steroids should not influence the conclusions drawn.

The results of the EOG cross-adaptation studies at 10^{-6} M (10^{-5} M TCD) confirmed that the two male tilapia urinary steroids 20α-P-3-G and 20β-P-3-G act through a shared receptor mechanism (Figure 3). They further suggested that 20one-P-3-G, as well as the androstane ETIO-3-G are detected by the same olfactory receptor mechanism, hereafter 3G-R-I (Figure 6), referring to the position of the glucuronate in the steroid. 20β-P-3-G and 20one-P-3-G consistently reduced EOG responses to all test steroids (except TCD) during adaptation to a point that they were not significantly different from the self-adapted control (SAC). Some slight anomalies were found, however, with 20α-P-3-G and ETIO-3-G as adapting steroids; both reduced the response to 20one-P-3-G only partially, to a level still significantly different from the SACs. Less pronounced response reduction was also observed when 20β-P-3-G was adapted to 20α-P-3-G. In contrast, responses to TCD could not be reduced below 80 % of the unadapted response, regardless of the adapting steroid. Surprisingly, however, when the olfactory epithelium was adapted to TCD, mean responses to all administered test-steroids were reduced by at least 57 %, although they remained significantly higher (except for ETIO-3-G) than the SAC (Figure 3).

Given the distinct concentration response curve of E2-3-G, we hypothesized this steroid to act via a mechanism other than 3G-R-I. To test this, cross-adaptation tests including E2-3-G, 20α-P-3-G and 20β-P-3-G were performed at 10^{-8} M, as EOG amplitudes of the three steroids were more similar at this concentration than at 10^{-6} M (Figure 1). E2-3-G could not reduce the responses to 20α-P-3-G or 20β-P-3-G below 70 % during adaptation (Figure 4). Reciprocal adaptation of the olfactory epithelium to 20α-P-3-G or 20β-P-3-G confirmed these results, as responses to E2-3-G were consistently much higher than the SACs and generally closer to the initial response. This indicates that the Mozambique tilapia is able to distinguish E2-3-G from other 3-glucuronidated pregnanes and androstanes via a distinct olfactory receptor mechanism, hereafter '3G-R-II' (Figure 6).

EOG binary mixture tests

The mean independent component index (I_{CI}) and mixture discrimination index (I_{MD}) of the binary mixture 20α-P-3-G/20β-P-3-G were around 0.5 and 1.0, respectively, and consistent with the cross-adaptation studies, strongly suggesting a shared olfactory receptor mechanism. Mean I_{CI} and I_{MD} values for 20α-P-3-G or 20β-P-3-G mixed with either 20one-P-3-G or ETIO-3-G were statistically similar to the 20α-P-3-G/20β-P-3-G mix (Figure 5). The mean 'within-group' I_{CI} and I_{MD} value was 0.49 and 0.99, respectively. The results support the cross-adaptation tests; 20one-P-3-G and ETIO-3-G are detected by the 3G-R-I, as are the urinary pheromonal steroids 20α-P-3-G and 20β-P-3-G.

Mean I_{CI} values for 20α-P-3-G or 20β-P-3-G mixed with E2-3-G were generally closer to 1 and significantly different ($P < 0.001$) from the 20α-P-3-G/20β-P-3-G mix, indicating that E2-3-G is detected by a different receptor mechanism 3G-R-II. These results were also confirmed by the respective mean I_{MD} values which were both above 1, although significant difference, as compared the 20α-P-3-G/20β-P-3-G mix, was only reached for the 20α-P-3-G / E2-3-G mix, but not the 20β-P-3-G / E2-3-G mix.

Both I_{CI} and I_{MD} values of TCD mixed with 20α-P-3-G, 20β-P-3-G or E2-3-G were close to 1 (I_{CI}) or clearly above 1 (I_{MD}) and significantly different ($P < 0.001$) from the 20α-P-3-G/20β-P-3-G mix, supporting the assumption that TCD acts via a separate receptor mechanism, and consistent with the cross-adaptation studies. The mean 'across-group' I_{CI} and I_{MD} values were 0.77 and 1.45, respectively, and significantly larger than the mean 'within-group' I_{CI} and I_{MD} values (Man Whitney rank sum tests, $P = 0.002$).

Discussion

This study demonstrates that the Mozambique tilapia possesses high olfactory sensitivity to several 3-glucuronidated steroids *via* two distinct olfactory receptor mechanisms; 3G-R-I selects C21 and C19 5β,3α-reduced steroids whereas 3G-R-II selects C18 aromatic steroids.

Cross-adaptation tests

EOG responses not only confirmed the sensitivity of females and males to the previously identified male tilapia sex pheromone components, 20α-P-3-G and 20β-P-3-G, but they also show that structurally related 3-glucuronidated pregnane(s) and androstane(s) produce similar concentration-response curves and act via the same olfactory receptor mechanism 3G-R-I. Some slight anomalies, however, were observed in cross-adaption tests with 20α-P-3-G or ETIO-3-G as adapting steroid and 20β-P-3-G and/or 20one-P-3-G as test odorant; responses were reduced but not to the extent of the self-adapted control (SAC). This may be explained by the lower apparent EC_{50} values obtained for 20β-P-3-G and 20one-P-3-G than for 20α-P-3-G and/or ETIO-3-G. When two odorants compete for the same receptor site but one odorant has a higher affinity (as indicated by the lower apparent EC_{50}), it is likely to replace the other odorant at the receptor binding site, thereby giving a partial olfactory response. Cross-adaptation tests reveal further that E2-3-G is detected through a separate olfactory mechanism 3G-R-II, producing a markedly differently concentration response curve.

The bile acid TCD was expected to act through an olfactory receptor mechanism separate from the steroid conjugates tested. Consistent with our previous work (Keller-Costa and others 2014) the response to TCD was never reduced below 80 % of the initial response, regardless of the adapting odorant. Surprisingly, however, with TCD as the adapting odorant, responses to the test steroids were considerably reduced (50 % or more) although never as low as the SAC. It is possible that TCD may act as partial agonist, or antagonist, at the 3G-R-I receptor sites when present at high concentrations.

Binary mixture tests

Results of binary mixture experiments were generally consistent with those of the cross-adaptation tests. Mean 'within-group' I_{CI} and I_{MD} values were 0.49 and 0.99, even lower than those obtained for *A. burtoni* (0.63 and 1.26; Cole and Stacey 2006) and fitting nearly perfectly the expected values (<1 and 1) for shared receptor groups. The mean 'across-group' I_{MD} value of 1.45 exceeded the predicted value of 1, suggesting receptor independence. However, the mean 'across-group' I_{CI} value of 0.77 was below the expected value of 1, and lower than 'across-group' values observed from *A. burtoni* (0.94; Cole and Stacey 2006) and the sea lamprey (0.97; Li and

Sorensen 1997). However, 'across-group' mixtures do not always reach the perfect I_{CI} value of 1, as seen by (Caprio and others 1989) with amino acid odorants in the channel catfish, *Ictalurus punctatus*. The authors suggested that different receptor site types present on the same receptor cell may not be as independent as different receptor site types on different cells leading to slightly reduced responses in binary mixture tests. It may be possible that the 3G-R-I and 3G-R-II receptor types of *O. mossambicus* are present in the same receptor cell.

The olfactory receptor mechanism '3G-R-I', detecting the tilapia sex pheromone is specific to 5β,3α reduced 3-glucuronidated steroids

All 3-glucuronidated steroids induced similar EOG responses in both males and females, which is consistent with earlier EOG studies in *O. mossambicus* (Keller-Costa and others 2014) and other teleosts, e.g. *A. burtoni* (Cole and Stacey 2006), goldfish (Sorensen and Goetz 1993) or round goby (Murphy and others 2001). In agreement with previous findings (Frade and others 2002), the olfactory epithelium of *O. mossambicus* did not respond to unconjugated-, nor to a variety of 17- or 20-conjugated steroids, nor to E2-3-S and it was insensitive to prostaglandins ($PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$). This suggests that the olfactory receptor mechanisms for steroid detection in *O. mossambicus* require a glucuronide at C3 position. Structure and 3-dimensional orientation of the cyclohexane ring 'A' seem to determine whether the ligand is detected by 3G-R-I or 3G-R-II. However, at least in case of 3G-R-I, some freedom in the functional group or aliphatic chain attached to C17 in cyclopentane ring 'D' of the steroid ligand is possible, although apparently this can affect affinity.

The role of 20one-P-3-G and ETIO-3-G as putative reproductive pheromones has been discussed previously in other teleost species. Testis-derived ETIO-3-G from black goby males (*Gobius jozo*) attracts ripe females (Colombo and others 1980). A similar observation was made from African catfish (*Clarias gariepinus*) males, where the most potent testicular odorant was found to be 20one-P-3-G (Lambert and Resink 1991). Androstanes and pregnanes with 5β,3α configuration are as well potent odorants for the round goby (*Neogobius melanostomus*; Murphy and others 2001) and recent studies have demonstrated that round goby males release several conjugated forms of these steroids via their urine (Katare and others 2011), eventually to attract females (Tierney and others 2012). However, in the round goby, the olfactory receptor mechanism detecting ETIO-3-G appears to be less specific than in tilapia, as several unconjugated androstanes, pregnanes and even androsten, are being detected by the same (ETIO-3-G) receptor mechanism.

20one-P-3-G and ETIO-3-G are not natural constituents of tilapia male urine (own unpublished observations). It remains to be seen if 20one-P-3-G and ETIO-3-G are able to activate the same signal cascade that triggers the endocrine response in females as 20α-P-3-G and 20β-P-3-G. If so,

ETIO-3-G or 20one-P-3-G could be valuable for future research, avoiding the time intensive and expensive synthesis of 20α-P-3-G and 20β-P-3-G, respectively.

Tilapia detects a putative social cue from females, E2-3-G, via a distinct olfactory receptor mechanism '3G-R-II'

This is the first report of a cichlid detecting E2-3-G. The low detection threshold shows that *O. mossambicus* is highly sensitive to E2-3-G and the low apparent EC_{50} value suggests high affinity to 3G-R-II. On the other hand, the low apparent I_{max} may indicate a relatively small number of receptor cells in the epithelium responding to this stimulus. Since 17β-estradiol is produced by the growing follicle, E2-3-G could act as a social cue released by female tilapia, providing information on reproductive condition. Males are capable of discriminating pre-ovulatory versus post-spawning females through the smell of the females' urine (Almeida and others 2005). Moreover, they drastically increase their own urination frequency in the presence of a female that is near ovulation but not as much with post-spawn females (Almeida and others 2005). Pre-ovulatory females release overall more E2 into the water than post-spawn females (Huertas and others 2014) and urine from pre-ovulatory females contains large quantities (100 - 150 $ng.ml^{-1}$) of 17β-estradiol (3 and/or 17)-glucuronide (unpublished observations). E2-3-G is also a potent odorant for the round goby, *Neogobius melanostomus*, where it increases ventilation rate (opercula movements per minute) in males, but not females (Murphy and others 2001). Future investigations will determine if E2-3-G is released by pre-ovulatory tilapia females into their urine and if it functions indeed as a chemical signal.

Comparison of *O. mossambicus* with a more recently derived African cichlid, *A. burtoni*

It seems that both *O. mossambicus* and *A. burtoni,* have one olfactory receptor for 5β,3α-reduced 3-glucuronidated steroids in common (3G-R-I) , but also show substantial difference in the steroid types they detect. In addition to the putative 3G-R I, *A. burtoni* possesses four other independent receptor sites recognizing 17-glucuronidated-, 3-sulphated-, 17-sulphated- and 3,17-disulphated steroids (Cole and Stacey 2006). The olfactory sensitivity of *O. mossambicus* to di-sulphated steroids has not been investigated, but it appears to be largely insensitive to the other steroid conjugates. However, Mozambique tilapia is able to distinguish E2-3-G *via* a distinct olfactory receptor mechanism (3G-R-II), an ability that *A. burtoni* lacks (Cole and Stacey 2006). Common to both cichlids is that they neither detect prostaglandins nor unconjugated steroids. In this they differ substantially from cypriniformes, such as goldfish (Sorensen and others 1988; Sorensen and others 1995) and carp (Lim and Sorensen 2011; Lim and Sorensen 2012), salmoniformes, e.g. Atlantic salmon, (Moore and Waring 1996), brown trout and brook trout (Essington and Sorensen 1995) and Arctic char (Sveinsson and Hara 2000), and even the perciform round goby (Murphy

and others 2001). It would be interesting to investigate more cichlid species to establish whether insensitivity to prostaglandins and free steroids is a general feature of this family.

Unfortunately, it is not known whether *A. burtoni* releases any of the five steroid types it is able to detect. One study reported that *A. burtoni* males increase serum testosterone levels in response to a mixture of representatives of the five steroid types (Cole and Stacey 2003) but not when presented only one type alone. The reproductive biology and social organization of *A. burtoni* and *O. mossambicus* are comparable in several ways; both are maternal mouth-brooders and arena spawners (Bruton and Boltt 1975; Fernald and Hirata 1977). In both species, males establish dominance hierarchies and increase urination frequency during aggressive encounters with rivals or when courting females (Barata and others 2008; Barata and others 2007; Maruska and Fernald 2012). It is therefore possible that *A. burtoni*, as *O. mossambicus,* releases the steroid types it detects (or at least some of them) *via* its urine, playing (a) similar pheromonal role(s) as in the Mozambique tilapia. However, the larger number of receptors suggests greater complexity and/or differences in the meaning of the steroidal 'message'.

Comparison of (only) two African cichlids shows that there is substantial variability in the types of conjugated steroids they detect, indicating substantial diversity in olfactory steroid receptors among different species. Clearly, future studies should include more representatives - sympatric and allopatric - from different genera and clades within the Cichlidae to assess whether there is any link between the diversity of steroid receptor types, ecology and phylogeny. In addition, the biological significance of these receptors, i.e. pheromonal function and release routes of detected steroids, needs to be explored. Such insights may shed light on the exiting question if chemical communication could have been among the drivers of African cichlid radiation.

In conclusion, the Mozambique tilapia has evolved high olfactory sensitivity and specificity to 3-glucuronidated steroids. Apparently, two distinct receptor sites are involved; one (3G-R-I) detecting a male sex pheromone (i.e. 20α-P-3-G and 20β-P-3-G) and a second (3G-R-II) detecting 17β-estradiol 3-glucuronide, which may function as a (pre-ovulatory) female pheromone.

Acknowledgements

The authors are grateful to Dr C. Paetz, Dr Y. Nakamura and Dr B. Schneider from the Max Planck Institute of Chemical Ecology in Jena, Germany for synthesising the steroid-conjugates 20α-P-3-G and 20β-P-3-G used in this study. The technical help of Vanessa Afonso and Sebastiaan Mestdagh is also acknowledged. T. K.-C. (SFRH/BD/46192/2008) received a research fellowship from the Science and Technology Foundation (FCT), Portugal.

Tables

Table 1 | Steroids tested in in this study.

Chemical class	Chemical group	Compound name	Abbreviation	Detection threshold[1]	Saturation[1]	Test conc.[2]
bile acid	24-carbon	taurochenodeocxcholic acid	TCD	10 nM	-	10 µM
prostaglandins (PG)	20-carbon	prostaglandin F2α	PGF2α	insensitive[3]	-	-
		prostaglandin 15keto-F2α	15k-PGF2α	insensitive[3]	-	-
unconjugated steroids	21-carbon	5β-pregnane-3α,17α,20β-triol	20β-P	insensitive[3]	-	-
		5β-pregnane-3α,17α,20α-triol	20α-P	insensitive[3]	-	-
		3α,17α-dihydroxy-5β-pregnan-20-one	20one-P	insensitive[3]	-	-
	19-carbon	etiocholan-3α-ol-17-one	ETIO	insensitive[3]	-	-
	18-carbon	17β-estradiol	E2	insensitive[3]	-	-
3-sulphated steroids	19-carbon	etiocholan-3α-ol-17-one 3-sulphate	ETIO-3-S	100 nM[4]	-	-
	18-carbon	17β-estradiol 3-sulphate	E2-3-S	insensitive[3]	-	-
17-sulphated steroids	19-carbon	testosterone 17-sulphate	T-17-S	insensitive[3]	-	-
20-sulphated steroids	21-carbon	17α,20β-dihydroxy-4-pregnen-3-one 20-sulphate	17,20β-P-20-S	insensitive[3]	-	-
3-gluc. steroids	21-carbon	5β-pregnan-3α,17,20β-triol-3-glucuronate	20α-P-3-G	1 nM	1 µM	1 µM
		5β-pregnan-3α,17,20α-triol-3-glucuronate	20β-P-3-G	1 nM	1 µM	1 µM
		3α,17-dihydroxy-5β-pregnane-20-one-3-glucuronate	20one-P-3-G	100 pM	1 µM	1 µM
	19-carbon	etiocholan-3α-ol-17-one-3-glucuronide	ETIO-3-G	1 nM	1 µM	1 µM
	18-carbon	17β-estradiol-3-glucuronate	E2-3-G	10 pM	1 nM	10 nM
17-gluc. steroids	19-carbon	testosterone 17-glucuronate	T-17-G	insensitive[3]	-	-
	18-carbon	17β-estradiol 17-glucuronate	E2-17-G	insensitive[3]	-	-
20-gluc. steroids	21-carbon	17α,20β-dihydroxy-4-pregnen-3-one 20-glucuronate	17,20β-P-20-G	insensitive[3]	-	-
3,17-digluc. steroid	18-carbon	17β-estradiol 3,17-diglucuronate	E2-3,17-diG	100 nM[4]	-	-

[1] read estimates based on mean concentration response curves.
[2] concentration used in EOG cross-adaptation and binary mixture tests.
[3] *O. mossambicus* does not possess any olfactory sensitivity to this steroid
[4] responses not consistent and EOG amplitudes small, therefore this steroid was excluded from further concentration-response, cross-adaptation and binary mixture tests

Figures and figure legends

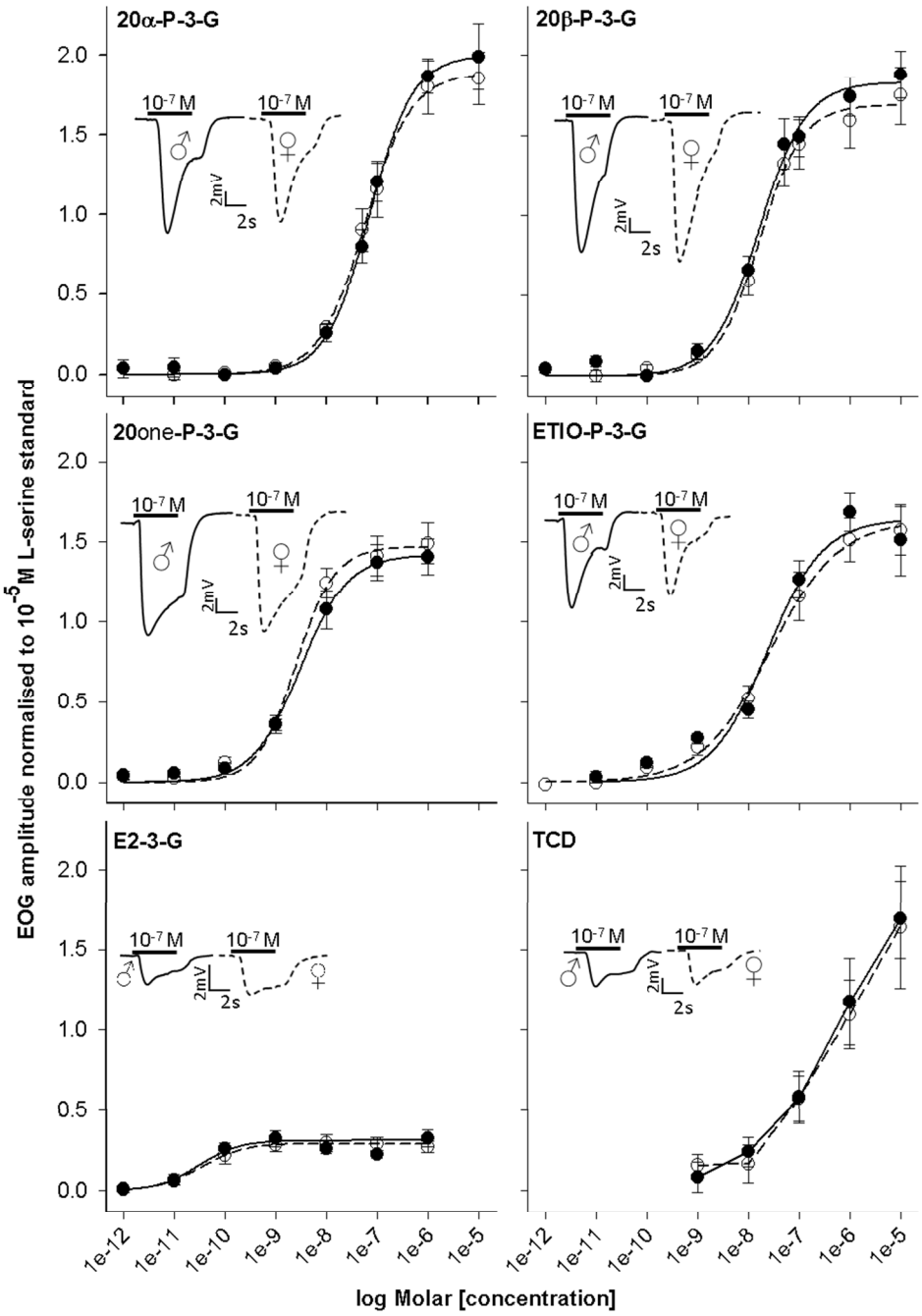

Figure 1 | EOG concentration response profiles. Normalised (to 10^{-5}M L-serine response) EOG concentration-response (CR) curves (semi-logarithmic plot, mean ± SEM) for the male tilapia sex pheromone, 20α-P-3-G and 20β-P-3-G, and other steroid-3-glucuronates and a bile acid (TCD). Responses of males (*N* = 8-14; filled circles) and females (*N* = 10-14; open circles) are shown. A sigmoidal (Hill-3-parameter) curve was fitted to the response profiles of the steroid-3-glucuronates of both sexes (males = solid, females = dashed line). Representative EOG traces of a

male (solid line) and a female (dashed line), recorded at 10^{-7} M odorant concentrations, are presented as inserts.

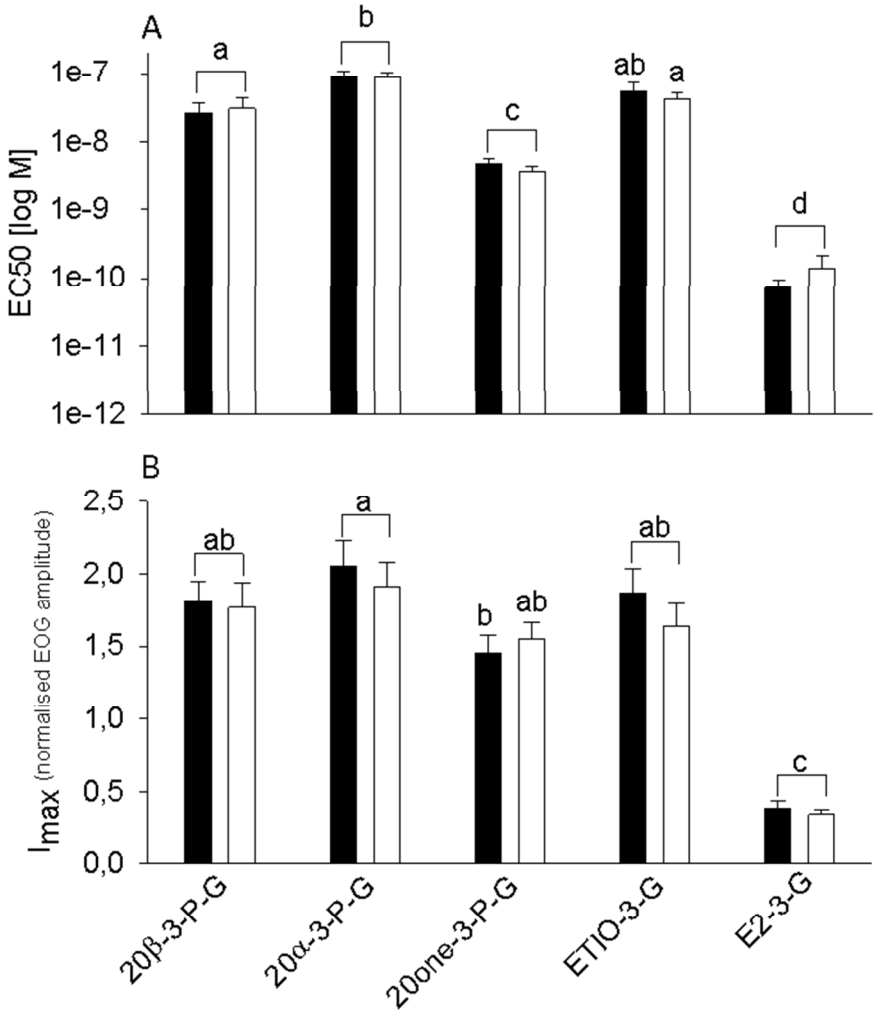

Figure 2 | Comparison of apparent EC_{50} and I_{max} values. Data (mean ± SEM) were calculated from sigmoidal concentration response curves of the male tilapia sex pheromone 20α-P-3-G and 20β-P-3-G, and other steroid-3-glucuronides. Apparent **A)** EC_{50} values (in nM; $\log_{10}(y)+2$ - transformed values) and **B)** I_{max} values for males (N = 7-14; black bars) and females (N = 10-14; open bars) for each steroid were similar. Different letters above bars indicate significant differences ($P < 0.001$) among steroids. Two-Way ANOVA followed by Holm-Sidak post-hoc test. F and P values were as follows: **A) apparent EC_{50} values** - sexes: $F_{1,\ 103} = 0.131$, $P = 0.781$; steroids: $F_{4,\ 103} = 177.968$, $P < 0.001$, interaction sexes x steroids: $F_{4,103} = 0.257$, $P = 0.905$. **B) apparent I_{max} values** - sexes: $F_{1,103} = 0.686$, $P = 0.409$; steroids: $F_{4,103} = 34.280$, $P < 0.001$; interaction sexes x steroids: $F_{4,103} = 0.397$, $P = 0.810$. All data were of equal variance.

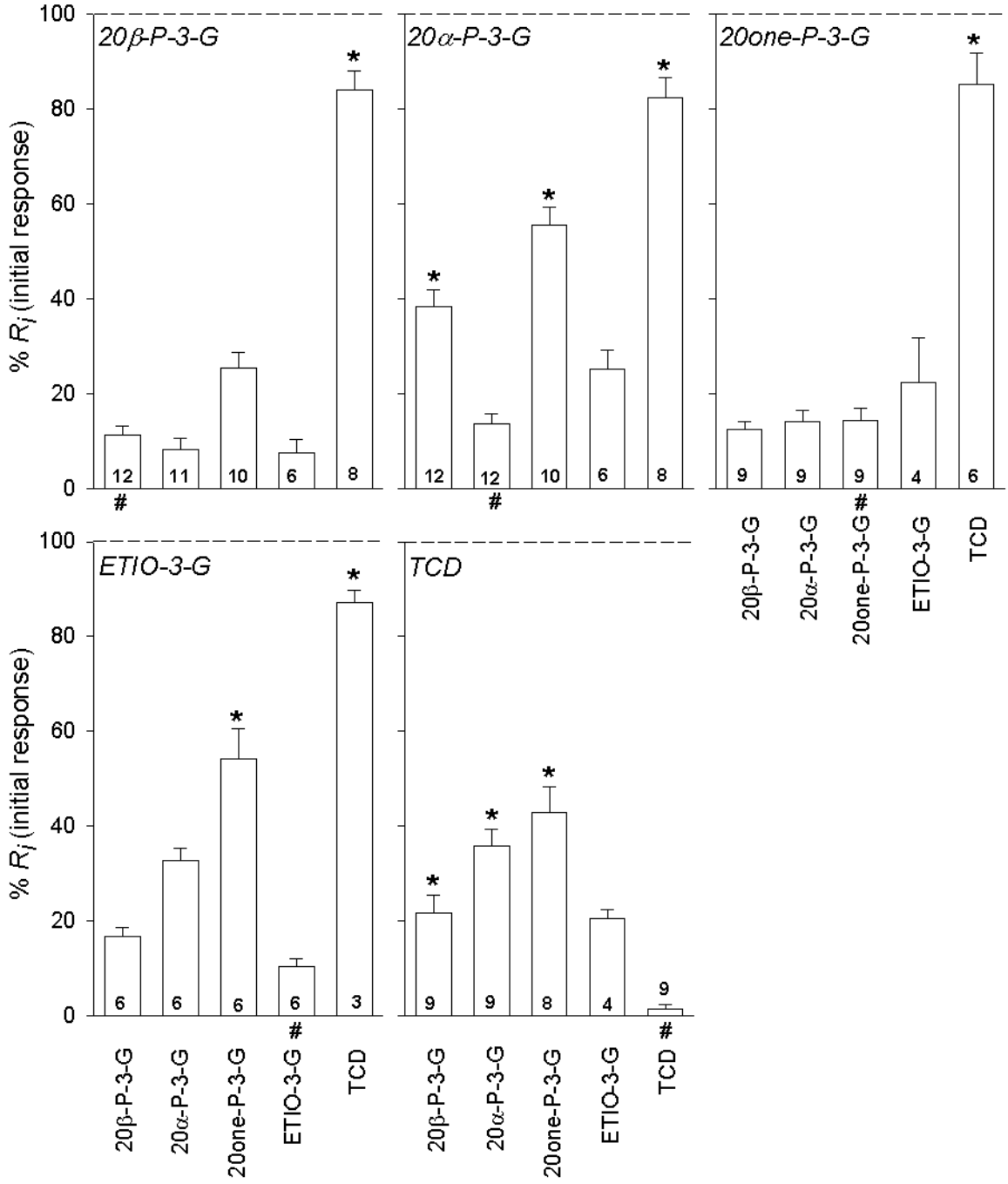

Figure 3 | EOG cross-adaptation studies. Relative EOG response (mean + SEM) to 10^{-6} M steroid conjugates (or 10^{-5} M TCD) expressed as percentage of the initial unadapted response (% R_I) to the same 10^{-6} M steroid (10^{-5} M TCD), delivered before cross-adaptation. Sharps **#** indicate the self-adapted controls (SAC). Numbers in bars indicate sample size. Asterisks ***** above bars indicate significant differences from the SAC ($P < 0.05$). Kruskal-Wallis ANOVA on ranks followed by Dunn`s method, multiple comparisons versus SAC as control group. **20β-P-3-G:** H = 28.951, df = 4, $P < 0.001$. **20α-P-3-G:** H = 38.624, df = 4, $P < 0.001$. **20one-P-3-G (10^{-6} M):** H =

16.136, *df* = 4, *P* = 0.003. **ETIO-3-G:** *H* = 23.243, *df* = 4, *P* < 0.001. **TCD:** *H* = 26.903, *df* = 4, *P* < 0.001.

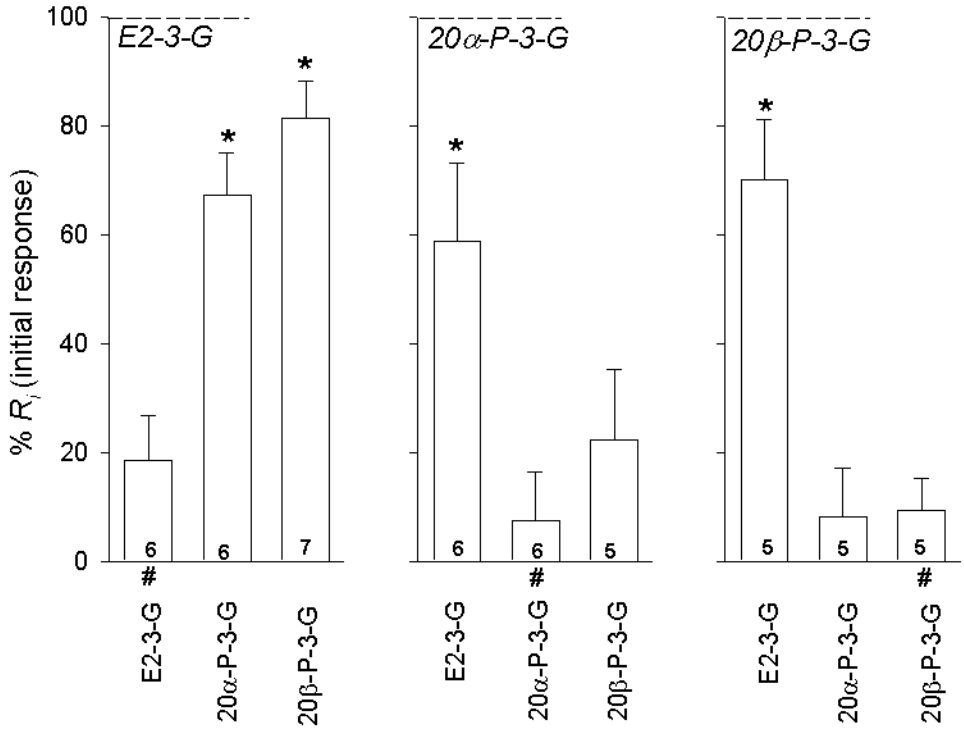

Figure 4 | EOG cross-adaptation studies involving 17β-estradiol-3-G (E2-3-G). Relative EOG response (mean + SEM) to 10^{-8} M steroid conjugates expressed as percentage of the initial unadapted response (% R_I) to the same 10^{-8} M steroid delivered before cross-adaptation started. Sharps # indicate the self-adapted controls (SAC). Numbers in bars indicate sample size. Asterisks * above bars indicate significant differences from the SAC ($P < 0.05$). Kruskal-Wallis ANOVA on Ranks followed by Dunn`s method, multiple comparisons versus SAC as control group. **E2-3-G:** *H* = 11.523, *df* = 2, *P* = 0.003. **20β-P-3-G:** *H* = 9.420, *df* = 2, *P* = 0.009. **20α-P-3-G:** *H* = 6.371, *df* = 2, *P* = 0.041.

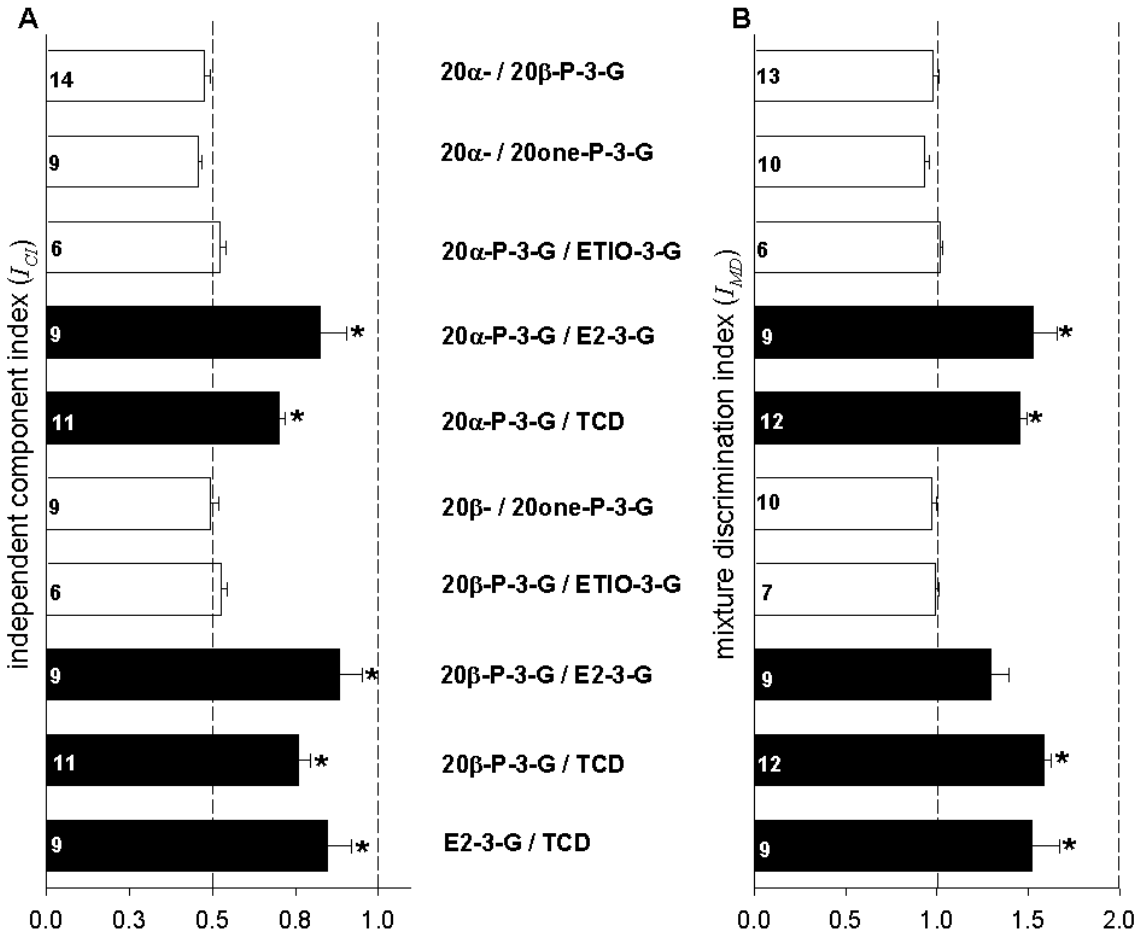

Figure 5 | Results of EOG binary mixture tests. A) Independent component (I_{CI}) and **B)** mixture discrimination (I_{MD}) indices (mean + SEM) calculated from binary mixture tests. Open bars ('within-group'): values for I_{CI} (~0.5) and I_{MD} (~1) indicate that the steroids in mixture interact with a common receptor mechanism. Black bars ('across-group'): values for I_{CI} *(~1)* and I_{MD} (>1) indicate that the steroids in mixture interact with different receptor mechanisms. Numbers in bars indicate sample size. Asterisks * above bars indicate significant differences. Kruskal-Wallis ANOVA on Ranks followed by Dunn`s method, multiple comparisons versus the mixture 20α-P-3-G/20β-P-3-G as control group; **A)** $\boldsymbol{I_{CI}}$**:** H = 67.6369, df = 9, P = <0.001; **B)** $\boldsymbol{I_{MD}}$**:** H = 62.672, df = 9, P = <0.001.

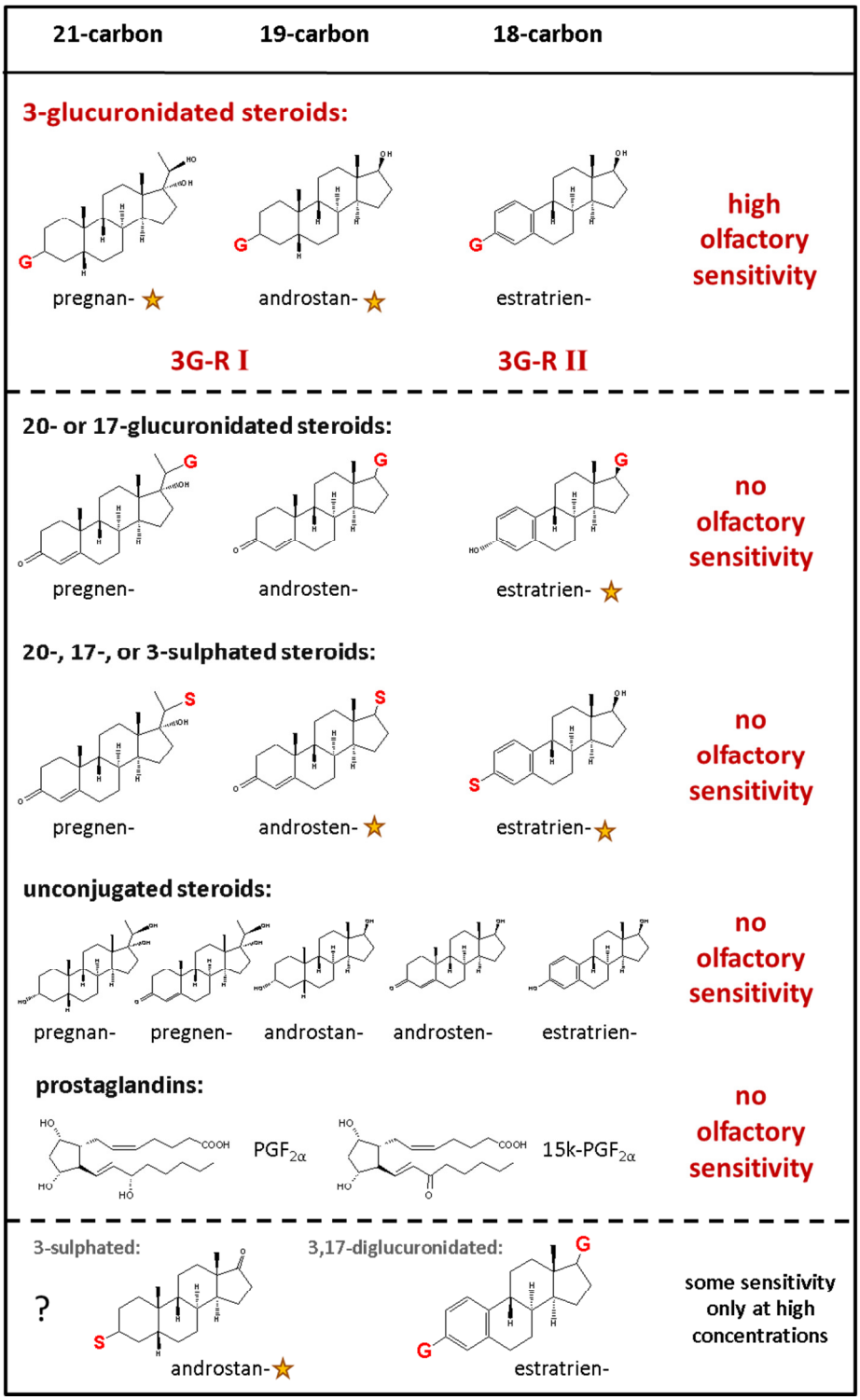

Figure 6 | Summary of olfactory sensitivity and receptor specificity to steroids in *O. mossambicus*. Red letters on steroid structures indicate conjugation position; G = glucuronate, S = sulphate. The Mozambique tilapia is highly sensitive to 3-glucuronidated steroids and cross-adaptation and binary mixture tests suggest two distinct olfactory receptor mechanisms 3G-R-I and 3G-R-II. It does not exhibit EOG responses to prostaglandins, unconjugated, 17- or 20-conjugated steroids or E2-3-S (this study, Frade and others 2002). Yellow stars indicate steroids detected by *Astatotilapia burtoni* (Cole and Stacey 2006).

Chapter V

Diplomacy is complex: the aggression-reducing signal from male tilapia urine is a multicomponent pheromone

This chapter investigated further the pheromonal function of the steroid 3-glucuronides that were identified as sex pheromone for females from the urine of dominant male tilapia in chapter III. It specifically addresses the question, if these two urinary steroids are also the major constituents mediating male-male aggression.

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Diplomacy is complex: the aggression-reducing signal from male tilapia urine is a multicomponent pheromone

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Abstract

Males often use scents to signal their social rank and mediate aggressive and breeding behaviours. In fish, however, the chemistry of dominance interactions still needs to be elucidated. Male Mozambique tilapia (*Oreochromis mossambicus*) use urine to signal social status, reduce aggression, and prime females to spawn. The urinary sex pheromone directed at females consists of 5β-pregnan-3α,17α,20β-triol 3-glucuronate and its α-epimer and their concentration is positively correlated with the social rank of the donor. The current study tested whether these pregnanetriol 3-glucuronates can modulate male-male aggression. Males were allowed to fight their mirror image and were either offered a water control or a chemical stimulus – dominant male urine (or fractions) or a mixture of the two steroids. Control males mounted an increasingly aggressive fight against their image over time. However, urine reduced this aggressive response. The two urinary pregnanetriol 3-glucuronates did not replicate the effect of whole urine. Neither did the C18 solid phase urine extract (containing the pregnanetriol 3-glucuronates) alone or the C18 solid phase urine flow-through to which the two pregnanetriol 3-glucuronates were added. Only reconstitution of both urine fractions (extract and flow-through) restored the aggression-reducing effect of whole urine. Olfactory activity was present in the urine extract and the hydrophilic flow-through fraction. We conclude that pregnanetriol 3-glucuronates alone have no effect on aggression and that the urinary signal driving off male competition is a multi-component pheromone, present both in the hydrophobic and hydrophilic urine components. Further investigations are needed to unravel the chemical code of inter-male diplomacy.

Key words | social behaviour, aggression, chemical communication, pheromone, urine, mirror, fish, *Oreochromis mossambicus*

Running title | Multi-component pheromone reduces aggression in tilapia

Introduction

Males of many animals, including several freshwater fishes, use scents to signal their social rank and mediate aggressive and breeding behaviours (Appelt and Sorensen 2007; Barata and others 2008; Barata and others 2007; Keller-Costa and others 2014; Keller-Costa and others 2012; Martinovic-Weigelt and others 2012; Maruska and Fernald 2012; Rosenthal and others 2011). These cues are often tactically released pheromones but research in fishes has mainly focussed on the role of reproductive or sex pheromones (reviewed by Stacey and Sorensen 2005). Fishes with complex social structures also actively release chemical cues to advertise social status and mediate aggression, and thus contribute to the hierarchical stability of a group. Recent studies on African cichlids (Barata and others 2007; Maruska and Fernald 2012) and fathead minnow (Martinovic-Weigelt and others 2012) provided evidence for the existence of such putative dominance pheromones. However, the identity and complexity of these signals, as well as their regulation and precise action, remains largely unknown.

Mozambique tilapia (*Oreochromis mossambicus*) males use chemical cues in their urine for multiple purposes (Barata and others submitted manuscript; Keller-Costa and others 2014) and are therefore a useful model to study urine-derived pheromones. In their natural habitat, Mozambique tilapia males establish social hierarchies in leks and aggressively defend a territory in the centre, while visiting females mate preferentially with the dominant males (Bruton and Boltt 1975). Male urine is a potent olfactory stimulus for conspecifics (Frade and others 2002) and the olfactory activity of urine increases with ascending social rank of the donor (Barata and others 2008). The muscular wall of the urinary bladder of dominant males is much thicker than that of subordinates (Keller-Costa and others 2012), which allows them to store large quantities of urine and control its release (Barata and others 2007). Dominant males tactically increase their urination frequency when courting females (Barata and others 2008) and the exposure of females to male urine stimulates the endocrine system to produce the oocyte maturation-inducing hormone 17,20β-dihydroxypregn-4-en-3-one (17,20β-P; Huertas and others 2014). Recently, we have 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 a sex pheromone present at high concentrations in male urine which stimulates the reproductive axis of females (Keller-Costa and others 2014). Moreover, male urine also has an immediate effect on the mating behaviour of the opposite sex; females ready to spawn clearly prefer to lay their eggs in the vicinity of males and nesting sites scented with dominant male urine (Barata and others submitted manuscript).

Dominant male tilapia also increase their urination frequency during aggressive disputes with rivals. In contrast, a male never releases urine when submissive to its opponent (Barata and others 2007). In male pairs wherein urination is prevented by constriction of the genital papillae, interactions escalate more frequently and rapidly into highly aggressive behaviours and escalating

fights than in controls (Keller-Costa and others 2012). Also, in control pairs, the male displaying the first aggressive behaviour usually wins the subsequent fight, which is not the case in male pairs prevented from urinating (Keller-Costa and others 2012). This strongly suggests that male urine contains chemical information about the sender's social rank, which likely dissuades males from engaging in costly energy-demanding escalating battles (Ros and others 2006) if they judge their rival to be of higher rank. Likely, the dominance signal in male urine 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. The aggression-reducing effect of dominant male urine has been recently investigated using a mirror assay (Barata and others submitted manuscript). Fishes are unable to recognize their own mirror image and attack it as if it was a rival and engage in escalating fights (Dijkstra and others 2012; Oliveira and others 2005). However, if dominant male urine is introduced next to the mirror, males' aggression towards their own image significantly decreases. In contrast, the addition of urine from subordinates has the reverse effect, amplifying aggression (Barata and others submitted manuscript). While these experiments demonstrate an aggression-reducing effect of male urine, the compounds involved are still unknown.

Here, we test the hypothesis that the two pregnanetriol 3-glucuronates, previously identified in dominant male urine as a sex pheromone for females, may serve a second function as a mediator of male aggression. For that purpose **1)** the pregnanetriol 3-glucuronates, dominant male urine, C18-cartridge urine extract, C18-cartridge urine flow-through and their combinations were applied in the mirror test and behaviours quantified, and **2)** the relationship between dominance status and olfactory sensitivity to urine and C18-cartridge urine extract and flow-through was assessed using the electro-olfactogram.

Materials and methods

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). Sexually mature males and females were kept together in 500 l stock tanks with a sandy bottom in aerated recirculating freshwater at 25 – 27 °C and 12L: 12D photoperiod until used either to setup social groups for urine collection or in behavioural or electrophysiological studies. Fish were fed once per day with commercial cichlid food pellets.

Assessment of social status and collection of urine

Each social group consisted of five females and five males and males were of similar standard length (SL, mm) and body weight (BW in g; coefficient of variation of BW < 5%). Males were colour tagged (T-Bar anchor FD94, Floy Tag Inc., Seattle, WA, USA) for systematic daily focal observation of their behaviour as previously described (Barata and others 2007; Keller-Costa and others 2012). A daily and 5-days average dominance index (DI), ranging from zero to one, was calculated for each male as the ratio between the sum of dominant behaviours and the sum of dominant and subordinate behaviours (Barata and others 2007; Keller-Costa and others 2012). Urine was collected from each male after each daily observation by gently squeezing the area above and anterior to the urogenital papilla and stored at -20°C until use.

Preparation of stimuli for the mirror assay

The following stimuli (Table 1) were prepared for the mirror assay (see below):

1) Water control;

2) Synthetic steroid mixture consisting of a 0.5 mM solution of a 4:1 mixture of 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 previously used on females (Keller-Costa and others 2014);

3) Dominant male urine (DMU) collected from 30 mature dominant males (BW = 81.8 ± 40.5 g, mean ± SD) with a 5-days average dominance-index greater than 0.5; a total of 30 ml urine was collected and partitioned into 1 ml aliquots;

4) DMU extract, the solid-phase-extraction (SPE; 500 mg C18 sorbent cartridges, 6 ml glass reservoir, Isolute®, Biotage) methanol eluate of a 34 ml pool of urine collected from 18 mature dominant males (BW = 194.8 ± 58 g, mean ± SD)

5) DMU aqueous flow-through from **(4)** plus synthetic steroid mix as in **(2)**;
6) DMU extract **(4)** plus DMU flow-through derived from **(4)**.
The non-polar (steroid containing) urine fraction (DMU extract, **4**) was eluted with a volume of methanol equal to the extracted urine volume (i.e. per 500 mg C18 sorbent, 5 ml of urine were passed through the cartridge and then eluted with 5 ml methanol). Both flow-through and the extract were each portioned into 1 ml aliquots in glass vials and all samples stored at -20 °C until the mirror assay. Immediately before the start of a behavioural trial, an aliquot was thawed and diluted 1:100 v/v in water, which was collected from the recirculating assay system at an outlet after the filter passage. The same water was also used as the water control stimulus (**2**).

Behavioural assays

Males for the behavioural assays were of similar size (Table 1) and different individuals from the urine donor males, and each performed the mirror trial only once. Before being transferred to the mirror aquarium, each male was housed for seven days together with a female-only group (3-5 individuals) in a 200 l tank to minimize possible effects from previous intra-sexual competitions. All males were reproductively active and spawning occurred spontaneously but eggs were removed from the female's mouth to stimulate the initiation of a new ovulatory cycle. The mirror assay was based on a previously described assay (Barata and others submitted manuscript), but used eight glass aquaria (39 x 26 x 29 cm; ca. 29 l) disposed in line and connected to a closed water circuit (Figure 1). De-chlorinated tap-water at 27 ºC 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 seven days to further standardize the (social) environment before testing and concede habituation to the test environment. This set-up allowed testing eight males consecutively on the same day without moving or disturbing the animals before the assay begun. At the start of the assay, the water inlet was closed and the sliding plate lifted, exposing the mirror. Each male was given up to 20 min to approach the mirror and/or show a first reaction towards it. Immediately after the first approach/reaction, the chemical stimulus was applied via a silicon tube and peristaltic pump, close to the mirror, during one minute, in pulses (20 $ml.min^{-1}$), followed by a one minute interval; this was repeated 5 times over a 10 min period. Male behaviour was recorded by a digital camera for 15 min after starting stimulus delivery. The experimenter was not visible to the male, but he/she was able to follow the fish's behaviour 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 twelve valid replicates (i.e. males showing an aggressive response to their mirror image) were obtained for each stimulus type.

Male behaviours recorded on digital video were quantified using The Observer XT software v. 8 (Noldus Information Technologies, The Netherlands). Behaviours and percentage of time in each of the following states were scored: *non-aggressive* (hovering in the water column, fins not erect, grey colour), *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* (circling fight; duration of consecutive mirror bites). In addition, mirror bites per min were counted (as single events). One-Way ANOVAs followed by the post-hoc Tukey-tests were used to compare the effect of the different stimuli on total number of mouth attacks, latency until the first mouth attack and total percentage of time dedicated to each state of aggression. Two-Way Repeated Measures ANOVAs followed by post-hoc Tukey-tests were used to compare the effects of the different chemical stimuli and time on aggressive behaviours.

Electro-olfactogram (EOG) recordings

Preparation of animals and recording of the EOG was carried out as previously described in detail for tilapia (Frade and others 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 1322A, 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 samples (pooled over five observation days) collected from dominant (DI $\geq$ 0.8; N = 6; mean ± SD, BW = 150 ± 31 g; SL = 168 ± 11.7 mm;), intermediate (DI $\geq$ 0.2 but $\leq$ 0.5; N = 5; BW = 156 ± 26.9 g; SL = 171 ± 12.3 mm) and subordinate (DI $\leq$ 0.16; N = 6; 150 ± 42.5g; SL = 170 ± 13.5 mm) males, as well as of the respective C18-cartridge urinary extracts and flow-through samples was assessed. Urine donors 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 on three adult males (mean ± SD; BW = 157.9 ± 19.1 g) and three females (BW = 110.3 ± 15.4 g) at a dilution of 1:10,000 in water (v/v). EOG data normalised to 10^{-5} M L-Serine standard and then log(y+1)-transformed, in which y is the normalized EOG amplitude, and dominance indices (DI) were arcsin-transformed. Linear regressions of EOG amplitudes on DIs were performed on pooled data from both sexes, since EOG amplitudes of male and female responses were statistically similar. One way ANOVA followed by the post-hoc Tukey-test was used to compare slopes and elevations of regression lines. Data were normally distributed and of equal variance.

Results

Male aggressive behaviour

In total, 176 males were exposed to their mirror image (Figure 1) but only 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 steroid mixture) showed clear mating behaviour (i.e. courtship, nest digging, deep black colouration) instead of aggression during the entire observation period, and were excluded from further analysis. Males reacting aggressively, usually first approached the mirror with slow and careful movements and an inconspicuous light grey colouration. At the mirror, some males immediately erected their fins and stated themselves lateral to the image, exhibiting a posture of low aggression. Progressively, their colour changed into darker shades of grey and they would exhibit lateral and frontal displays which with time may descend into high aggression. Other males instead first spent several seconds to 1-2 min exploring the mirror, swimming up and down and along it while touching it gently with their mouth closed, before they would assume colour changes and a similar behaviour pattern as described above.

The various stimuli affected aggressiveness of receiver males differently. However, latencies (Table 1) and aggressive behaviours within the first five minutes of observation were similar among all different stimuli (Figure 2). In DMU exposed males, the percentage of time allocated to the different behavioural states (Figure 2 A, C, E) and the frequency of mouth attacks (Figure 2 G), remained relatively constant over time, whereas in water control exposed males, non- and low-aggression behaviours decreased, and highly aggressive behaviours increased (Figure 2 A and E). Moreover, males spent in total less time on being highly aggressive when exposed to DMU (mean ± SEM; 11.4 ± 4.6 %) as compared to the synthetic steroid mix (34.4 ± 8.3 %) or water control (25 ± 3.3 %; One-Way ANOVA, $F_{2,29} = 4.239$, $P = 0.024$). Males exposed to synthetic steroid mix had a significantly higher number of mouth attacks towards the mirror (mean ± SEM, 181 ± 43; $P = 0.02$) than DMU exposed males (59 ± 23). Also, the percentage of time allocated to non- and low-aggression behaviours was overall lower in synthetic steroid 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 behaviours and frequency of mouth attacks increased with time (Figure 2 E and G). However, there was no significant difference in agonistic displays between water control males and DMU or steroid exposed males, although the *P*-value ($P = 0.054$) was at the limit of the significance level (5 %).

Because aggression was not reduced by the synthetic steroid mix alone, we investigated whether additional components in dominant male urine are necessary to elicit the aggression-reducing effect. Indeed, the DMU extract combined with the DMU flow-through (representing reconstituted DMU urine) had the lowest number of mouth attacks (Table 1) while the overall

pattern of aggressive behaviour was relatively similar to that of DMU alone (Figure 2). In contrast, males exposed with only the DMU extract or the DMU flow-through combined with the steroids spent in total significantly more time being highly aggressive (One Way ANOVA, $F_{2,31}$ = 6.13, P = 0.006), and also had a higher frequency of mouth attacks during the last 10 minutes of observation (P = 0.003, Figure 2 H), than males exposed to DMU extract combined with DMU flow-through.

Olfactory responses to male urine and its C18-SPE fractions

Male urine was, as expected, the most potent olfactory stimulus, followed by urine extract and, to a smaller extent, the urine flow-through samples (Figure 3 A). The olfactory potency of all the three stimuli increased with ascending social ranks of the donor males (Figure 3 B), but this increase was less pronounced in the male urine flow-through than in raw urine or urine extract, as revealed by significantly different slopes of regression lines ($P < 0.05$; Figure 3 B). No difference was found between the EOG responses of the two sexes to any of the stimuli.

Discussion

The present study shows that, consistent with previous work (Barata et al., submitted manuscript), male tilapia exposed to DMU are less aggressive towards their mirror image. It also shows that a steroid mix composed of the previously identified male tilapia pheromone (a natural constituent of DMU extract) by itself is not enough to restore the aggression-reducing properties of DMU, contrasting our initial hypothesis. Furthermore, neither the DMU extract on its own, nor the DMU flow-through combined with the synthetic steroid mix of pregnanetriol 3-glucuronates reduced aggressive escalation towards the mirror. Only reconstituted DMU (i.e. DMU extract and DMU flow-through combined) clearly reduced aggression.

Although statistical significance was not reached, DMU- and reconstituted DMU-stimulated males had by tendency higher latencies until the first mouth-attack while control water stimulated males tended to have the shortest latencies. This is in accordance with our previous study showing that urogenital papillae-tied male pairs prevented from urine release engage significantly faster in highly aggressive fighting than unrestricted, urinating controls (Keller-Costa et al., 2012). Males stimulated with the steroid mix and/or only one fraction of DMU had somewhat medium latencies until the first mouth-attack in this study. It may suggest that these males perceived and processed the chemical signal in some way but the message was incomplete and could not be fully interpreted.

Interestingly, males receiving the synthetic steroid mixture tended to show the highest increase in number of mouth attacks over time as compared to all other stimuli. Maybe, when only synthetic steroids were introduced, the males did not sense the stimulus as coming from another competitor, but instead, as their own chemical signal that has become even more 'powerful' due to a higher total steroid concentration in the water. One could speculate that this gave the steroid-stimulated males an even higher resource holding potential and so explain the tendency for more mouth-attacks. A mechanism must exist by which a male distinguishes his rival's chemical signal from its own during encounter. One possibility is that other identifying chemicals are released by males in concert with the steroid 3-glucuronates which allows males to subtract their own individual chemical message from their rivals'. Clearly, there is great potential for future studies to test these hypotheses and gain understanding on how complex chemical/olfactory information is integrated in the brain.

Altogether, our results strongly suggest that multiple urinary components are involved in the modulation of male aggression**.** Often, pheromones effecting animal behaviour are odour 'bouquets' rather than only one or two substance(s) (Wyatt 2003d). When the multiple pheromonal constituents are combined in a particular ratio, they then act in synergy. For example, 2,3-dehydroxy-exo-brevicomin and 2-sec-butyl-4,5-dohydrothiazole in male mice (*Mus*

musculus) urine act synergistically and challenge aggression in receiver males. But they are inactive on their own (i.e. when simply added to the water) and become only active when added to urine of castrated animals (which, in turn, is inactive alone; Novotny and others 1985).

Both male and female tilapia have olfactory sensitivity to the hydrophilic DMU flow-through and responses were positively correlated with the donor's social status. Moreover, although most olfactory activity in the DMU extract is attributed to the pregnanetriol 3-glucuronates (Keller-Costa and others 2014), there is evidence that other compounds are present in the DMU extract, eliciting olfactory responses as well (unpublished observations). Those additional compounds present in the DMU extract and the hydrophilic odorants present in the DMU flow-through are possibly 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. So far, the only evidence that hydrophilic urinary components – likely trimethylamine – could play a role in the communication of social status comes from a report in the fathead minnow (Martinovic-Weigelt and others 2012). However, small major histocompatibility complex (MHC) peptides are potent odours which occur in body fluids including urine and have been shown to influence mate choice decision in stickleback (Milinski and others 2005; Milinski and others 2010) and olfactory imprinting on kin in zebrafish (Hinz and others 2013). The polar fraction of ovulated female carp holding water synergizes the attracting effect of prostaglandin to males; these polar products may confer species-specific information and amino acids as putative constituents were hypothesised (Lim and Sorensen 2011). Species-specific polar metabolites have been similarly discussed also for the goldfish (Levesque and others 2011). All fishes possess broad olfactory sensitivity to amino-acids and several can be detected in the hydrophilic urinary flow-through fraction of *O. mossambicus* males (unpublished observations). Further research is clearly necessary to shed light on the identity and function of additional chemical cues released into the urine of tilapia males. Future investigations should also clarify whether the function of two identified pregnanetriol 3-glucuronates is restricted to its priming role in females and their presence in the urine is irrelevant to male aggressiveness, or if they still play a role but as discussed here in a blend with additional, as yet unidentified, compounds. The tight positive correlation of the urinary concentration of these steroids to the social rank of the donor male (Keller-Costa and others 2014) suggests the latter. Therefore, we would hypothesize that additional urine signals act, when combined at a certain ratio, in synergy with the previously identified pregnanetriol 3-glucuronates to influence inter-male aggression in *O. mossambicus.*

In this study, not all the test individuals reacted to their image on the mirror. Similar observations were made previously from African cichlids (Barata and others submitted manuscript; Dijkstra and others 2012), although the reason remains unclear and could be manifold. It may perhaps echo a lower aggressive motivation, a different stress coping style

(Øverli and others 2004) or 'shyness' (Coleman and Wilson 1998; Wilson and others 1993). Interestingly, 45 % of males exposed with control water showed clear mating behaviour (courtship, nest digging, black colouration) towards the mirror. Courting behaviour 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 1998b). In circumstances of high competition, dominant males are more likely to attract any neutral or light coloured individual that looks like a potential mate and leave discrimination to a later stage (Oliveira and Almada 1998b). 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 an unsuspicious light grey (female-like) coloration before changing to a darker shade. In contrast, courting behaviour was rarely observed in males exposed to the synthetic steroid mix and never in males exposed to male urine or urinary fractions. This underpins the emerging evidence that chemical cues facilitate discrimination of conspecifics and their interactions in this cichlid (Almeida and others 2005; Barata and others 2008; Barata and others 2007; Keller-Costa and others 2012; Miranda and others 2005).

We conclude that the two urine-derived pregnanetriol 3-glucuronates, a sex pheromone stimulating the female reproductive system, are not sufficient to reduce male-male aggression. The urinary signal mediating inter-male aggression is most likely a multi-component pheromone comprised of compounds from both polar and non-polar urine fractions. Thus, male-male aggression is modulated by a chemical signal different from that of ovulation priming in females.

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Tables

Table 1

Stimulus	*N* Males[1]	SL (mm)[2]	BW (g)[3]	Latency MA (min)[4]	Total *N* MA[5]	
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 ab	Group 1
Steroids	10	119 ± 6.8	43.3 ± 4.0	5.4 ± 1.8	181 ± 43 b	
DMU extract	12	112 ± 3.2	40.5 ± 2.5	4.4 ± 1.5	121 ± 30 b	
Steroids + DMU flow-thr.	11	112 ± 3.8	43.6 ± 4.1	4.3 ± 1.2	123 ± 19 b	Group 2
DMU extract + flow-thr.	11	108 ± 3.1	--	8.5 ± 1.8	29 ± 12 a	

[1] number of male replicates [2] standard body length [3] body weight
[4] latency until first mouth attack; ANOVA; Group 1: $P = 0.196$; Group 2: $P = 0.100$, no significance.
[5] total number of mouth attacks during experiment; letters (a,b) behind values indicate significant differences; ANOVA followed by Tukey-test; Group 1: $F_{2,29} = 4.511$, $P = 0.02$; Group 2: $F_{2,31} = 5.706$, $P = 0.008$.
DMU = dominant male urine DMU flow-thr. = C18-SPE flow through of dominant male urine

Figures and figure legends

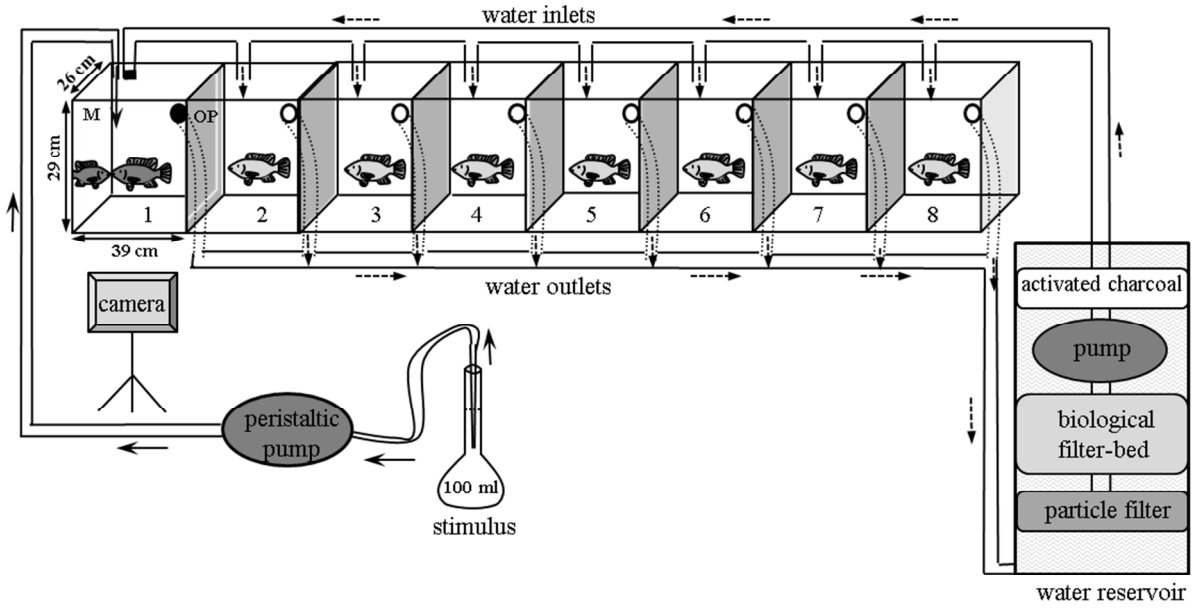

Figure 1 | Experimental set-up to study stimulus dependent mirror-elicited behaviour in male tilapia. Eight aquaria, connected to a recirculating freshwater system, allowed consecutive testing of eight male replicates at the same day. Males were socially isolated in their aquaria without visual contact to each other for seven days 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 minutes 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 pulses, five times for one minute at one minute intervals. The animal's behaviour was video recorded for 15 min.

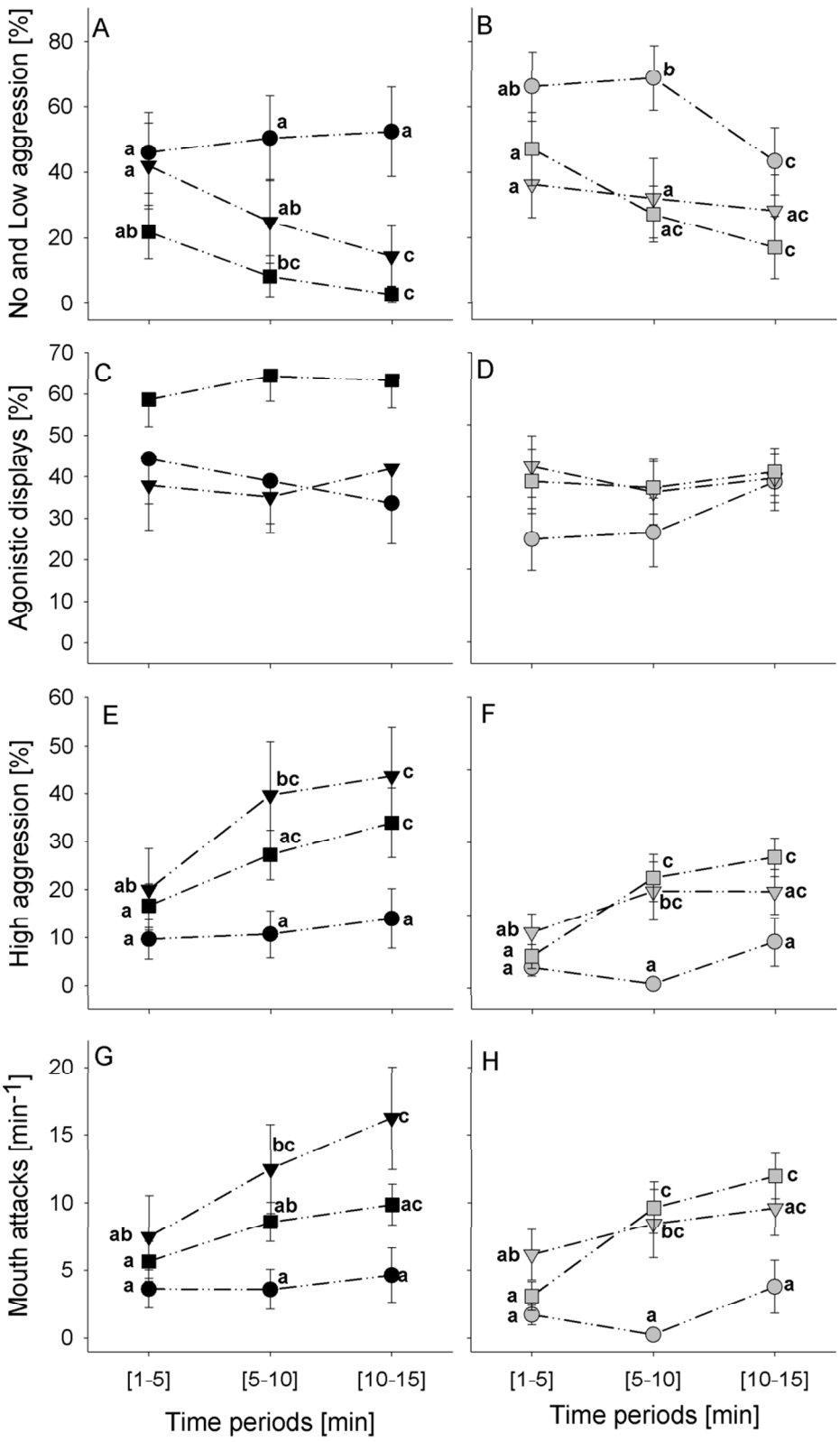

Figure 2 | Development of aggressive behaviours of receiver males over time. Relative duration of not or lowly aggressive **(A, B)**, agonistic display **(C, D)** and highly aggressive **(E, F)**

states, and frequency of mouth attacks **(G, H)** of males exposed to their mirror image and exposed to the synthetic steroid 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-cartridge urine extract (open triangles, N = 12), C18-cartridge urine flow-through plus synthetic steroid mix (open squares, N = 11), or reconstituted DMU, i.e. extract plus flow-through (open circles, N = 11) **(B, D, F, H**). All values are means ± SEM of time (%) or frequency (min^{-1}) observed in each of the three 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 - $F_{2,29}$ = 3.952, P = 0.003; time - $F_{2,29}$ = 5.703, P = 0.005; interaction - $F_{4,58}$ = 3.12, P = 0.022; **(B)** stimulus - not significant n.s.; time - $F_{2,31}$ = 9.44, P < 0.001; interaction - n.s.; **(C, D)** n.s.; **(E)** stimulus - $F_{2,29}$ = 4.275, P = 0.024; time - $F_{2,29}$ = 6.132, P = 0.004; interaction - n.s.; **(F)** stimulus - $F_{2,31}$ = 4.724, P = 0.016; time - $F_{2,31}$ = 13.914, P < 0.001; interaction - $F_{4,62}$ = 4.135, P = 0.005; **(G)** stimulus - $F_{2,29}$ = 4.511, P = 0.02; time - $F_{2,29}$ = 7.397, P = 0.001; interaction - n.s.; **(H)** stimulus - $F_{2,31}$ = 5.706, P = 0.008; time - $F_{2,31}$ = 15.13, P < 0.001; interaction - $F_{4,62}$ = 4.314, P = 0.004.

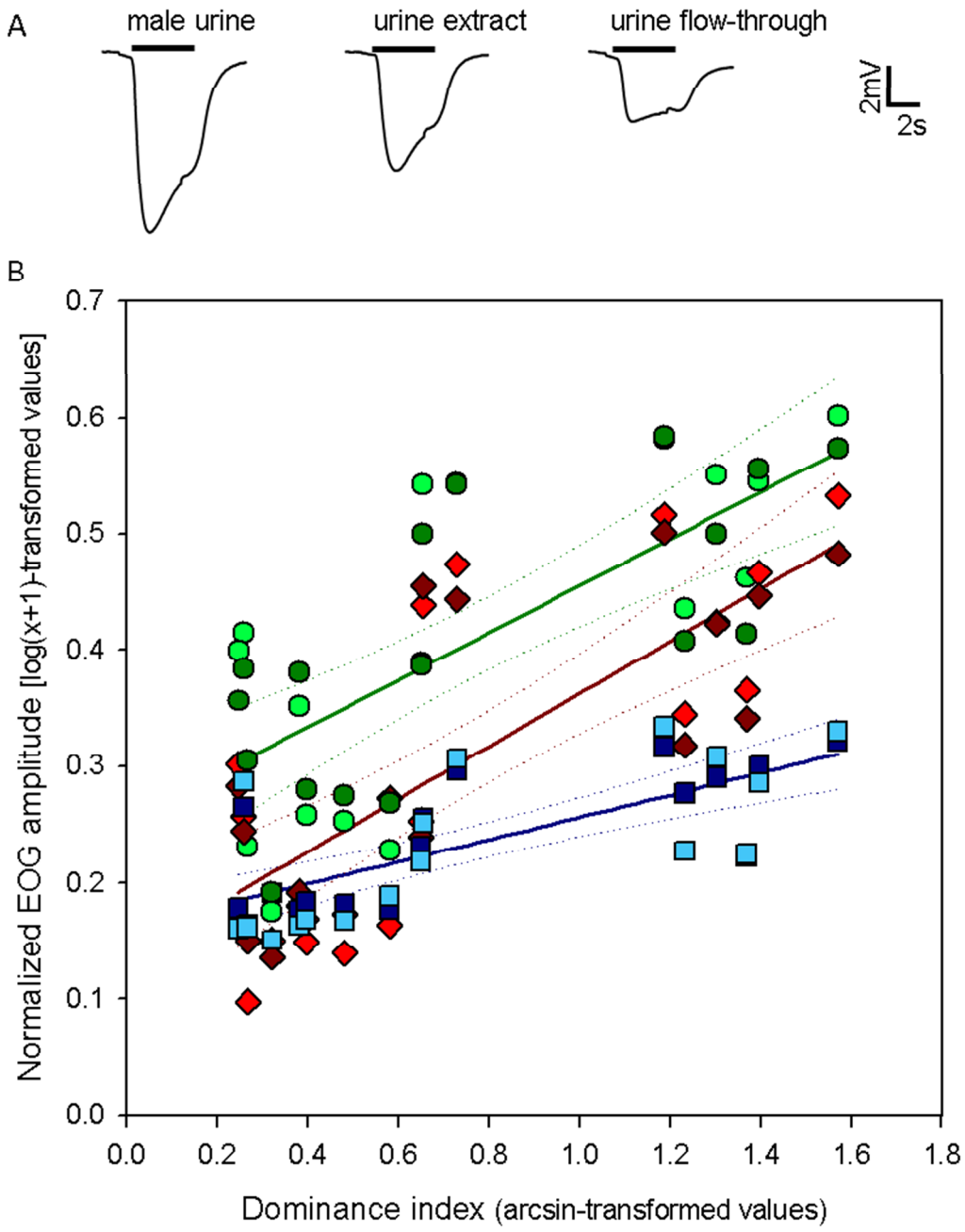

Figure 3 | Olfactory responses to male urine and its C18-SPE fractions. Typical electro-olfactograms (**A**) recorded in response to a urine sample and its C18-cartridge urine extract and C18-cartridge urine flow-through from a dominant donor male at 1:10,000 v/v dilution in water. Mean EOG responses (**B**) normalised to 10^{-5} M L-serine standard (log(y+1)-transformed) of males (dark symbols; *N* = 3) and females (light symbols; *N* = 3) to urine samples (green circles) and corresponding urine extracts (red diamonds) and flow-through samples (blue squares) from tilapia males (*N* = 17) of different social rank (all samples at 1:10,000 v/v dilution). EOG responses are plotted over the donor male`s social status (expressed as dominance index DI in arcsin-transformed values). Linear regression analysis was performed on pooled data from both sexes

(solid lines and 95% CI dashed lines). Urine [green lines, $R^2 = 0.53 \pm 0.09$ (±SE), $F_{1,32} = 35.5$, $P < 0.001$, log(EOG+1) = 0.25 (±0.03) + (0.20 (±0.03) x arcsin-DI)] was the most potent stimulus followed by the extract [red lines, $R^2 = 0.59 \pm 0.09$, $F_{1,32} = 46.0$, $P < 0.001$, log(EOG+1) = 0.14 (±0.03) + (0.23 (±0.03) x arcsin-DI)] with regression lines of similar slopes but of significantly different elevations ($P < 0.01$). The urine flow-through [blue lines, $R^2 = 0.52 \pm 0.04$, $F_{1,32} = 35.1$, $P < 0.001$, log(EOG+1) = 0.16 (±0.01) + (0.10 (±0.02) x arcsin-DI)] also elicits olfactory responses although it was the least potent stimulus with a regression line of lower slope ($P < 0.05$) and different elevation ($P < 0.001$) than urine and extract.

Chapter VI

General discussion, conclusions and future directions

This chapter integrates the major findings from the individual chapters into a final discussion and overall conclusion. It also delineates unanswered questions giving an outlook into future research.

General Discussion and future directions

1. General Discussion

1.1 The Mozambique tilapia – a new, promising model in fish pheromone research

The results of this thesis establish the Mozambique tilapia as the fourth species, after goldfish (Dulka and others 1987), sea lamprey (Li and others 2002; Sorensen and others 2005) and masu salmon (Yambe and others 2006) for which the identity of pheromone signals, release route and mechanism, olfactory perception and biological response have been unveiled. The 'tilapia journey' started twelve years ago, when it was shown that holding water and body fluids, especially male urine, were strong olfactory stimuli for conspecific females (Frade and others 2002). Three years later, it was found that males increase the urination frequency in the presence of females, and even more drastically when the female was close to ovulation. EOG recordings confirmed that males are indeed able to distinguish pre- from post-spawn females by their sense of smell (Almeida and others 2005; Miranda and others 2005). Along with emerging proof for male-female chemical communication, evidence arose for urine-derived chemical signals mediating male-male aggressive encounters (Barata and others 2007). A first attempt to track down the chemical identity of the involved compounds pointed towards a sterol-like odorant present in the 'less-polar' urine extract (Barata and others 2008). Now, six years later, we have unraveled this odorant to consist of a mixture of 5β-pregnane-3α,17α,20β-triol 3-glucuronate and its 20α-epimer. We suggest that they act via a common receptor mechanism highly specific for the detection of 5β,3α-reduced steroid 3-glucuronates. Thus, we have also resolved the riddle of what types of hormonal steroids the Mozambique tilapia smells. Meanwhile, multiple pheromonal functions were assigned to urine from dominant males, such as reduction of male aggression, attraction of females to nesting sites (Barata and others submitted manuscript), and stimulation of the females' reproductive system, leading to an increased release of the maturation-inducing steroid 17,20β-P (Huertas and others 2014). For the latter, we were able to demonstrate that the two identified pregnanetriol 3-glucuronates mimic this effect, establishing their role as male sex pheromone. In contrast, the aggression-reducing effect of dominant male urine cannot be attributed to these steroids (at least not alone), and behavioural experiments with different urine fractions suggest that multiple, likely hydrophilic, compounds must be involved. From the work herein presented, we also conclude that the ability of dominant males to store large urine volumes over long periods is linked to a more muscular urinary bladder and not to higher urine production rates *per se*. Such a morphological adaptation shows that the differential urination pattern and storage ability of dominant males comes indeed with an energetic cost. Last, but not least, we found that Mozambique tilapia possesses (a) specific olfactory receptor mechanism(s) for

estradiol 3-glucuronate of high sensitivity, in the picomolar range. This compound may explain the olfactory preference of males for pre-ovulatory over post-spawn females and function as putative female pheromone; something to be examined in the future.

Table 1 | Comparison between the sex pheromone systems of lamprey, goldfish, masu salmon and tilapia.

		Ancestral fish		Teleosts	
		Sea lamprey[a] *Petromyzon marinus*	**Goldfish**[b] *Carassius auratus*	**Masu salmon**[c] *Oncorhynchus masou*	**Mozam. tilapia** *Oreochromis mossambicus*
reproductive pheromones	territoriality	yes	no	yes	yes
	direction	male → female	female → male	female → male	male → female
	compound class	C24 bile acid	C19 & C21 hormonal steroids C20 prostaglandins	C10 amino acid	C21 hormonal steroid metabolites
	name of chemical(s)	3K-PS	AD 17,20β-P 17,20β-P-S $PGF_{2\alpha}$ 15K-$PGF_{2\alpha}$	L-kynurenine	20α-P-3-G 20β-P-3-G
	site of release	gills	gills & urine	urine	urine
	producing organ	liver	gonads	not clear	likely gonads
	detection threshold (EOG)	10^{-12} - 10^{-10} M	10^{-12} - 10^{-10} M	10^{-14} M	10^{-9} M
	signaling mechanism	passive	passive or active advertisement	not clear	active advertisement
	response (primer effect)	sexual maturation	sperm & seminal fluid production	plasma 17,20β-P increase	oocyte maturation
	response (releaser effect)	search behavior attraction nesting behaviour	arousal sexual behaviour	attraction	not clear yet attraction (?)

a (Chung-Davidson and others 2013; Johnson and others 2009; Li and others 2002; Li and others 2003; Siefkes and Li 2004; Siefkes and others 2003; Siefkes and others 2005)
b (Appelt and Sorensen 2007; Bjerselius and others 1995a; Defraipont and Sorensen 1993; Dulka 1987; Sorensen and others 1988; Sorensen and others 1995; Stacey and others 1989)
c (Yambe 2008; Yambe and others 2006; Yambe and others 2008)

1.2 Four different model species – four different sex pheromonal systems

Comparing the tilapia, goldfish, masu salmon and lamprey pheromonal systems (Table 1), there is some degree of overlap, but many differences. Whereas sterols in general may be widely used as sex pheromones by fishes, the Agnatha use bile salts produced by the liver, and teleosts tend to use hormonal steroids and/or prostaglandins produced by the gonads and/or accessory glands (Stacey 2003; Stacey and others 2003), with masu salmon females releasing an amino acid as a (so far) notable exception.

Within the teleosts, the type of reproductive hormones used as pheromones varies among different species. In many cyprinids with a scramble-competition-type mating system, such as goldfish (Kobayashi and others 2002), common carp (Lim and Sorensen 2011) and crucian carp (Bjerselius and others 1995a), but also several salmonids (Laberge and Hara 2003a; Moore and Scott 1992; Moore and Waring 1996; Sveinsson and Hara 2000), the prevailing olfactory active or attracting pheromonal compounds are (F-type) prostaglandins and/or 17,20β-P and its immediate metabolite 17,20β-P-S. These compounds have direct endogenous functions in the sender and are either passively released through the gills (e.g. unconjugated steroids diffuse readily from the blood stream; Scott and others 2008) or are, with no or little modification, excreted via the urine (reviewed in Stacey and Sorensen 2002).

In mating strategies in which males compete for social dominance and territories and wherein female mate choice prevails, chemical communication systems seem to be more specialized and pheromones appear to be instead hormonal precursors or metabolites – derived from a supposedly specialized production route in the testes. 5β,3α-Reduced 3-glucuronidated steroids (pregnanes and androstanes) were shown to be either potent olfactory odorants (from EOG measurements) or behavioural stimuli (from attraction/preference tests). The African catfish, for example, produces several 3-glucuronidated steroids in its testes (especially in the seminal vesicles; accessory glandular organs at the posterior end of the testis) and shows high olfactory sensitivity to 20one-P-3-G; females are attracted to steroid glucuronide-containing extracts from the seminal vesicles (Resink and others 1987; Van den Hurk and others 1987). Further support, that 5β,3α reduced 3-glucuronidated, testis-derived steroids may act as male sex pheromone comes from the black and round gobies; ETIO-3-G, 11K-ETIO-3-G and related free and conjugated androstanes act as female attractants (Colombo and others 1980; Katare and others 2011) and potent olfactory stimuli (Murphy and others 2001). Analogous to gobies and African catfish, steroid glucuronates secreted during the breeding season by accessory testicular glands (blind pouches) have been hypothesized to act as hormonal pheromones in the peacock blenny (Salaria pavo; Lahnsteiner and others 1993). But - so far - only in the Mozambique tilapia were the release site and signaling mechanisms determined and a physiological (i.e. priming) effect on the receiver demonstrated for these compounds.

However, teleosts are a species rich taxon, with many different mating strategies and diverse reproductive behaviours. Although, gonad-derived steroids and prostaglandins are most likely the predominating compound classes functioning as reproductive pheromones in teleosts, other types of hormonal and non-hormonal sex pheromones are possible, as evidenced by L-kynurenine from the masu salmon. Also, non-hormonal sex pheromones seem to be released from the exocrine glands at the anal fin (anal glands) of spawning peacock blenny males to attract females to nests. These compounds are hydrophilic odorants of less than 500 Dalton and a mixture of amino acids and/or small peptides was eventually suggested (Serrano and others 2008). However, the need for coordination of reproduction and spawning synchronization in external fertilizers probably led to development of a tight link between the sexual maturation state and sex pheromone production/release, potentially conferring an important evolutionary advantage (confirmed at least in goldfish), and therefore a widespread trait.

Olfactory detection thresholds of lamprey and goldfish for 3-keto-petromyzonol 24-sulphate (3K-PS) and 17,20β-P, respectively, are markedly lower (10^{-12} M) than those of tilapia for 20β-P-3-G and 20α-P-3-G (10^{-9} M). The highly soluble lamprey pheromone has a large active space as it functions as an attractant in streams over long distances, i.e. > 650 m (Johnson and others 2009). On the other hand, the higher threshold for the tilapia sex pheromone may suggest that its active space is smaller and its priming function is most important in closer interactions when the female is already in the proximity to the nest. Whether the two pregnanetriol 3-glucuronates also attract females to nesting sites or stimulate sexual behaviours has not yet been investigated. According to the few studies in natural habitats, females usually shoal together away from territorial males and only enter leks for reproduction (Neil 1966). Thus, exogenous and endogenous signals inform females of where and when to enter spawning areas. Investigating the functions of sex pheromones in more natural settings/habitats beyond the laboratory could help to better understand their mechanisms of actions and evolutionary significance as well as relative importance in the context of other signals such as vision or sound, and influence of environmental factors.

1.3 Sex pheromones and species specificity

The previous sections illustrate how identical or similar hormonal compounds may function as sex pheromones in various teleost species. This is likely, because the number of hormones (and precursors and metabolites) as well as enzyme pathways to create hormone-derived pheromonal candidates is limited. This leads to several intriguing questions. Firstly, if many fishes can presumably be attracted or 'primed' by the same compounds, why or how they do not get 'confused' and assure species integrity over time? Or, looking at it from another angle, is interbreeding in closely related species facilitated by shared pheromone systems? Several factors that could prevent 'confusion' in natural environments are obvious; geographic or habitat

separation, or, in overlapping habitats, different reproductive seasons, biology and behaviours or varying diurnal patterns. Inputs from other sensory channels, such as vision and audition, may reinforce or validate the chemical message, at least for certain releaser pheromones. Priming effects, however, can occur independently from multimodal signals; the female tilapia responding to male urine or pregnanetriol 3-glucuronates in this study (chapter III) had neither visual nor acoustic contact with a male. It has been shown in several species that anosmia leads to reduced reproductive behaviour and gonad regression (de Souza and others 1998; Liley and others 1993; van Weerd and others 1990), emphasizing the predominant role of smell. However, phylogenetically very closely related (freshwater) fishes with similar reproductive biology frequently hybridize, as seen for instance in various cyprinid or tilapia species. In captivity and in nature the Mozambique tilapia mates easily with its allopatric close relative, the Nile tilapia (*Oreochromis niloticus*), despite the contrasting breeding colours of dominant males (courting Mozambique tilapia become black, whereas Nile tilapia become white). A comparative study revealed that dominant Nile tilapia males release the same pregnanetriol 3-glucuronates via their urine and possesses similar olfactory sensitivities to those two compounds as the Mozambique tilapia (Hubbard and others 2014). Frequent interbreeding occurs also between crucian carp (*Carassius carassius*) and goldfish (*Carassiu auratus*), two clear allopatric species, both using 17,20β-P as pre-ovulatory pheromone (Bjerselius and others 1995b; Dulka and others 1987). The question on species-specificity of hormonal pheromones was recently addressed in goldfish and common carp (*Cyprinus carpio*) (Lim and Sorensen 2011), also capable of interbreeding, yet less frequently than goldfish x crucian carp (Hänfling and others 2005). Common carp and goldfish females release the same types of prostaglandins as post-ovulatory pheromone, although at slightly different rates and ratios, and male common carp are attracted to the heterospecific prostaglandin mix just as well as to the conspecific mix. Together these findings point out three things: Firstly, (hormonal) sex pheromones in fishes are not necessarily species-specific signals. Secondly, observed interbreeding in closely related species is likely facilitated through similar pheromonal compounds. Thirdly, phylogenetically close but allopatric species may have had no selective pressure to diverge with regard to their pheromonal compounds in order to maintain species integrity.

Nevertheless, in the common carp (Lim and Sorensen 2011), the effect of the prostaglandins is synergized by other released (polar) substances which may confer species-specificity. Even though female goldfish holding water, when presented on its own, was attractive to carp males, when they had the choice between carp and goldfish female water, they chose conspecific water over heterospecific (Lim and Sorensen 2011). This suggests that fish can integrate complex odours conveying different information, and that species-specific odours released in concert with less-specific pheromonal compounds may provide the means to maintain species integrity in the

wild, given that access to conspecifics is not a limiting factor. Furthermore, it highlights the importance of studies in more complex settings, such as the natural habitat, as mentioned above.

Phylogenetically closely related sympatric species with similar reproductive strategies must have evolved a variety of mechanisms to avoid interbreeding in order to retain their genetic integrity. One such mechanism could be chemical communication. Sympatric African cichlids from the East African lakes provide an excellent model for investigation into this possibility. As discussed in chapter IV, in concert with their nuptial colours, chemical communication is likely to play an important role in reproduction. For example, females of the Malawi cichlid *Pseudotropheus emmiltos* (now *Maylandia emmiltos*) only show preference for spawning with conspecific males over the closely releated *P. fainzilberi* males if olfactory cues are present (Plenderleith and others 2005). Furthermore, the number and types of putative olfactory receptor mechanisms detecting conjugated steroids varies greatly among African cichlid genera (Norman Stacey, personal communication). A systematic comparison of chemical fingerprints from urine and/or holding water samples of several sympatric African cichlids, together with EOG-guided fractionation is a promising approach to obtain some insight into the presence and relative importance of putative signature- and/or pheromonal compounds.

1.4 The tilapia sex pheromone – an honest signal about the male's reproductive condition?

As mentioned above, it is likely that the pregnanetriol 3-glucuronates identified here are testis-derived. Tilapia do not possess accessory testicular glands, which are responsible for the production of steroid glucuronates in catfish and gobies. However, *in vitro* incubation experiments with steroid precursors have shown that, in fishes without additional reproductive glands, conjugation, including glucuronidation indeed occurs in the testis itself, especially in the interstitial Leydig cells (Kime and Hyder 1983; van den Hurk and Resink 1992). Incubation of testes derived from sexually mature Mozambique tilapia, with labelled (tritiated) testosterone at 22-30 °C (breeding temperature of this species), yields large amounts of testosterone- and androstane glucuronates (Kime and Hyder 1983).

Pregnanetriols are the result of an enzymatic reduction of the double bond in the A-ring, giving rise to 5β-reduced forms of progestagens (Scott and others 2010). 17,20β-P is the major progestagen in fish, mainly produced in the testis under the control of luteinising hormone (LH; Scott and others 2010). In male fish, 17,20β-P has been suggested to be involved in the onset of meiosis during spermatogenesis, and is able to enhance sperm motility and milt production. In some species, its epimer 17,20α-P was also identified, although no physiological role has yet been found (Scott and others 2010). 17,20β-P and 17,20α-P (including sulphate and glucuronate conjugates) are present at higher concentrations in the urine of dominant male Mozambique tilapia than in subordinates (Oliveira and others 1996). Interestingly, when males move from social isolation into social groups and become subordinate, their 1720β-P levels drop

significantly. Indeed, other studies with African cichlids have shown that long-term subordinate males possess smaller testis, fewer groups of Leydig cells and lower sperm motility, although spermatogenesis continues (Kustan and others 2012; Maruska and Fernald 2011; Oliveira and Almada 1999; Pfennig and others 2012). Thus, the high levels of 17,20β-P in dominant male urine are very likely a direct reflection of the male's sperm quality and reproductive ability. However, the olfactory epithelium of the Mozambique tilapia is insensitive to 17,20β-P, 17,20α-P and their conjugates (this study; Frade and others 2002). Metabolism of 17,20α-P and 17,20β-P into pregnanetriols and subsequent 3α-glucuronidation originates a water soluble signal which carried in the urine can be detected by the females. The increased availability of 17,20β-P during spermiation in dominant males may provide the substrate for the increased ratio of 20β-P-Gluc/20α-P-Gluc. Thus, it seems that the pregnanetriol 3-glucuronates from dominant males are an 'honest' and reliable signal, informing the female that the maturation-inducing steroid pathway has been activated and thus, about the male's reproductive potential. Clearly, this hypothesis should be tested in future studies focusing on the biosynthetic pathway of the pregnanetriol 3-glucuronates, their endogenous regulation and exogenous stimulation (see also next section on future directions V). If the substrate for the pregnanetriol 3-glucuronates is indeed 17,20α-P/17,20β-P this raises the question, why is tilapia not using 17,20β-P itself as a pheromone and why has it not evolved olfactory sensitivity to 17,20β-P conjugates instead? Clearly, much needs still to be learnt about the evolution of pheromones in teleosts.

A related, interesting aspect is the transport of the pregnanetriol 3-glucuronates into the urinary bladder. Assuming, that the pregnanetriol 3-glucuronates are produced in the testis, they should be carried to the bladder via the blood stream (unless there is an as yet uncovered direct link from the testis to the bladder). In the Mozambique tilapia, there are some indications that the very posterior testis end is attached to urinary bladder tissue, which makes castration without damaging the urinary bladder difficult in this species (Almeida and others 2014). Moreover, a portal system (blood vessels) leading from the testis to the urinary bladder is present (unpublished observations). Collection of male urine by catheterization of the urinary bladder drastically reduces the olfactory potency of urine and concentration of the pregnanetriol 3-glucuronates drops below HPLC-ELSD detection limit (personal observation). Thus, a thought-provoking hypothesis for future exploration is that the pheromone might enter the urine post renal formation and through a direct testis-bladder portal system.

2. Main conclusions

I. The ability of dominant males to store large urine volumes over long periods is facilitated by a more muscular urinary bladder but not linked to higher urine production rates *per se*.

II. The olfactory most active urine fraction of male tilapia consists of a mixture of 5β-pregnane-3α,17α,20β-triol 3-glucuronate and its 20α-epimer.

III. The two identified pregnanetriol 3-glucuronates act via a shared olfactory receptor mechanism and stimulate the endocrine axis of females, which promotes oocyte final maturation. This establishes their function as a sex pheromone that synchronizes spawning and increases thereby the reproductive success of the species.

IV. The Mozambique tilapia has evolved high olfactory sensitivity and specificity to 3-glucuronidated steroids through two distinct olfactory receptors; one detecting a male sex pheromone and a second detecting 17β-estradiol 3-glucuronide, a putative chemical signal from females.

V. Males can judge the social rank and/or aggressive motivation by smelling their opponents' urine. However, the aggression-reducing effect of dominant male urine cannot be attributed to the two pregnanetriol 3-glucuronates alone. Multiple, yet unidentified, likely also hydrophilic compounds are responsible for this effect.

VI. This thesis presents not only the first chemical identification of a cichlid sex pheromone from the first principle, but also the first sex pheromone from a teleost with a mating system in which territorial males signal to females, and that is strongly driven by female mate choice.

VII. Knowing now the chemical cues that mediate reproduction may provide a tool towards improvement of tilapia-culture and, even more importantly, control of invasive Mozambique tilapia populations in the future.

3. Future directions

This Ph.D. thesis certainly contributed to an advancement of our understanding on chemical signaling in the Mozambique tilapia. However, it also gave rise to a multitude of questions, some of which were already briefly addressed throughout the discussion. A selection of the five most important is summarized below.

I) Despite the 'priming effect', is there also a 'releaser effect' of the pregnanetriol 3-glucuronates in females? Tilapia females prefer to spawn in nesting sites scented with urine from dominant males over those scented with urine from subordinate males (Barata and others submitted manuscript). Are the two pregnanetriol 3-glucuronates the attractants responsible, or do they otherwise stimulate sexual behavior in females?

II) The two pregnanetriol 3-glucuronates identified in tilapia male urine as sex pheromones for females, are not, on their own, responsible for the aggression-reducing effect of dominant male urine. The question remains, then, what are the compounds involved? Do the pregnanetriol 3-glucuronates play a role at all in male-male encounters? EOG results suggest that polar odorants could be involved. Amino acids, mainly L-arginine and L-glutamate are present in dominant male urine and their concentrations are positively correlated with the social rank of the donor male (unpublished data). Could these amino acids play a role? Identification of male urinary odorants mediating male aggression is crucial and will give highly valuable insights into the role of pheromones in intra-sexual competition.

In addition to the observed immediate behavioural effect of dominant male urine on receiver males, there might also be a 'priming' effect on the endocrine system. Preliminary results from the mirror experiment showed that control-water stimulated males had lower levels of 11K-T in their urine after the challenge than 24 hours before. However, no reduction was observed in DMU stimulated males. Another pilot assay revealed that exposing males to DMU with neither a mirror image nor visual/ acoustic contact with another male increased 11K-T release to the water significantly within one hour. Future studies should confirm whether the identified male sex pheromone elicits such an endocrine response in male competitors, and what could be its function.

III) One question that was not yet directly addressed in this thesis but is rather salient is if the two pregnanetriol 3-glucuronate epimers are equally important for activity. In other words, are both necessary and, how important is a specific ratio or is there redundancy? Males release several fold more 20β-P-3-G into their urine than 20α-P-3-G and the ratio seems to increase with ascending rank of the donor male. However, both steroids share the same olfactory receptor

mechanism(s), although 20β-P-3-G likely has a slightly higher affinity. If both compounds are activating the same ORNs it seems conceivable that both compounds also lead to stimulation of the same brain areas and endogenous signaling cascades, thus evocation of a similar physiological or behavioural response. However, to date we have little understanding on how olfactory stimuli are processed and perceived in the fish brain. Furthermore, we cannot rule out completely the possibility that 20α-P-3-G may act as a partial antagonist to the receptor, although this seems unlikely given that 20α-P-3-G on its own gives strong EOG responses.
To settle this query, the female priming assay as well as the behavioural essay(s) on males (and females) should be used with each compound alone and in different ratios with the other. In addition, the 'priming assay' should be further refined testing the pregnanetriol 3-glucuronates at various concentrations and measuring 17,20β-P release rates in females, to obtain a concentration-response curve and establish the threshold and maximum effectiveness. This should indicate how adjusted is the 'priming effect' to the amounts released by dominant males and how large is the active space.

IV) Having identified the chemical nature, release site and biological function of a male tilapia pheromone, the next step is to gain understanding on the neural processing of the chemical stimuli (partially reviewed in Derby and Sorensen 2008). Several questions need answer: **1)** what ORNs types detect the two steroids, i.e. ciliated, crypt or microvilli? **2)** Into which glomeruli region(s) in the olfactory bulb are the ORNs converging? **3)** Which olfactory tracts, e.g. lateral or medial, pass on the information to the forebrain? Since the effect of the male tilapia sex pheromone on females is to stimulate 17,20β-P production and thus stimulation of oocyte final maturation, the pheromone must activate the females' brain-pituitary-gonad axis (Nagahama 1994). Thus, increased gene expression and production of GnRH (gonadotropin-releasing hormone), shall be detectable in the hypothalamus, and gonadotropins, i.e. FSH (follicle-stimulating hormone) and LH, in the pituitary.

V) Earlier in the discussion, the likelihood of pregnanetriol 3-glucuronates being produced in the testis and metabolites of 17,20α-P and 17,20β-P was elaborated. To confirm this hypothesis in vitro cultures of testis explants with or without stimulation with gonadotropins allow measurement of endogenous steroids and conjugates in the culture media. Other approaches could be incubation of testes segments with tritiated percursors such as pregenenolone or 17α-hydroxyprogesterone (Lambert 1986; Schoonen and others 1987). If production of the tilapia sex pheromone is indeed coupled to gonadal 17,20β-P/17,20α-P production, then it should occur under LH control and via the brain-pituitary-gonad axis. Indeed, recent studies on *Haplochromis burtoni* showed that subordinates males receiving the opportunity to ascend in social rank undergo a rapid surge in LH and FSH expression in the pituitary and release to the blood

(Maruska and others 2011). In *H. burtoni*, Nile tilapia and Mozambique tilapia, plasma 11KT levels rise quickly as males are acquiring a dominant position and sperm quality enhances within few days (Maruska and others 2011; Oliveira and others 1996; Pfennig and others 2012). This leads to the speculation that production of the sex pheromone in Mozambique tilapia males may be triggered by social information and achievement of a high social position; interesting thoughts to follow up in the future.

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