UNIVERSIDADE DE LISBOA
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Dominance pheromones in male and female cichlid fish

“ Documento Definitivo”

Doutoramento em Biologia
Especialidade de Etologia

Samyar Ashoori

Tese orientada por:
Prof. Doutor Adelino V. M. Canário
Doutor Peter C. Hubbard
Prof. Doutor Paulo J. Fonseca

Documento especialmente elaborado para a obtenção do grau de doutor

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Nota Prévia

A presente tese contém, na íntegra, três (3) artigos científicos publicados e dois (2) em preparação para publicação em revistas internacionais indexadas de acordo com o Regulamento do Ciclo de Estudos conducente ao Grau de Doutor no Despacho n.º 3098/2018, de 26 de março. Tendo os trabalhos mencionados sido efetuados em colaboração, o autor da tese esclarece que:

(1) No artigo do capítulo II participou na recolha e análise de dados, discussão dos resultados e redação do manuscrito.

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(3) No artigo do capítulo V, participou integralmente na análise de dados, discussão dos resultados e redação do manuscrito.

Faro, Junho de 2025

“If I sit silently, I have sinned.”

Mohammad Mossadegh

I dedicate this thesis to my father, in memory of his love, and to my mother, for her unwavering support.

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Abstract

Dominance hierarchies are formed within groups of fish in response to social competition for limited resources such as food, mates and space. In a dominance hierarchy, a fish displays dominant behaviours toward individuals lower in rank and submissive behaviours toward those higher in rank. Dominant fish exhibit agonistic behaviours, such as chasing and biting while defending a nest, and sexual behaviours, such as more frequent courtship. During and after formation of dominance hierarchies, a variety of physiological (e.g., endocrine), morphological (e.g., coloration), and behavioural (e.g., courtship) changes can occur. In Mozambique tilapia (*Oreochromis mossambicus*), dominant males release urine containing pheromones when interacting with other males or pre-ovulatory females. Male urine has been shown to contain two molecules, 20α- and 20β-pregnanetriol-3-glucuronates (P3Gs), which act as sex pheromones and stimulate ovulation in females. Males also appear to be able to discriminate the sexual state of females using olfactory cues present in urine and faeces. Furthermore, behavioural experiments, such as those using the mirror assay, have shown that urine from dominant males can reduce aggression in focal males, but P3Gs alone do not affect aggression. The identity of dominance signals present in urine is largely unknown. However, there is evidence that the signal in the urine of dominant males that controls aggression may be a multicomponent pheromone. In the laboratory, during the mouthbrooding period, females have occasionally been observed to display dominant behaviours and create territories for themselves, suggesting that females may compete with each other. We hypothesized that these behaviours might be mediated by chemical signals. This thesis aimed to identify the urinary dominance pheromones released by dominant males and pre-ovulatory females of Mozambique tilapia, using analytical chemistry, electrophysiology and behavioural analysis. The basic methodology consisted of collecting urine and faeces samples from dominant and subordinate males and pre-ovulatory and post-spawning females and using solid phase extraction (SPE) and high-performance liquid chromatography (HPLC) to fractionate the odorants involved. Electrophysiological methods tested the olfactory potency of the fractions, and those with the highest bioactivity were isolated to identify their chemical structure using liquid chromatography associated with mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR). The effect of putative pheromones on modulating aggression was tested using the mirror assay. Furthermore, it was tested whether mature males use chemical signals to discern the ovulation status of females and for mate choice through a preference assay. The results showed that dominant males release amino acids through urine, but high-performance liquid chromatography (HPLC) fractions containing amino acids did not affect male aggression. Similarly, a mixture of amino acids did not induce a significant preference response in focal males. Tilapias had olfactory sensitivity to extracts obtained from different solid phase media, including C18 eluate, C18 filtrate, mixed-mode cation exchange (MCX) bases, and mixed-mode anion exchange (MAX) acids—but none of the extracts separately affected male aggression, supporting previous suggestions of a multicomponent dominance pheromone. Mass spectrometry identified higher concentrations of cholic acid and taurocholic acids in the faeces of pre-ovulatory females than in post-spawning females. To assess the possible involvement of faeces in modulating aggressive behaviours in Mozambique tilapia, mirror assays were conducted; however, HPLC fractions containing bile acids (used as stimuli) did not affect aggression in focal males. However, male tilapia showed a preference for pre-ovulatory conditioned water, as well as their faeces (and bile acids within), compared to post-spawning females. However, males preferred equally pre-ovulatory female-conditioned water and water containing 17β-estradiol 3-glucuronate (released by pre-ovulatory females), suggesting this compound could be part of a pheromone bouquet to synchronise reproductive activity between males and females or as a token of female quality. Taken together, the findings of this thesis significantly enhance our understanding of the nature of chemical compounds involved in chemical communication in Mozambique tilapia. Moreover, it offers valuable information for future strategies in identifying dominant pheromones, from designing the behavioural experiments and monitoring the experimental fish to isolation and fractionation of samples for structural analysis.

Keywords: Mozambique tilapia, Chemical communication, Behaviour, Olfaction, Dominance index

Resumo

As hierarquias de dominância são formadas dentro dos grupos de peixes em resposta à competição social por recursos limitados, como alimento, parceiros e espaço. Devido a estas interações, os peixes dominantes são os primeiros a ter acesso aos recursos, criando inclusive territórios nos locais mais adequados. Numa hierarquia, um peixe comporta-se de forma dominante em relação aos que estão abaixo dele e de forma submissa em relação aos que estão acima dele. Os peixes dominantes exibem comportamentos agonísticos, como perseguir e morder enquanto defendem um ninho, e comportamentos sexuais, como o cortejo mais frequente. Durante e após a formação de hierarquias, pode ocorrer uma variedade de alterações fisiológicas (por exemplo, endócrinas), morfológicas (por exemplo, coloração) e comportamentais (por exemplo, cortejo). Na tilápia moçambicana (*Oreochromis mossambicus*), os machos dominantes armazenam e libertam urina em diferentes contextos sociais quando interagem com outros machos ou com fêmeas antes da desova. Foi demonstrado que a urina dos machos contém duas moléculas, 20α- e 20β-pregnanetriol-3-glucuronatos (P3Gs), que atuam como feromonas sexuais e estimulam a ovulação nas fêmeas. Os machos parecem também ser capazes de discriminar o estado sexual das fêmeas utilizando sinais olfactivos presentes na urina e nas fezes. Além disso, experiências comportamentais, como as que utilizam o ensaio de espelho, mostraram que a urina dos machos dominantes pode reduzir a agressividade dos machos focais, mas os P3G por si só não afetam a agressividade. A identidade dos sinais de dominância presentes na urina é em grande parte desconhecida. No entanto, existe evidência de que o sinal na urina dos machos dominantes que controla a agressividade pode ser uma feromona multicomponente. Em laboratório, durante o período de incubação bocal, observou-se ocasionalmente que as fêmeas apresentam comportamentos dominantes e criam territórios para si próprias, sugerindo que as fêmeas podem competir entre si. A nossa hipótese é de aquele comportamento possa ser mediado por sinais químicos.

Esta tese teve como objetivo identificar as feromonas de dominância libertadas por machos dominantes e fêmeas pré-ovuladas da tilápia moçambicana utilizando química analítica, eletrofisiologia e análise comportamental. A metodologia base consistiu na recolha de urina e fezes de machos dominantes e subordinados e de fêmeas pré-ovulatórias e em pós-desova, e utilizando a extração em fase sólida (SPE) e a cromatografia líquida de alta eficiência (HPLC) para fracionamento dos odorantes envolvidos. Os métodos eletrofisiológicos testaram a potência olfativa das frações, e aquelas com maior bioatividade foram isoladas para identificar a sua estrutura química através de cromatografia líquida associada a espectrometria de massa (LC-MS) e de ressonância magnética nuclear (RMN). O efeito das putativas feromonas na modulação da agressão foi testado através do ensaio de espelho. Além disso, foi testado se os machos maduros utilizam sinais químicos para discernir o estado de ovulação das fêmeas e para a escolha do parceiro através de um ensaio de preferência.

Os resultados do ensaio do espelho confirmaram que a urina dos machos dominantes reduz o comportamento agressivo dos machos focais expostos à sua imagem no espelho. Os estudos com LC-MS confirmaram a existência das feromonas sexuais previamente identificadas (P3Gs), com maior concentração na urina dos machos dominantes do que na de machos subordinados. Embora estudos anteriores tenham mostrado que os machos dominantes libertam aminoácidos através da urina, os ensaios comportamentais de frações de HPLC contendo aminoácidos permitiram rejeitar a hipótese de que estes contribuam para a sinalização de dominância na tilápia. Igualmente, os machos focais não mostraram preferência por uma mistura de aminoácidos. Por outro lado, foram identificadas concentrações mais elevadas dos ácidos biliares, ácido cólico e ácido taurocólico, nas fezes das fêmeas pré-ovuladas do que em fêmeas pós-desova. Quando testados no ensaio do espelho, os ácidos biliares não modularam a agressão em machos focais. No entanto, os machos de tilápia mostraram preferência por fêmeas pré-ovuladas, incluindo água por estas condicionada (água em que as fêmeas permaneceram e que continha produtos de excreção) do que água condicionada de fêmeas pós-desova. A adição de 17β-estradiol 3-glucuronato (libertado pelas fêmeas pré-ovuladas) fez aumentar a preferência da água condicionada de fêmeas pós-desova para níveis semelhantes à da água condicionada de fêmeas pré-ovuladas, sugerindo que este composto pode fazer parte de um bouquet de feromonas para sincronizar a atividade reprodutiva entre machos e fêmeas ou pode ser um sinal de qualidade das fêmeas. Tomados no seu conjunto, os resultados da presente tese melhoram significativamente a nossa compreensão da comunicação química na tilápia moçambicana e oferecem informações valiosas para estratégias futuras na identificação de feromonas de dominância, desde a conceção de experiências e monitorização dos peixes até ao isolamento e fraccionamento de amostras para análise estrutural.

Palavras-chave: Tilápia moçambicana, Comunicação química, Comportamento, Olfato, Índice de dominância

Content

CHAPTER I

GENERAL INTRODUCTION

1. Social hierarchies in fish

Social hierarchies are formed in groups of fish in response to social competition for limited resources such as food, mates and space. Males and females in group-living species usually establish and maintain social hierarchies (Maruska, 2014). The consequence of these interactions can lead to the creation of territories by dominant fish that occupy the best territories (Chapman, 1966). After the formation of social hierarchies, a variety of physiological (*e.g.,* endocrine responses), morphological (*e.g.,* colouration), and behavioural (*e.g.,* courtship) changes can occur based on the rank and position of each fish in the hierarchy (Burmeister et al., 2005; Maruska, 2014).

In many fish species, dominance hierarchies are established, and the social rank of individuals in a hierarchy can directly influence the reproductive potential by regulating the activity of the hypothalamic-pituitary-gonadal (HPG) axis (Sapolsky, 2005). Dominance hierarchies are often created through aggressive and submissive behaviours when individuals start interacting with each other. In some fish species, males in the hierarchies compete fiercely towards subordinates to obtain better access to food resources (Dara et al., 2022). Males rainbowfish (*Melanotaenia duboulayi*) within the Melanotaeniidae family engage in intensive territorial battles, where the victorious males benefit from dominance by, for example, easier access to mates (Colléter & Brown, 2011). Members of Cichlidea (cichlids) typically form a linear hierarchy wherein dominant individuals control the available resources by establishing territories and typically keep their position through strong, aggressive posturing and visual displays (Oliveira & Almada, 1996). A similar dominance hierarchy has also been observed among members of the Poeciliid family. Dominant male guppies exhibit courtship behaviours and increase the time spent near ovulated females after excluding the subordinate males from access to these females (Kodric-Brown, 1993). Therefore, the social network of males can effectively affect their reproductive success, as males in higher social positions are more likely to secure mating opportunities and pass on their genes to the offspring (Tamilselvan & Sloman, 2017). On the other hand, females play a critical role in forming social hierarchies, using strategies that often differ from males to increase their reproductive efficiency and survival (Milewski et al., 2022). For example, in the family Pomacentridae, not only males create feeding territories, but also females establish their own territories to form colonies (Karino & Nakazono, 1993). Female cichlids influence social hierarchy formation by displaying aggressive behaviour and maternal care. Wild dominant female *Neolamprologus pulcher*, an African cichlid, defend territories and protect their offspring through aggressive behaviours (Culbert et al., 2021; Desjardins et al., 2008). In Mozambique tilapia (*Oreochromis mossambicus*), after spawning, females carry and brood their eggs in the mouth until hatching. When the mother releases the larvae from her mouth, it aggressively protects them from conspecifics, showing maternal care (Kasumyan & Levina, 2023).

Several studies have demonstrated that dominance behaviours are influenced by the circulating plasma concentration of sex steroids after the formation of hierarchies and *vice-versa*, and a positive correlation between plasma androgen levels and social status was found in Mozambique tilapia (Oliveira et al., 1996). A higher social position in a hierarchy can also influence the social behaviours of conspecifics through the release of hormones or hormone metabolites (pheromones) into the water as excretory products (Barata et al., 2007).

2. Chemical communication and pheromones

Chemical communication is the most frequently used term in the literature to refer to various chemical interactions between individuals. Chemical communication between members of the same species or conspecifics involves transmitting and receiving information *via* chemical substances, known as pheromones (Karlson & Lüscher, 1959). Chemical communication in a hierarchy starts soon after chemical recognition, leading to changes in a fish's behavioural and physiological response. Due to the complex nature of aquatic environments, such as turbidity caused by heavy rainfall, the ability of fish to detect and distinguish between water-soluble chemicals and chemical cues released by conspecifics becomes more important. Fishes are exposed to countless chemicals (Sumpter, 2009), which trigger a wide range of responses ranging from growth and survival to social interaction and sexual attraction (Chung-Davidson et al., 2010). Fish communicate and gather information from the environment through different sensory channels (Ward, 2014). Chemicals (pheromones) can underlie social organisation by providing information about status or sexual receptiveness to conspecifics. An updated definition of pheromone was proposed by Wyatt (2010): a molecule that is an evolved signal eliciting a specific reaction, released and detected by conspecifics. Pheromones can be released in different contexts, triggering various physiological and behavioural responses in conspecifics. Fish pheromones are released in the water and possess structural properties that affect solubility and simplify excretion. The addition of polar functional groups such as carboxylic acid (-COOH), hydroxyl (-OH), and amide (-NH2) or bioconjugation with ionic groups such as sulphate, glucuronide or taurine increases the polarity of the pheromones and eventually promote a substantial increase in their water solubility (Stewart et al., 2013).

Furthermore, conjugation – especially of steroids – does increase water solubility, but it also reduces their binding affinity with plasma proteins, so increasing their renal clearance. And, in the case of hormonal pheromones, deactivates their *endocrine* activity.

Different chemical substances with pheromonal activity, such as amino acids, bile acids, prostaglandins and steroids (free and conjugated), have been identified (Keller-Costa et al., 2014b; Kobayashi et al., 2002; Li et al., 2002; Lim & Sorensen, 2012; Yambe et al., 2006). These chemicals, released *via* urine, faeces, mucus, or gill epithelia, are used as cues to signal the senders' physiological and behavioural states. It has also been shown that amino acids may serve as the potent olfactory stimulus of homing migration in salmonids (Hara et al., 1984). Bile acids, an important class of compounds from which pheromones can be derived, can enhance mating success and orient the reproductive migration (Buchinger et al., 2024; Li et al., 2002; Li et al., 2007). Moreover, there is evidence that conjugated steroids may be actively excreted into the water through a specific transport mechanism in the gills (Siefkes et al., 2003). However, few studies have directly addressed these issues in fish despite their importance for physiology and behaviour.

Initial studies, mainly using the electro-olfactogram technique (Stacey, 2011; Stacey & Sorensen, 2002), showed that urine (Olsén, 1987), intestinal fluid (Fisknes & Døving, 1982), gill (Scott & Sorensen, 1994) and skin mucus (Stabell et al., 1982) are potential release routes of pheromones in various teleost fish species, including Salmoniformes, Cypriniformes, Siluriformes, Characiformes and Perciformes. Before explaining the roles of the different types of pheromones and chemical cues, it is necessary to understand the sense of smell and the neurobiology of fish's olfactory system.

3. Olfactory system of fishes

Fishes have evolved an olfactory system specialised in the detection of odorants, discrimination between different chemicals, and the mediation of the appropriate responses to these chemicals (Kasumyan, 2004).

The anatomy of the olfactory epithelium of fishes is organised into a pair of peripheral structures called olfactory rosettes, which consist of leaf-like lamellae that increase the surface area available for chemical detection, enhancing olfactory efficiency (Elsheikh et al., 2024; Triana-Garcia et al., 2021). The olfactory epithelium contains olfactory sensory neurons (OSNs), which detect odorant molecules and initiate neural signals. These signals are transmitted, *via* the olfactory nerve, to the olfactory bulb in the brain (Mori et al., 1999). The olfactory bulb processes the information and sends it to target forebrain processing centres for further interpretation and mediation of behavioural and/or physiological responses (Miyasaka et al., 2014; Nikonov & Maruska, 2019). One primary target is the dorsal-posterior part of the telencephalon, responsible for distributing and discriminating odours (Yaksi et al., 2009). Moreover, olfactory signals can be directed to the dorsal telencephalic area, analogous to the amygdala, influencing aggression-related behaviours (Portavella et al., 2002). In some species, olfactory information reaches areas in the telencephalon, hosting the medial pallium, which is homologous with the mammalian hippocampus and plays a crucial role in spatial memory and navigation, allowing fish to use olfactory cues for orienting in their home stream (Gerlach & Wullimann, 2021; Rodríguez et al., 2021). The hypothalamus is the other region of the teleost brain that receives olfactory signals. It has been shown, at least in zebrafish, that hypothalamic cell types mediate the reproduction and social behaviour through the regulation of secretion of gonadotropin-releasing hormone, neuropeptide Y, and hypocretin (Kermen et al., 2013; Machluf et al., 2011).

The three main types of OSNs in fish are ciliated olfactory sensory neurons, microvillous olfactory receptor neurons, and crypt cells. Each type has different receptor proteins that bind to specific odorants (Lastein et al., 2014). As in other vertebrates (Buck & Axel, 1991), olfactory receptor proteins belong to the G-protein coupled receptor class (Ngai et al., 1993). Supporting cells are non-neural cells interspersed among the sensory cells, providing structural support and maintaining the extracellular environment by secreting mucus and other substances. These supporting cells play a role in protecting and nourishing the sensory neurons. Additionally, basal cells are present in the olfactory epithelium, acting as progenitor cells that can differentiate into new sensory or supporting cells, thereby contributing to the regeneration and maintenance of the olfactory epithelium (Poncelet & Shimeld, 2020).

The olfactory system of fishes enables them to detect small molecules in their aqueous environment (Laberge & Hara, 2001). The olfactory capabilities of fish can vary widely among species, often correlating with their ecological niches and life history (Dymek et al., 2021). For instance, species that rely heavily on smell for locating food or mates, such as catfish and eels, tend to have highly developed olfactory systems (Kasumyan, 2004). In Senegalese sole (*Solea senegalensis*), it has been shown that the upper (right) olfactory epithelium exhibited higher olfactory sensitivity to food-related odorants compared to the lower (left) epithelium, indicating a specialised function in detecting certain bile acids (Velez et al., 2009). Previous studies on anosmic fish have also confirmed that deprivation in the olfactory epithelium would cause an inability to evoke an appropriate behavioural response to a given stimulus. Westerberg (1982) showed that movements of anosmic salmon differed from those of intact salmon navigating using olfactory cues during their homing migration. The same behavioural failure of anosmic sea lamprey (*Petromyzon marinus*) has been reported when navigating rivers towards a larval odour (Meckley et al., 2014). In Mozambique tilapia, anosmic males behave differently from intact males, and urine pulses did not increase in the presence of sexually ovulated females, suggesting that the sense of smell must be involved in response to pheromones released by pre-ovulatory females (Almeida et al., 2005; Miranda et al., 2005).

4. Functional roles of fish pheromones

Before explaining the functional role of pheromones, it is necessary to define some terminology in the field of chemical communication that has been frequently used in the literature to refer to chemicals with biological or behavioural effects between conspecifics or heterospecifics.

Chemical cues, according to Sorensen (2014b) are any stimulus that produces a reaction (a response) in the sensory nervous system. These conspecific cues can be classified as alarm cues, reproductive cues, and social cues (Sorensen & Stacey, 2004). Chemical signals, also known as chemosignals, are a type of chemical cue in which its chemical identity has been changed through an evolutionary process; therefore, they may elicit a specific response in receivers' physiological or behavioural status (Sorensen, 2014b; Stacey & Sorensen, 2002). Pheromones are a specific type of signal that are released by a conspecific (as a single or mixture of compounds) and understood to reflect the physiological status of senders (Sorensen, 2014b).

4.1 Hormonal and non-hormonal pheromones

Hormonal pheromones are chemical signals that convey information about an individual's hormonal or physiological status to other members of the same species. These hormonal pheromones, which include hormonal precursors and their metabolites, often play different roles in forming social hierarchies and eliciting reproductive behaviours (Stacey & Sorensen, 2006). In teleost fish, hormonal pheromones can involve chemical compounds related to reproductive hormones. For instance, in goldfish (*Carassius auratus*), females during the reproductive cycle release pre-ovulatory sex pheromones containing androstenedione (AD), 4-pregnen-17,20β-diol-3-one (17,20β-P), and its sulphated metabolite, 17,20β-P-s, signalling their readiness to mate. At the final stage of oocyte maturation, post-ovulatory pheromones containing prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its metabolite 15K-$PGF_{2\alpha}$ are released into the water to stimulate male spawning behaviours (Kobayashi et al., 2002).

Similarly, males release steroids or other compounds indicating their reproductive status (Katare et al., 2011). These hormonal cues help attract potential mates, synchronise reproductive activities, and facilitate successful mating. In Mozambique tilapia, two steroid glucuronates have been identified in the urine of dominant males that act as sex pheromones and prime the final ovulation in females (Keller-Costa et al., 2014b).

Non-hormonal pheromones are chemical signals that convey information without directly being tied to an individual's hormonal state (Buchinger et al., 2024). An excellent example of this chemical class of pheromones is the non-hormonal sex pheromone of female masu salmon (*Oncorhynchus masou*) that is released *via* the urine to advertise female readiness for mating and to elicit male-specific behaviour (Yambe et al., 2006). Also, stream-dwelling larval lamprey (*Petromyzon marinus* L.) release sulphated biliary sterols that serve as non-hormonal migratory pheromones by guiding the adult and mature sea lampreys to suitable spawning sites (Sorensen et al., 2005).

4.2 Kin recognition

Kin recognition is the ability of fishes to detect and respond to specific chemical signals released by conspecifics (kin), which convey information about genetic relatedness (Olsén, 1992). Fishes typically exhibit a diverse range of social behaviours, from cooperative breeding to hierarchical structures within shoals, all of which can be influenced by their ability to recognise kin (Godwin & Thompson, 2012; Taborsky, 1984). The dynamics of social behaviour such as kin recognition within a wild aquatic ecosystem can help us to understand the evolutionary specialization of fish population (Villegas-Ríos et al., 2022).

The mechanisms underlying kin recognition in fishes primarily involve sensory perception, mainly through chemical cues. By detecting and interpreting these chemical cues, fishes can discriminate between kin and non-kin based on genetic relatedness. Close genetic relatives share more genetic markers, resulting in similarities in their chemical profiles that allow fish to identify family members (Mehlis et al., 2008). Behavioural familiarity also contributes significantly to kin recognition. Fishes that grow up together or share the same environment develop social bonds and recognise each other through repeated interactions. This familiarity enables them to distinguish kin from non-kin and influences their social behaviours, such as cooperative foraging or defending territories (Sorensen & Baker, 2014).

Kin recognition helps fishes avoid inbreeding and its negative genetic consequences. By selectively associating with genetically unrelated individuals for mating, fishes reduce the risk of genetic disorders and maintain genetic diversity within the population (Arnold, 2000).

4.3 Alarm cues

Chemical cues that are released by injured tissues of fishes to deter predators or warn conspecifics of potential danger are anti-predator cues (Wisenden, 2014). These chemical cues play a crucial role in fish communication and survival strategies, evoking escape behaviour. Alarm cues can be released through the damaged cells and tissues (Maniak et al., 2000). Upon detecting these cues, prey fish may alter their behaviour to reduce predation risk. It has been shown that the epidermal club cells in Ostariophysan fishes act as a source of alarm signals and protect the fish against predators (Smith, 1992). The recognition and identification of the chemical structure of alarm cues has received less attention because the release of these chemicals is mainly context-dependent and can be categorised as postattack and post-ingestion (Wisenden, 2014). However, several chemical compounds have been suggested as having a role in assessing predation risk. Hypoxanthine-3-*N*-oxide is a core component of chemical substances that convey antipredator signals (Brown et al., 2000) and glycosaminoglycan chondroitin in the mucus of zebrafish induces a mild alarm reaction Mathuru et al. (2012). However, antipredator cues are not limited to the release of the chemicals. It has been shown that conspecific tissues such as blood in Nile tilapia (*Oreochromis niloticus*) can evoke defensive behaviours in receivers (Barreto et al., 2013).

On the other hand, predator associated cues (*i.e.*, chemical cues released by the predators) can evoke anti-predator responses, that not only cause behavioural changes but also can induce morphological changes in the prey. This has been pointed out as predator-induced behavioural plasticity, which helps fishes avoid predation risk by changes in their morphology (Stabell & Lwin, 1997). For instance, the fathead minnow (*Pimephales promelas*) can detect chemical cues from predatory fish like northern pike (*Esox lucius*) and respond by avoiding areas with high predator odour concentrations (Mathis & Smith, 1993). Antipredator behaviours can also be elicited when predators release diet cues containing odours of the ingested conspecifics (Manassa & McCormick, 2012).

4.4 Dominance cues

In fishes, dominance cues are crucial in establishing and maintaining social hierarchies within a group. Dominant individuals release these chemical signals to assert their position and influence the behaviour and interactions of other fish within the same social group (Gonçalves-de-Freitas et al., 2008). Dominance relationship between members of a social hierarchy is normally influenced by two important intrinsic (*e.g.*, size and sex) and extrinsic (*e.g.*, social context) factors of individuals. For example, it has been found that the subordination of individuals is directly related to their body size (Cleveland & Lavalli, 2010). Nile tilapia also form dominance hierarchies and dominant individuals signal their rank not only visually by making their bodies darker but also chemically, signalling their social status (Gonçalves-de-Freitas et al., 2008). The dominance hierarchy has also been seen in group-living zebrafish where individuals are attracted to aggressive interaction between conspecifics (Abril-de-Abreu et al., 2015).

Dominant individuals often use chemical signals (dominance pheromones) to mark and defend their territory, communicate their status, and regulate the behaviour of other group members. This helps in reducing the potential for conflict and maintaining social order within social hierarchies (da Silva et al., 2021). However, no dominance signal has been identified yet, but there is clear evidence that in some territorial fishes, including Mozambique tilapia, excretory products such as urine carry a dominance signal. Senders might use this dominance signal (pheromone) to overcome high energy-demanding activities such as defending the territory or competing for a mate (Barata et al., 2007; Loranger & Bertram, 2016; Maruska & Fernald, 2012; Spence & Smith, 2005). Chemically-mediated dominance was also observed in bullhead catfish (*Ictalurus nebulosus*), in which body odours signal the social rank of resident dominant fish and this allows them to react aggressively toward chemical odours coming from non-resident conspecifics (strangers) (Bryant & Atema, 1987). Fathead minnows, like Mozambique tilapia, use urine containing dominance cues including bile acids and volatile amines that were hypothesized as the signal of dominance (Martinovic-Weigelt et al., 2012)

As a highly social species, Mozambique tilapia is an ideal candidate to study how individuals from different social ranks influence each other physiologically and behaviourally in their social hierarchies. It has been shown that Mozambique tilapia increase urine release as pulses in two different contexts: 1) in the presence of a pre-ovulatory female and 2) in the presence of a male intruder (Barata et al., 2008; Barata et al., 2007). The sex pheromones (*e.g.,* steroid glucuronates) identified in the urine of males had no aggression-reduction effects on interaction between male rivals when given alone. Furthermore, mirror studies have shown that polar and non-polar urine fractions alone are insufficient to reduce aggression in rival males. Therefore, a multicomponent pheromone must be responsible for signalling the dominance status and reducing the aggressive interaction between the dominant sender and the intruder-receiver in their social hierarchies (Keller-Costa et al., 2016).

5. Mozambique tilapia (*Oreochromis mossambicus*)

For almost a century, tilapia family members have been introduced to different countries for aquacultural purposes. Among the various species of tilapia, Mozambique tilapia (*Oreochromis mossambicus*) is the second cichlid species most produced in aquaculture after the Nile tilapia (*Oreochromis niloticus*) (Arumugam et al., 2023). The global production of Mozambique tilapia reached almost 24,000 tons, contributing 0.04% of total inland aquaculture until 2020. Tilapia are crucial in providing food to thousands of people in regions such as South Africa and China (El-Sayed, 2006). Besides the nutritional and commercial importance of tilapia, there are other advantages, such as biological control of aquatic weeds and insects, biodiversity, and conservation, that tilapia has over other common aquaculture species (D'Amato et al., 2007; Russell et al., 2012). In contrast, the tilapia is listed as invasive by the International Union for Conservation of Nature red list (iucnredlist.org) due to its ability to adapt to a wide range of environmental conditions (Casal, 2006). Following its introduction to regions where it is not native, it has established populations in ecosystems, leading to concerns about its impact on local biodiversity. In general, invasive species can negatively affect native ecosystems by outcompeting native species for resources (Champneys et al., 2021), altering habitats (Sowersby et al., 2016), transmitting diseases (Gozlan et al., 2010), and sometimes leading to hybridisation (Huxel, 1999).

The tilapia is a maternal mouthbrooder native to flowing rivers of southeastern and central Africa. Dominant males in their social hierarchies defend a territory associated with the areas where they dig, nest, and shelter. During spawning, dominant males use urinary signals to attract ovulated females. These urinary signals contain a mixture of two epimeric steroids, 20α- and 20β-pregnanetriol 3α-glucuronates (P3Gs), acting as sex pheromones and priming final maturation and ovulation in pre-ovulatory females within 1 hour after exposure (Huertas et al., 2014; Keller-Costa et al., 2014b). Urine pulses from dominant males also signal the sender's social status and prevent males from engaging in highly aggressive interactions with rivals

(Barata et al., 2007; Saraiva et al., 2017). Furthermore, behavioural bioassays have shown that neither of those P3Gs hormonal pheromones nor other excretory products, such as faeces, had an aggression-reduction effect on dominant males (Ashouri et al., 2023; Keller-Costa et al., 2016).

6. Identification of a dominance pheromone in Mozambique tilapia

Using pheromones for the control of invasive species involves synthesising or mimicking them to disrupt communication and behaviour (Sorensen, 2014a; Sorensen & Stacey, 2004). By strategically placing synthetic dominance pheromones, it would be possible to interfere with mating patterns, territorial behaviours, migration, or other vital aspects of the invasive species' life cycle (Sorensen & Johnson, 2016; Teeter, 1980). This disruption can suppress their ability to reproduce or establish dominance, contributing to effective population control. A good example of this is the use of a migratory pheromone for the management of invasive populations of sea lampreys (*Petromyzon marinus*) in the Laurentian Great Lakes (Johnson et al., 2009). This strategy might be helpful to apply in controlling the invasive population of Mozambique tilapia. In general, several methods could be used to manage and control invasive tilapia populations, including physical, chemical, biological, and genetic (Russell et al., 2012). Identifying such a dominance signal (*e.g.*, pheromone) in tilapia may not only be used to limit and reduce their invasive population but could also be applied to mono-sex rearing facilities to reduce aggression among males.

7. Analytical methods of pheromone identification

Chemical identification refers to a series of treatments that facilitate isolation and characterization of the molecular structure of a target chemical (Maltez et al., 2008; Stewart et al., 2013). Over the last two decades, the rapid expansion of technology and development of new analytical instruments have allowed chemists to isolate and identify pheromones with greater precision. In this section, we do not intend to discuss chemical identification techniques in detail, but we will briefly describe the main techniques for separating and identifying chemicals.

7.1 Solid phase extraction

Solid phase extraction (SPE) is a sample preparation method, in which molecules (*e.g.,* putative pheromones) are separated and concentrated by passing a fluid through SPE cartridges. Solid-phase cartridges contain an adsorbent (stationary phase) that selectively retain sample components when a solvent (mobile phase) is applied through the cartridge (Poole, 2003), components that can then be eluted with a different solvent. Depending on the purpose and origin of the samples, different SPE cartridges can be used to separate and isolate the target analytes such as C2, C18, MCX (Mixed-mode Cation Exchange), and MAX (Mixed-mode Anion Exchange) cartridges. C2 cartridges are non-polar due to their stationary phase with short-chain carbons that allow them to retain a wide range of hydrophobic compounds (Velez et al., 2007). C18 cartridges are used because of their strong non-polar analyte-matrix interactions. Since many fish pheromones identified to date are derived from steroid hormones, C18 cartridges provide an effective absorbent to retain and isolate such non-polar compounds (Benedetti et al., 2022). To separate and isolate compounds with basic properties, MCX cartridges are normally used. These cartridges contain a mix-mod polymeric ion exchange (stationary phase), and they possess a strong cation-exchange capability that can effectively extract the basic compounds from the matrices (Domínguez-Romero et al., 2014). In contrast, MAX cartridges provide a stationary phase that not only functions as a reversed phase but also possesses strong anion-exchange properties. These cartridges are used to separate the acidic compounds from the complex matrices (Kakimoto et al., 2008).

7.2 High-performance liquid chromatography (HPLC)

High-performance liquid chromatography is a chromatographic technique widely used to separate complex mixtures. The new HPLC systems offer advantages such as increased speed, higher sensitivity (*e.g.*, detectors), enhanced precision, and the ability to use various types of column (Reuhs, 2017). A high-pressure pump connected to the HPLC system forces the mobile phase (*i.e.*, carrier of samples) along the stationary phase (column). Different components within a mixture interact with column matrix with different affinities and thereby elute from the column at different time. At the end, a detector converts the amount of each component to an electronic signal.

Two main techniques are typically used for separation: 1) normal phase and 2) reversed phase. The key difference is the polarity of the stationary phase through which the samples are passed. While in the normal phase technique, a non-polar mobile phase is pumped through a polar stationary phase (*e.g.*, C6), in the reversed phase, a polar mobile phase goes through a non-polar stationary phase (*e.g.*, C18). These techniques were successfully applied for the identification of pheromones in round goby (*Neogobius melanostomus*), Mozambique tilapia (*Oreochromis mossambicus*), and masu salmon (*Oncorhynchus masou*) (Katare et al., 2011; Keller-Costa et al., 2014b; Yambe et al., 2006).

7.3 Gas chromatography (GC)

Gas chromatography is a sample separation technique that, like other chromatography methods, also requires stationary and mobile phases. In GC, the mobile phase is typically an inert gas, such as helium, that does not interfere with the volatile compounds that must be analysed (McNair et al., 2019). The use of GC to identify fish pheromones is uncommon because, unlike terrestrial animals and insects, fish pheromones are usually less volatile and more soluble in water. Thus, an inert gas carrier would not be expected to separate small quantities of a highly water-soluble pheromone.

7.4 Thin layer chromatography (TLC)

TLC is an analytical chemistry procedure used to separate non-volatile compounds. However, this method has limitations for the identification of fish pheromones because of 1) its low sensitivity, which makes it difficult to detect low concentrations of pheromones, and 2) its lower resolution and lower quantitative detection ability in comparison with HPLC (Fuchs et al., 2011).

7.5 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR is a modern analytical chemistry technique with high sensitivity, which increases the probability of detecting low-concentration components with the further advantage of not requiring purification. NMR is a non-destructive method that makes it easier to analyse small sample quantities. The concept of NMR stands on the atoms' nuclei behaviour under a strong constant magnetic field. The feature of nuclear spin is fundamental to NMR. Briefly, the nuclei of isotopes have a nuclear spin, which is ½ for isotopes common in organic chemistry, such as 1H, 13C, and 9F, and the spinning nucleus generates a magnetic moment (μ). Upon applying an external magnetic field, an energy shift occurs in the state of the atoms and, consequently, changes the NMR spectrum. For further detailed information about NMR spectroscopy, see (Claridge, 2016).

8. Electro-olfactogram (EOG)

The electro-olfactogram is a specialised electrophysiological technique designed to measure the negative electrical potential recorded as a direct current (DC) voltage at the surface of the olfactory epithelium in response to chemical stimuli (Scott & Scott-Johnson, 2002). This method is useful in identifying pheromones in fishes, where chemical communication plays a vital role in various behaviours, as many different compounds can be tested on the same fish. Furthermore, pheromones are often complex mixtures, and several different components must be present to evoke the full biological or behavioural response, whereas the EOG can respond to each component individually. In fish, the EOG involves placing the recording electrode near the olfactory epithelium, the tissue that detects odorants by olfactory receptor neurons (ORNs). When these sensory cells are exposed to chemical stimuli, such as pheromones, they generate electrical signals caused by the influx of cations (Ca^{2+}) through cyclic nucleotide-gated (CNG) channels that eventually lead to cell depolarization (Lapid & Hummel, 2013; Reuter et al., 1998).

The EOG technique is particularly effective for studying pheromone detection in freshwater due to its sensitivity (Keller-Costa et al., 2014a). By recording the changes in the electrical potential of the olfactory epithelium when exposed to different chemical stimuli, we can identify which compounds elicit strong olfactory responses. The amplitude and pattern of the EOG signals provide valuable information about the sensitivity and specificity of the olfactory receptors. The EOG set-up is shown in Figure 1.

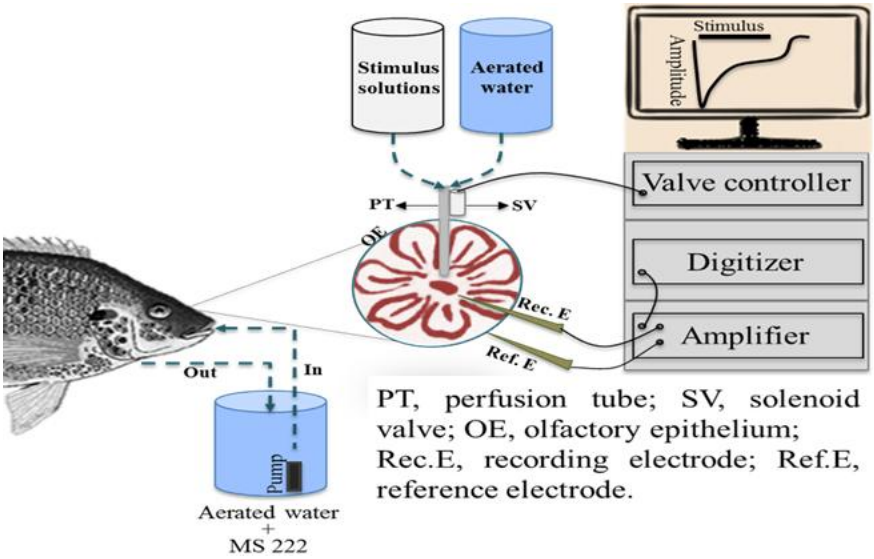

Figure 1 During an EOG experiment, the fish is anaesthetised in an aqueous solution of 50 mg/L 3-aminobenzoic acid ethyl ester (MS222) and placed in a padded V-clamp, with aerated water pumped over the gills. The perfusion tube irrigates the nostril with either water or stimulus-containing water. The recording electrode is positioned close to the centre of the olfactory rosette, and the reference electrode is placed lightly on the skin outside the nostril. The EOG response to stimuli is amplified, filtered, digitized, and recorded on a PC running Axoscope v10.6 (Molecular Devices).

9. Behavioural bioassay

Behavioural bioassays are used to evaluate the effects of various environmental factors, such as pollutants and pharmaceuticals, on fish behaviour (Dutra Costa et al., 2020). Behavioural bioassays are also crucial in assessing behavioural changes in response to chemical signals (pheromones) (Johnson & Li, 2010).

The advantage of behavioural experiments in the field of chemical communication is that fish behaviours are observed and recorded in a non-invasive way, and quantitative data on the frequency and duration of a specific behaviour can be obtained (Martins et al., 2012). Behavioural responses typically include changes in locomotion, feeding behaviour, aggression, preference, and avoidance responses (Castillo & Arce, 2021; da Silva et al., 2021; Giaquinto et al., 2010; Manassa & McCormick, 2012). For example, in the bio-assay guided fractionation of urine to identify the dominance signal in Mozambique tilapia, a mirror assay has been frequently used to evaluate the effects of urine fractions on male aggression against their mirror image (Keller-Costa et al., 2016).

In this study, we used the mirror assay to assess whether dominant male tilapia use urinary and/or faecal pheromones as a mediator of male-male aggression. Then we tested the hypothesis that this signalling depends on social rank (dominant and subordinate) when only a visual stimulus (mirror image) is available. Furthermore, we tested whether mature males could discern, by using chemical and/or visual cues, the ovulation status of females and if these could mediate the mate choice. For this, a preference experiment was conducted (for more details see chapter 3 and 5, materials and methods).

10. Physiological bioassays

Physiological bioassays play an important role in the identification and characterization of fish pheromones. These bioassays are typically used to measure the response of tissues to chemical cues (pheromones), providing further support to identify and isolate the fish pheromones. Endocrine bioassays measure changes in hormone levels in response to exposure to potential pheromones, linking chemical signals to physiological changes within the fish. Hormones can be measured using chemical methods (*e.g.,* mass spectrometry as indicated above) or immunological methods such as radioimmunoassay (RIA) or enzyme linked immunoassay (ELISA). These are competition assays in which the competitor ligand is either radioactive or is conjugated to an enzyme or fluorescent label that can be quantified (Stewart et al., 2013). For example, in the study of tilapia urinary sex pheromone, RIA was used to analyse changes in plasma and urine steroid levels in female tilapia after exposure to male urine. The increase in the maturation-inducing steroid (17,20β-P) indicated a primer effect of male pheromone on the endocrine system of females, facilitating synchronization of spawning (Huertas et al., 2014). Similar observations have been made in goldfish males exposed to female pheromone (Poling et al., 2001). RIA has also been effectively used to measure plasma 15α-hydroxyprogesterone (15α-P) concentrations in sea lamprey after exposure to the mating pheromone 3 keto-petromyzonol sulphate (Chung-Davidson et al., 2013). ELISA has been used to measure the release rate of 3α-hydroxy-5β-androstane-11,17-dione-3-sulphate (11-O-ETIO-3-S), putative pheromonal component in reproductive male round goby (*Neogobius melanostomus*) conditioned water and urine (Farwell et al., 2017).

11. Objectives and thesis overview

The objective of the present thesis is to identify pheromone(s) released by dominant male and pre-ovulatory female tilapia *via* the urine and faeces and advance our knowledge on the pheromonal activity of odorants released by different body fluids and their biological function(s).

This thesis is composed of five chapters, each addressing different aspects of the study of Mozambique tilapia chemical communication.

CHAPTER 1: Introduction

This chapter provides an overview of fish chemical communication and its importance in aquatic ecosystems. It discusses the different types of chemical signals, and their roles in various behaviours including mating, territoriality, and predator avoidance. The chapter also introduces the different methods of pheromone identification in fish species.

CHAPTER 2: Minimising time to evaluate pheromone-mediated reduction of aggressive behaviour in Mozambique tilapia (*Oreochromis mossambicus*).

This chapter assessed two experimental approaches designed to identify dominance signals in Mozambique tilapia chemicals: male-male interactions with real opponents and the mirror assay. Both approaches confirm the aggression-modulating effect of dominant male urine. But, based on optimal use of time, we suggest that the mirror assay is the better choice. The chapter was published in the *Journal of Fish Biology* (Ashouri et al., 2024).

CHAPTER 3: Bioassay-guided methodology for aggression pheromone identification in Mozambique tilapia (*Oreochromis mossambicus*).

This chapter investigated the olfactory responses to urine from dominant and subordinate males, focusing on various solid-phase extraction fractions in order to identify the chemicals involved. Dominant male urine evoked higher electro-olfactogram responses compared to subordinate males. Although dominant male urine and some of its fractions had higher olfactory potency in comparison to subordinate male urine, individually these did not reduce male aggressive behaviour in the mirror assay. These findings suggest that multiple chemical compounds may signal dominance rather than acting independently. Indeed, pheromones are frequently complex mixtures.

CHAPTER 4: Bile acids as putative social signals in Mozambique tilapia (*Oreochromis mossambicus*).

This chapter assessed the potency of faeces from males and females as a potential source of pheromones. The faeces were fractionated, and bioactive components were analysed by mass spectrometry. We identified a group of amino acids, and the bile acids (cholic acid and taurocholic acid) released in higher amounts by dominant males and pre-ovulatory females (compared to subordinate males and post-spawning females, respectively). We have shown that the olfactory potency of faecal extracts is mainly due to amino acids and bile acids. Furthermore, we have shown that cholic and taurocholic acids released in pre-ovulatory female faeces may act as a signal to attract reproductive males. This chapter was published in *Physiology and Behaviour* (Ashouri et al., 2023).

CHAPTER 5: A putative pheromonal role for 17β-estradiol 3-glucuronate in Mozambique tilapia (*Oreochromis mossambicus*).

This chapter provides information about the role of females with different ovulation status in creation of social hierarchies and how chemical and visual cues of females can play a role in this process. In this chapter we assume that due to the higher plasma concentration of 17β-estradiol during the vitellogenesis in pre-ovulatory females, the glucuronidation form of 17β-estradiol is released by them to convey information about their reproductive status. We have shown that conspecific male tilapia can distinguish the visual and chemical cues sent by females with different ovulation status. Also, the frequency of reproductive behaviours of focal males were markedly higher in response to visual stimulus in comparison to only chemical stimulus of females. Although 17β-estradiol 3-glucuronate was not effective enough to evoke reproductive behaviours such as nest-digging and urine pulses, focal males preferred to spend more time in the zones that received 17β-estradiol 3-glucuronate. This chapter has been submitted to the journal *Behavioral Ecology and Sociobiology*.

CHAPTER 6: General discussion and conclusions

In summary, this thesis aims to deepen our understanding of chemical communication in Mozambique tilapia by exploring various aspects of its communication system, including sex-dependent differences, ovulation status, and the role of metabolic byproducts. Our findings not only support previous results but also provide stronger molecular and behavioural evidence of the aggression-reducing effects of dominant male urine. This thesis has validated the use of the mirror assay as an important behavioural experiment in the identification of dominance pheromones in male tilapia, and it has been determined the optimal timing for conducting this assay to achieve the most accurate results. Additionally, the chemical isolation and fractionation of tilapia faeces suggest that it is only the urine of dominant males, rather than other excretory products, that plays the role in reducing aggression among tilapia conspecifics. This thesis also investigated the role of visual signals in tilapia communication, beside the olfactory system. Visual cues can work simultaneously with chemical signals to modulate different social behaviours, such as digging and urination, highlighting the complex and multi-modal nature of communication in this species. Taken together, the outcomes of this PhD thesis provide information into the mechanisms underlying the role of urine in reduction of aggression in tilapia, as well as the role of faeces in attraction of conspecifics. These findings have practical applications for managing the aggression in mono-sex tilapia cultures and for controlling invasive tilapia populations, since they are real threat for native fish species.

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CHAPTER II

Minimizing the time to evaluate pheromone-mediated reduction of aggressive behaviour in Mozambique tilapia (*Oreochromis mossambicus*)

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Minimizing the time to evaluate pheromone-mediated reduction of aggressive behaviour in Mozambique tilapia (*Oreochromis mossambicus*)

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Abstract

Some cichlid fishes release urine-containing chemical cues that lower aggression in their opponents. Bioassays to identify the aggression-modulating pheromone include assessing the effect of urine fractions on the behaviour towards a mirror image or in interactions with another male. However, many of these methods can be timeconsuming and require many fish. The objective of the present study was to assess the behaviour of male Mozambique tilapia (*Oreochromis mossambicus*) towards male urine using two methods with the intent of simplifying the bioassays: aggression towards a mirror image (mirror assay) and real opponents in which the urogenital papilla was tied using surgical silk to prevent urination. The results confirm the aggression-reducing effect of dominant male urine in both experimental approaches. Ten minutes of biting or 15 min of tail-beating behaviours in the mirror assay, or 5 min of opercular expansion or 15 min of lateral display in interactions with real opponents were necessary to detect a statistically significant reduction in aggressive behaviour towards dominant male urine. We also found that males with subordinate status had lower latency to initiate aggressive behaviours towards the mirror than dominants in the same condition, even though fish had been isolated for 1 week. However, no such differences in latency were found in the real opponent assay. We conclude that 5 min of opercular expansion behaviour in real opponent fights or 10 min of biting behaviour in the mirror assay are the shortest times necessary to test aggressive behaviour in urine fractions in bioassay-guided identification of pheromones.

Keywords: aggressive interaction, chemical communication, dominance index, dominance pheromone, urine

1. Introduction

Among the multiple sensory channels teleost fish use to communicate, chemosensory communication relies on odorants, often hormones and their derivatives, released into the water and detected by the olfactory system of conspecifics. These chemicals can provide information about the sender's physiological and/or social status and may influence the dominance hierarchy (Wisenden, 2014). In such a hierarchy, the social interaction to compete for the limited resources such as food, mates, and space can lead to the creation of a territory by dominant fish that occupy the most suitable places associated with an asymmetric aggression towards subordinate individuals (Kaufmann, 1983). This territorial pressure from dominants to subordinates is generally applied through species-specific aggressive behaviours, including chasing, tail beating, and biting. However, using chemical signals to convey status information reduces the energetic costs and risk of injury associated with aggressive behaviour (Baran & Streelman, 2020; Bayani et al., 2017; Earley, 2010).

In social fishes such as members of the family Cichlidae, aggressive interactions are often positively correlated with the position of individuals in a dominance hierarchy (Maruska, 2014). For example, subordinate non-territorial males are usually reproductively suppressed by dominant males (Kustan et al., 2012). This suppression from dominant males may be mediated by chemical cues, as illustrated by both the Mozambique tilapia *Oreochromis mossambicus* and *Astatotilapia burtoni*, which actively signal their reproductive and dominance status through the release of urine pulses (Barata et al., 2007; Maruska & Fernald, 2012)

In the Mozambique tilapia, hereafter ‘tilapia’, a multicomponent dominance pheromone is responsible for reducing the aggressive behaviour between rivals (Keller-Costa et al., 2016). The dominance signal consists of polar and non-polar components, although the exact chemical nature is still to be established (Keller-Costa et al., 2016). Bioassay-guided chemical isolation and identification of pheromones is generally cumbersome and time-consuming, and its success can be affected by pheromone concentration, bioassay variability, and possible multicomponent nature (Sorensen & Baker, 2014; Stewart et al., 2013). Simplifying bioassays and reducing the time necessary for behavioural experiments can accelerate pheromone identification.

Tilapia live in aggregations with dominant males acquiring territories and digging nests, which they defend aggressively by displaying various sexual and aggressive behaviours. Pre-ovulatory females are attracted to the nests by a urinary sex pheromone (pregnanetriol 3-glucuronates) released by dominant males that prime the females' neuroendocrine system to stimulate oocyte final maturation and ovulation (Keller-Costa et al., 2014). The dominant males have a larger and thicker urinary bladder than subordinate males, allowing them to store large volumes of urine, which they release in pulses when conspecific intruders try to enter their territory (Barata et al., 2007; Keller-Costa et al., 2012; Saraiva et al., 2017).

The study of aggressive behaviour in tilapia has relied mainly on the mirror assay, in which the fish spontaneously challenges its mirror image, providing a standardized procedure that avoids fights between opponents and allows experimental manipulation of chemical stimuli. However, limitations to the mirror assay have also been indicated. Firstly, the mirror image often fails to trigger a behavioural or physiological response in the focal fish, making it less effective than a live opponent (Oliveira et al., 2005). Secondly, the focal fish's interaction with its mirror image—frontal display (head-to-head)—immediately initiates aggressive behaviour, unlike the time lag when there is a live opponent. Thirdly, aggression escalates quickly in front of the mirror image, and some aggressive behaviours, such as lateral displays, are less frequent than in a real challenge with an opponent (Elwood et al., 2014). Balzarini et al. (2014) reported that the intensity of aggressive reactions might differ in response to a mirror or a live opponent, as may molecular and endocrine changes (Desjardins & Fernald, 2010). They suggested that the mirror assay should be validated for each species. Furthermore, studies with cleaner fish suggest they are self-aware and recognize their own reflection, eventually starting self-inspecting in the course of the experiments (Kohda et al., 2023; Kohda et al., 2019). However, such behaviour has not yet been reported for tilapia. In contrast, some studies also indicate that the mirror assay is reliable for measuring fish sociability (Cattelan et al., 2017).

We have previously observed that in male tilapia pairs with urination prevented by temporary constriction of the genital papillae, social interaction escalated to high aggression (mouth-to-mouth fighting) more rapidly and frequently than in unrestricted control pairs (Keller-Costa et al., 2012). This suggests an alternative to the mirror assay in which the focal fish can see and smell an opponent in the same tank through a perforated separation to avoid direct contact (Balzarini et al., 2014), while their urogenital papilla is constricted to prevent modulation by the subjects' urinary odorants. In both paradigms, urine from dominant and subordinate tilapia can be tested for their effects on aggression.

The objective of the present study, therefore, was two-fold: (1) to assess the behavioural response of males to urine in the mirror assay and towards opponent males in which the urogenital papilla had been tied to stop urination and (2) to determine a relatively fast measure that best discriminates aggressiveness among dominant and subordinate males.

2. Materials and methods

2.1 Ethics

Fish maintenance and experimentation were carried out according to Directive 2010/63/EU on protecting animals used for scientific purposes under license number 0421/000/000/2020 granted by the Directorate-General for Food and Veterinary of Portugal.

2.2 Fish

Sexually mature male and female tilapia from a well-established broodstock maintained at the University of Algarve (Faro, Portugal), descendants of fish from River Incomati (Mozambique), were randomly distributed in four 1000-L square tanks equipped with a closed water recirculating aquaculture system to establish social hierarchies. Two of the stock tanks provided fish for urine collection and the other two stock tanks provided fish for the behavioural experiments to eliminate any possible effect of odour familiarity. Each tank contained 10 size-matched males (mean ± standard error of the mean, weight (W) = 56.25 ± 1.49 g, standard length [SL] = 12.69 ± 0.16 cm) and 10 females (W = 54.80 ± 1.74 g, SL = 12.80 ± 0.26 cm). Each male was tagged with a coloured plastic tag (plastic T-Bar anchor FD94; Floy Tag) inserted into the muscle near the dorsal fin. The water was kept at 27°C under a natural photoperiod between January (10 h of light) and September (14 h of light) in Portugal. Fish were fed daily with a commercial cichlid diet (Sparos Lda.).

2.3 Urine collection

After the formation of hierarchies (within 7 days after group formation) in the large tanks, the total number of interactions (bites, chasing, and mouth fighting) was counted for each male daily for 30 min for 5 days. A dominance index (D_I) was calculated as the ratio between the number of dominant behaviours divided by the sum of dominant and subordinate behaviours (hovering in the water column in the absence of dark coloration) (Oliveira et al., 1996). Urine was collected from the most dominant (N = 8, $D_I = 1$, $W = 63.00 \pm 1.43$ g, SL = 13.31 ± 0.20 cm) and subordinate males (N = 10, $D_I = 0$, $W = 50.80 \pm 0.62$ g, SL =1 2.19 ± 0.11 cm) by gently squeezing the area above and anterior to the urogenital papilla and stored at −20°C. Two pooled urine samples were prepared from dominant and subordinate males, respectively, where each individual contributed the same volume, which was divided into 1-mL aliquots.

2.4 Mirror assay

Dechlorinated tap water was pumped from a filtration tank after passing through filter sheet plates, a biological filter bed (bio-balls), and activated charcoal into the four experimental aquaria (39 × 26 × 29 cm, ca. 30 L) (Figure 1) used in the mirror assay. The water in the filtration tank was at 27°C, aerated, with a flow rate at the water inlet of 650 L h^{-1}. Each aquarium had a sandy substrate with a mirror (26 × 29 cm) occupying the right side of the tank (facing the observer), hidden by an opaque plastic partition. Focal males were transferred from stock tanks to the experimental aquaria, where they were maintained isolated for 7 days before the experiment.

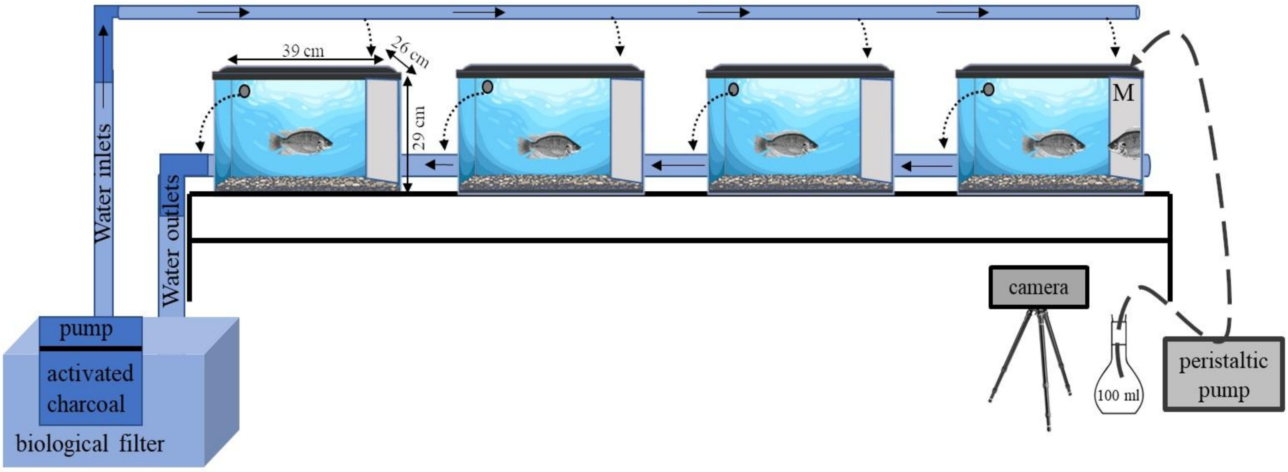

Figure 1 Experimental set-up of the mirror assay to study the behavioural responses of male tilapia to dominant and subordinate male urine.

Four hours before the experiment, focal males were anesthetized with 200 mg L^{-1} 3-aminobenzoic acid ethyl ester (MS222; Sigma-Aldrich) and injected with 100 µL of phenol red (50 mg mL^{-1} dissolved in 0.9% saline) into the dorsal musculature and returned to their aquarium. The phenol red is eliminated through the urine, allowing urination to be seen with the naked eye or camera (Barata et al., 2007). One hour before the experiment, the pump of the recirculating system was switched off, and a camera (SPK-HCD, Waterproof; Sony) was placed in front of the aquaria. The dominant male urine (DMU) and subordinate male urine (SMU) diluted 1:100 (*v/v*), and the control (filtered water) were randomly assigned to an individual focal male (one stimulus per individual) and administered by a multichannel peristaltic pump at 20 mL min^{-1} flow rate in the corner of the tank on the mirror side. The opaque partition was lifted manually to start the experiment, and the focal male was exposed to his mirror image. The latency was recorded as the time to the first reaction towards the mirror image. The male was removed from the experiment if no reaction was seen within 30 min (approximately 51% of 47 focal males reacted to their mirror image). After the first reaction towards the mirror image, the behaviours were recorded for 20 min: the first 5 min without stimulus, followed by 10 min with five 1-min of the stimulus separated by 1-min intervals with no stimulus, and the last 5 min without stimulus. After each set of experiments, the focal males were transferred to the stock tanks and the aquaria were washed with tap water.

2.5 Real opponent interactions

The assessment of the effect of urine on the aggressive interaction of urogenital papilla-tied males used a closed circulation experimental tank (79 × 35 × 45 cm), at 27°C, aerated and with a sandy substrate, divided by an opaque plastic plate into two similar-size sections (Figure 2).

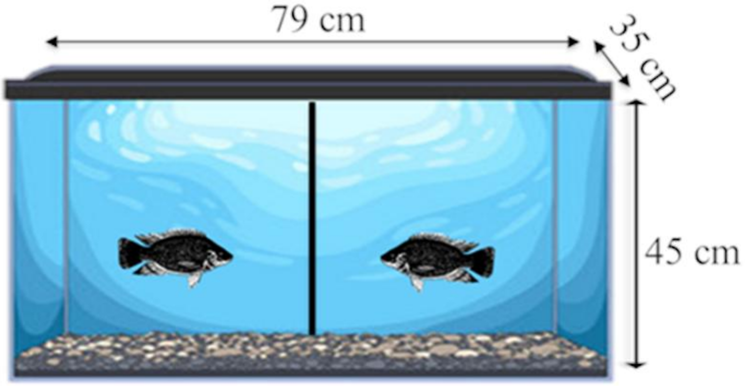

Figure 2 Set-up for interaction between urogenital papilla-tied males. Each pair of males matched for size and social status was exposed to dominant male urine, subordinate male urine, or filtered water to record the frequency of aggressive behaviours. Focal pairs were housed for 12 h in the aquarium before testing. Each experiment consisted of a 10-min stimulus-delivery period followed by 5 min without delivery.

Focal pairs (n = 20 pairs) matched for status and size were removed from stock tanks, anesthetized with 200 mg L^{-1} MS222, their urogenital papillae tied using surgical silk (Keller-Costa et al., 2012) and maintained in isolation for 12 h in the experimental tanks. The camera was positioned normal to the largest side aquarium in its middle region to record the behavioural interactions between focal pairs. To start the observations, the opaque plate between focal pairs was lifted, and focal males were exposed to each other visually and chemically. As with the mirror assay, a multichannel peristaltic pump was used to deliver a randomly assigned 20 mL min^{-1} stimuli of DMU, SMU (each diluted 1:100 *v/v* in distilled water) or filtered water (control) *via* a flexible plastic tube to the centre of the aquarium. Each pair only received one stimulus assigned randomly and fish were not reused. The experiment started immediately with a period of 10 min of stimulus delivery followed by 5 min without delivery. Latency was recorded as the time to the first aggressive reaction. Pairs that did not show aggressive interaction after 30 min (approximately 31% of 29 focal pairs) were removed from the experiment.

2.6 Quantification of behaviours

The video recordings were analysed using Observer XT software v. 8 (Noldus Information Technologies). Latency, aggressive behaviours [lateral display, tail beating, opercular expansion, biting; (Baerends & Baerends-van Roon, 1950; Oliveira, 1995)] and urine pulses were quantified as individual events (Barata et al., 2007).

2.7 Statistical analysis

The results are presented as mean ± standard error of the mean. We conducted two-way repeated-measures ANOVA (rmANOVA) to investigate the effects of stimulus and time on behaviour, followed by the Holm–Sidak (H-S) a posteriori test with comparisons to control stimulus and the first period in the time series. Data were tested for normality (Shapiro–Wilk test) and equality of variance (Brown–Forsythe test). Whenever the data were not normally distributed, they were log-transformed and zeros were replaced by a constant (0.5). Student's *t*-test was used to compare latency time between dominant and subordinate males. The level of significance was 5%. Statistical analyses were performed using SigmaPlot 14.0 (Systat Software, Inc.).

3. Results

3.1 Quantification of aggressive behaviours in the mirror assay

In the mirror assay, except for biting, fish behaviours decreased in the control and subordinate urine groups after 10–15 min (5–10 min for opercular expansion) of the start of the interaction with the mirror (Figure 3).

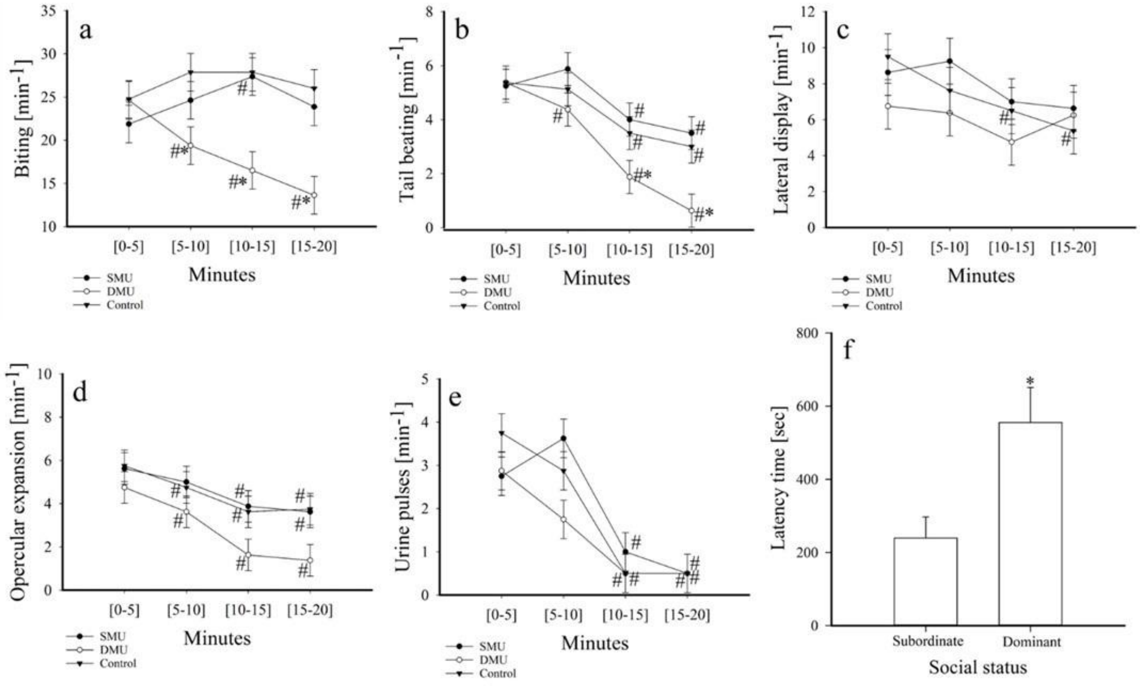

Figure 3 The aggressive behaviours of focal males in the mirror assay to subordinate male urine (black circles), dominant male urine (white circles), and control water (black triangles). # indicates a statistically significant difference ($p < 0.05$) from the first 5 min and * indicates a significant statistical difference of SMU or DMU in comparison to control (a–e) and in latency time between social status (subordinate and dominant males).

Biting behaviour (Figure 3a) was significantly affected by the stimuli and the effect changed over time with significant interactions between the two (rmANOVA effect of stimuli $F(2,21) = 4.58$, $p < 0.022$; effect of time $F(3,63) = 4.74$, $p < 0.005$; interaction stimuli x time $F(6,63) = 10.30$, $p < 0.001$). DMU reduced biting significantly compared to control after from 5 to 10 min onwards, while SMU caused a transient increase in biting at 10–15 min compared to control (H-S, $p < 0.05$).

Tail beating (Figure 3b) reduced progressively over time in all groups with the effect of the stimuli dependent on time (rmANOVA effect of stimuli $F(2,21) = 2.35$, $p < 0.10$; effect of time $F(3,63) = 57.75$, $p < 0.001$; interaction stimuli x time $F(6,63) = 4.23$, $p = 0.001$). The reduction in tail beating over time was faster for DMU, with statistical significance from 5 to 10 min compared to the initial 5 min (H-S $p < 0.05$) and statistical significance compared to control from 10 to 15 min onwards (H-S $p < 0.05$).

Lateral display (Figure 3c) was not statistically significant between treatments, and only in the control was there a steady reduction of this behaviour with time with statistical significance starting at 10–15 min (rmANOVA effect of stimuli $F(2,21) = 0.69$, $p = 0.51$; effect of time $F(3,63) = 8.26$, $p < 0.001$; interaction stimuli × time $F(6,63) = 1.65$, $p = 0.148$, H-S, $p < 0.05$). Opercular expansion (Figure 3d) was not statistically significant between treatments. However, for all the groups, there was a decline in this behaviour with time with statistical significance from 10 to 15 min (rmANOVA effect of stimuli $F(2,21) = 2.04$, $p = 0.16$; effect of time $\mathrm{F}(3,63) = 36.83$, $p < 0.001$; interaction stimuli × time $F(6,63) = 1.41$, $p = 0.226$, H-S, $p < 0.05$). The frequency of urine pulses (Figure 3e) was not statistically significant between treatments. However, for all the groups there was a steady reduction of urination with time with statistical significance starting at 5–10 min (rmANOVA effect of stimuli $F(2,21) = 1.31$, $p = 0.29$; effect of time $F(3,63) = 33.57$, $p < 0.001$; interaction stimuli × time $F(6,63) = 1.77$, $p = 0.119$, H-S, $p < 0.05$).

Although a comparison of the focal males' background (dominant or subordinate) was not initially planned, we noticed that males that were subordinate before the 7 days isolation period had significantly lower latency to start an interaction with the mirror than those that had been dominant (Figure 3f, two-tailed Student's *t*-test, $t = -2.926$, $df = 24$, $p = 0.007$). The coefficient of variation was lowest in biting (17%–22%), lateral displays (28%–38%), and opercular expansion (4%–63%).

3.2 Quantification of aggressive behaviours in real opponent interactions

Chasing was significantly different for stimuli effects (rmANOVA effect of stimuli $F(2,17) = 6.50$, $p = 0.008$; effect of time $F(2,34) = 0.52$, $p = 0.60$; interaction stimuli × time $\mathrm{F}(4,34) = 1.71$, $p = 0.17$) with temporarily significantly increased chasing after 5 min of SMU stimulus (H-S, $p < 0.05$). Biting between tied males was not statistically significant between groups or time (Figure 4b). Lateral displays decreased in all groups after 5–10 min and were significantly reduced by DMU compared to control after 10–15 min (Figure 4c; rmANOVA effect of stimuli $F(2,17) = 5.28$, $p = 0.016$; effect of time $F(2,34) = 3.53$, $p = 0.040$; interaction stimuli × time $F(4,34) = 0.624$, $p = 0.65$, H-S, $p < 0.05$). Opercular expansion was significantly lower in DMU compared to control with no significant changes with time (Figure 4d; rmANOVA effect of stimuli $F(2,17) = 4.551$, $p = 0.026$; effect of time $F(2,34) = 0.930$, $p = 0.404$; interaction stimuli × time $F(4,34) = 0.203$, $p = 0.935$, H-S, $p < 0.05$). There were no significant differences in latency between pairs with different social experiences (Figure 4e). The average coefficient of variation for each of the behaviours was never smaller than 110%.

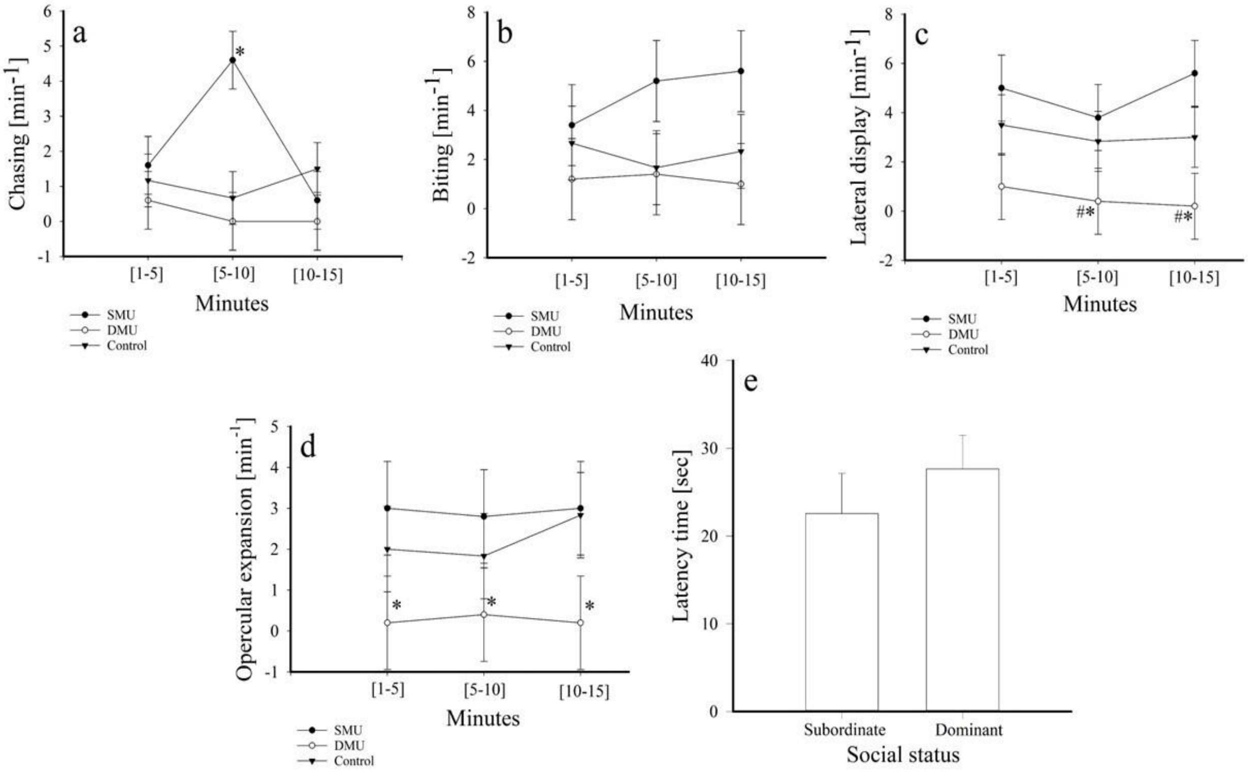

Figure 4 The aggressive behaviours of tied males to subordinate male urine (black circles), dominant male urine (white circles), and control water (black triangles). # indicates a statistically significant difference ($p < 0.05$) from the first 5 min and * indicates a significant statistical difference of SMU or DMU in comparison to the control (a–e).

4. Discussion

This study confirmed the aggression reduction effect of dominant male urine over time in both experimental approaches. Ten minutes of biting, 15 min of tail-beating in the mirror assay, or 5 min of opercular expansion or 15 min of lateral display in the real opponent interactions were the minimum necessary to detect a statistically significant reduction in aggressive behaviour in response to dominant male urine. Previous work had found urogenital papilla-tied males to be more aggressive than control intact males (Keller-Costa et al., 2012). In the mirror assay, biting, tail beating, and lateral displays are the most common aggressive behaviours, whereas urogenital papilla-tied males use mostly chasing and lateral displays. Additionally, in the mirror assay, aggressive interaction might be escalated compared to a real opponent under natural conditions because of space-limitation to withdraw from fighting and move away (Josi & Frommen, 2021).

Among aggressive behaviours of tilapia during an agonistic encounter, tail beating, and biting have been recognized as most aggressive to an intruder when defending a territory (Oliveira, 1995; Oliveira & Canário, 2000). We found that both tail beating and biting frequency of focal males exposed to DMU significantly decreased compared to the control in the mirror assay, as previously shown (Keller-Costa et al., 2016). However, real opponent males did not respond to urine and had low biting frequency and tail beating. While in the mirror assay, some behaviours may be amplified, as judged from the overall higher behavioural frequencies, the coefficient of variation of the behaviours was relatively low compared to the real opponent experiment. This could be advantageous for bioassay-guided fractionation as with a smaller variation, fewer individuals may be required to find significant differences between stimuli.

Like biting, chasing is a highly aggressive behaviour. It involves one male aggressively pursuing another male or female, often during territory formation or mating invitation, and can be recognized by rapid movement and physical contact, such as nipping (Arnott & Elwood, 2009). However, as with biting, there were no significant differences in chasing behaviour between groups or over time in the real opponent interactions.

Lateral displays decreased significantly with time in the mirror and real opponent experiments but, unlike biting, lateral displays were not significantly different between groups in the mirror assay. However, DMU reduced lateral displays in tied males compared to controls after 10–15 min. Lateral displays typically involve the focal male orienting sideways to the opponent so that it appears larger (Arnott & Elwood, 2009). Tilapia frequently show lateral displays during aggressive contests before engaging in mouth-to-mouth fights. A pair of males involved in a violent conflict, alternately change their direction from head to head (in the same direction) to head to tail (opposite direction), at the same time curving and turning, to gather information about each other's resource-holding potential (Arnott & Elwood, 2009; Enquist et al., 1990; Reddon & Balshine, 2010). This configuration is not displayed in a mirror image and might explain the reduction in this behaviour over time (Arnott et al., 2011).

Opercular expansion of focal males during the mirror assay also reduced over time. In aggressive male–male interactions, tilapia open their opercula widely simultaneously with lateral display, sometimes accompanied by erection of the dorsal fin, to appear larger to their opponent (Keller-Costa et al., 2016; Simpson, 1968). Focal males' ability to show the lateral display in the mirror assay is limited, which may also explain the reduction in opercular expansions. In contrast, real opponent males maintained a similar frequency of opercular expansion events over time, albeit significantly reduced by DMU as soon as the stimulus was added. Thus, opercular expansion in real opponent fights seems to be a good indicator of the level of dominance pheromone in the water.

In tilapia, tail beating is typically used to evaluate the fighting ability of rival males before engaging in a prolonged fight, and has been defined as a rapid slap of the tail and is usually accompanied by lateral display and opercular expansion (Gennotte et al., 2017; Neil, 1964; Ros et al., 2006). Although incapable of displaying lateral displays, focal males used tail beating and opercular expansion against their mirror image, and DMU significantly reduced the number of tail beats in focal males. This indicates that the aggression-lowering effect of DMU is effective at the highest aggression levels.

In the mirror assay, the frequency of urine pulses decreased in parallel with the overall decrease in the different aggressive behaviours over time in control and treatment groups. This supports the previously established relationship between urine release and aggression (Almeida et al., 2005; Barata et al., 2007). When facing other males or females, dominant males release urine more frequently, and if anosmic, they do not increase urination frequency, indicating olfaction is involved (Almeida et al., 2005; Barata et al., 2007). However, during aggressive displays or when the opponent becomes submissive, dominant males stop urinating (Barata et al., 2007). It is therefore somewhat surprising that although DMU suppressed some aggressive behaviours, urination frequency decreased similarly in the three groups. One possibility is that interactions with the mirror do not reach a winner/looser outcome, unlike interactions with real opponents (Barata et al., 2007).

Although males were placed in social isolation for 7 days, their social status before isolation appears to affect latency to start interacting with their mirror image but not a live opponent. That subordinate males reacted faster to their mirror image than dominant males could be linked to a reduction in the chronic stress they face in a social setting (Bessa et al., 2021; Øverli et al., 1999; Tea et al., 2019). The isolation period may reduce social stress and associated dominance inhibitory factors (such as the urinary dominance pheromone) and induce a compensatory dominance-seeking behaviour to establish their own territory. Social isolation may influence fish behaviour and social dynamics, but it does not necessarily remove their previous social experiences entirely. It has been shown that several social events, such as number of mates and social interactions during the early-life stages of fish, may affect adult behaviours (Fernald & Maruska, 2012; Harmon et al., 2024). Fish may still retain some memory of past social interactions even after a period of isolation (Anders, 1978).

In conclusion, our study identified that 10 min of biting in the mirror assay or 5 min of opercular expansion in the real opponent interaction were the shortest times necessary to detect a statistically significant reduction in aggressive behaviour in response to DMU. These findings will help us accelerate the pheromone identification process by bioassay-guided fractionation of dominant male urine.

Author contributions

Samyar Ashouri, Peter C. Hubbard, and Adelino V.M. Canário conceived the study. Samyar Ashouri carried out experiments and data analysis. Samyar Ashouri wrote the first draft. Peter C. Hubbard, and Adelino V.M. Canário edited the manuscript.

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Conflict of interest statement

The authors declare no conflict of interest.

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CHAPTER III

Bioassay-guided methodology for aggression pheromone identification in Mozambique tilapia (*Oreochromis mossambicus*)

Samyar Ashouri, José P. Silva, Peter C. Hubbard, and Adelino V.M. Canário

Bioassay-guided methodology for aggression pheromone identification in Mozambique tilapia (*Oreochromis mossambicus*)

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Abstract

Mozambique tilapia (*Oreochromis mossambicus*) release urinary odorants to signal dominance in their well-stablished hierarchies to prevent aggressive interaction with conspecific intruders. Although a pheromone with multi-component structure has been proposed to explain this urinary effect, its exact chemical nature remains unknown. The present study investigated the olfactory potency and behavioural responses of tilapia to untreated urine from dominant and subordinate males and their C18, mixed-mode cation (MCX), and mixed-mode anion (MAX) exchange solid phase extraction (SPE) fractions. The olfactory potency of C18 high-performance liquid chromatography (HPLC) fractions was also assessed. Electro-olfactogram (EOG) responses to C18 HPLC fractions using a gradient of water and methanol showed that the majority of olfactory activity was contained in the initial and final HPLC fractions (. EOG responses to untreated dominant male urine and C18 filtrate and eluate fractions evoked significantly higher EOG amplitude in comparison to subordinate males. Similarly, three fractions of MCX and MAX cartridges from dominant males, including eluate MCX bases, eluate MAX acid, and filtrate MCX bases, had higher olfactory sensitivity compared to subordinate males. However, neither of these three fractions, nor the reconstitution of the bases and acids fractions from MCX and MAX, evoked aggression-modulating effects like those of dominant male urine and the reconstituted C18 SPE fractions. These results not only confirm previous studies on the aggressions-modulating effect of dominant male urine, but also provide supporting evidence that Mozambique tilapia show high olfactory sensitivity to various chemical compounds involved in MCX and MAX fractions. However, these chemical compounds do not signal dominance individually but may work together in cooperation like reconstitution of C18 fractions.

Keywords: Solid-phase extraction, mixed-mode ion exchange, chemical communication, urine, dominance pheromone, mirror assay

1. Introduction

It is almost one century since members of the tilapia family have been introduced into different countries for biological control and aquaculture (D'Amato et al., 2007; Russell et al., 2012). Among the various species of tilapia, the Mozambique tilapia (*Oreochromis mossambicus*) is the second most cultured species after Nile tilapia (*Oreochromis niloticus*) based on its production (Arumugam et al., 2023). The global production of Mozambique tilapia reached almost 24 thousand tons, contributing 0.04% of total inland aquaculture until 2020 (FishstatJ, 2020). Mozambique tilapia, hereafter 'tilapia', plays a crucial role in providing food to millions of people in regions such as South Africa and China (El-Sayed, 2006). Besides the nutritional and commercial importance, tilapia is important for its use in biological control and conservation (D'Amato et al., 2007; Russell et al., 2012). However, tilapia is listed as invasive by the International Union for Conservation of Nature (IUCN) red list (iucnredlist.org) due to their ability to adapt to a wide range of environmental conditions and compete with local populations (Casal, 2006). Following its introduction to regions where it is not native (i.e., north and South America, Australia and Asia with references), it has sometimes established populations in sensitive ecosystems, leading to concerns about its impact on local biodiversity (Champneys et al., 2021; Gozlan et al., 2010; Huxel, 1999; Sowersby et al., 2016).

The tilapia is a maternal mouthbrooding cichlid native to flowing rivers of southeastern and central Africa. Males establish social hierarchies and defend their territory, within the areas where they dig nests; the 'lek'. During spawning, ripe females visit these leks and dominant males display well-characterised behaviours (Baerends & Baerends-van Roon, 1950) including the release of urine pulses to signal their dominance status to the females (Barata et al., 2008; Barata et al., 2007; Keller-Costa et al., 2012). The urinary signals contain a mixture of 20α- and 20β-pregnanetriol 3α-glucuronates (P3Gs), which prime the endocrine system to promote final oocyte maturation in females (Huertas et al., 2014; Keller-Costa et al., 2014). Urine pulses from dominant males also signal social status to other males, reducing highly aggressive interactions with rivals (Barata et al., 2007; Saraiva et al., 2017). This aggression-reduction effect of urine has been hypothesized to be elicited by (a) pheromone(s), consisting of both polar and non-polar (steroid-containing) components (Keller-Costa et al., 2016). Furthermore, behavioural bioassays have shown that neither P3Gs alone nor other excretory products, such as faeces, have an aggression-reduction effect on males (Ashouri et al., 2023; Keller-Costa et al., 2016).

Using pheromones for the control of invasive species involves synthesizing them, or their analogue mimics, and adding them to the environment to disrupt the species' communication and behaviour (Sorensen, 2014; Sorensen & Stacey, 2004). By strategically placing synthetic dominance pheromones, it may be possible to interfere with mating patterns, territorial behaviours, migratory period, or other vital aspects of the invasive species' life cycle (Sorensen & Johnson, 2016; Teeter, 1980) in a species-specific manner. This disruption can suppress their ability to reproduce or establish dominance, contributing to effective population control. An example of this practice is the use of a migratory pheromone for the management of invasive populations of sea lampreys (*Petromyzon marinus*) in the Laurentian Great Lakes (Johnson et al., 2009). Thus, identifying male dominance pheromones in tilapia could have applications in controlling invasive populations and reducing aggression among males in aquaculture.

The main objective of the present study was to develop procedures to identify the chemical identity of the dominance signal associated with the reduction of aggressive behaviour. We used different analytical methods, such as solid-phase extraction and preparative liquid chromatography to characterize the component(s) of the tilapia dominance pheromone.

2. Materials and methods

Experimental fish

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

Mozambique tilapia from a broodstock maintained at the Centre for Marine Science, Faro, Portugal, were used in experiments. Mixed-sex groups of sexually mature tilapia (2 males and 4 females) were kept in 300 L tanks with a glass front, sandy substrate and under-gravel filtration. The water temperature was maintained at 27° C under 12L:12D photoperiod. Fish were fed once daily with commercial fish pellets (Sparos Lda., Olhão, Portugal). In these conditions, dominance hierarchies were formed within a week, and a daily dominance index for each individual was measured as the ratio of the sum of dominance behaviours over the total number of dominant and subordinate behaviours (Oliveira et al., 1996).

Urine collection

Fresh urine samples were collected from males with a dominance index ranging from 0 (i.e., subordinate) to 1 (i.e., dominant) by gently squeezing the abdominal region anterior to the urogenital papilla and stored immediately at −20° C for up to one week until analysis. Dominance index was calculated as it previously described by (Oliveira et al., 1996). Briefly, after hierarchies were established in the large tanks within 7 days of group formation, the total number of interactions (bites, chasing, and mouth fighting) was recorded for each male daily for 30 minutes over 5 days. A dominance index (DI) was calculated as the ratio of dominant behaviours to the sum of dominant and subordinate behaviours.

C 18 solid-phase extraction

Urine samples were thawed at room temperature, pooled with equal volumes from each male, and vortexed slightly. The pooled samples from dominant and subordinate males were run through a C18 solid phase extraction (SPE) cartridge (Waters Sep-Pak™, Milford, U.S.A) following the manufacturer's instructions. Briefly, the total volume of pooled urine samples was measured (mean ± SE; dominant male urine, 2.5 ml; fish weight: 111.00 ± 6.78 g fish standard length L_S: 16.08 ± 0.28 cm; $N = 5$; subordinate male urine, 1.5 ml; weight: 62.40 ± 9.46 g; L_S: 13.00 ± 0.82 cm; $N = 5$) and an equal volume of methanol was used to activate the SPE cartridges. After activation, an equal volume of distilled water was used to wash the cartridges. The pooled urine samples were run through the cartridges to obtain the filtrate (polar) fraction. Finally, methanol was used to eluate the retained compounds.

Mixed-mode anion (MAX) and cation (MCX) exchange cartridges

C18 eluate and filtrate fractions were run separately through MAX and MCX cartridges to extract the acidic and basic compounds. According to the manufacturer's instructions (Waters Sep-Pak™, Milford, U.S.A, www.waters.com), an MCX cartridge was initially loaded with 2 ml of C18 urine fractions. The cartridge was then washed with 2 ml of 2% formic acid. A neutral fraction was then obtained with 2 ml of 100 % methanol. Finally, 2 ml of 5% NH_4OH was run through the cartridge to elute the basic compounds. Acidic compounds of the C18 eluate and filtrate fraction were obtained using a MAX cartridge. Initially, samples were loaded into the cartridge and washed with 5% NH_4OH. The neutral fraction was obtained with 2 ml of 100 % methanol. The acidic compounds were obtained by running 2 ml of 2% formic acid in methanol through the cartridge. For controls, miliQ water was run through C18 cartridge and eluate and filtrate fractions were run through the MCX and MAX cartridges as above.

Electro-olfactogram (EOG)

The electro-olfactogram is a record of an extracellular voltage change on the surface of olfactory epithelium considered to be a summation of generator receptors of those receptor cells responding to an odorant. The EOG responses to SPE (C18, MCX, and MAX), HPLC fractions, and non-treated urine samples were recorded as described by Frade et al. (2002). Briefly, male tilapia ($N = 5$; L_S: 18.4 ± 0.2 cm; weight:175.2 ± 6.6 g) were anesthetized with 100 mg L^{-1} MS-222 (3-aminobenzoic acid ethyl ester, Sigma-Aldrich) and placed in a padded V-clamp. Aerated water containing 50 mg L^{-1} MS222 was pumped over the gills through a tube in the fish's mouth. The right olfactory rosette was exposed by removing the skin surrounding the nostril. The recording electrode was positioned close to the centre of the olfactory rosette, and the reference electrode was positioned lightly on the skin outside the nostril. The perfusion tube for delivering the stimuli was placed over the olfactory epithelium. The stimulus solutions were fed by gravity through a three-way solenoid valve (in 4s pulses). The EOG signal was pre-amplified using a DC pre-amplifier and head-stage (NL102, Digitimer Ltd; www.digitimer.com) and filtered above 50 Hz (NL125, Digitimer Ltd), amplified (x100; NL106, Digitimer Ltd), digitized (DigiData 1440A, Molecular Devices, USA) and recorded on a PC running Axoscope (10.6; Molecular Devices). Untreated urine samples and C18, MCX, and MAX fractions were diluted 1:1000 from 10 µl aliquots, and HPLC fractions were diluted 1:100 before recording EOG.

Mirror assay

SPE fractions from dominant male urine that elicited a significantly greater EOG response than those from subordinate males were used in the mirror assay. The mirror assay was conducted to examine whether these fractions could mitigate the aggressive behaviour of focal males towards their mirror image (Keller-Costa et al., 2016). Briefly, focal males were taken from the stock tanks and moved to experimental aquaria. They were kept isolated for five days before the experiments. A camera was placed in front of the 30 L aquarium to record behaviours. Before the recording, the aeration was cut off to prevent air bubble disturbances to the mirror image. A 1 ml aliquot of different stimuli (distilled water as control, dominant male urine, eluate C18 fraction, filtrate C18 fraction, eluate MCX bases, eluate MAX acids, filtrate MCX bases, eluate + filtrate, eluate MCX bases + filtrate MCX bases, and eluate MAX acids + filtrate MAX acid) were thawed and diluted 1:100 *v/v* in distilled water and randomly assigned for each experimental aquarium. Equivalent amounts of urine were used in each extract. To initiate the experiment, an opaque plastic plate covering the mirror was lifted, and after the first reaction of the focal male (Fig. 3a $N = 8$ for each stimulus; Fig. 3b $N = 10$ for control, dominant male urine, eluate C18 fraction, filtrate C18 fraction, eluate MCX bases, eluate MAX acids, filtrate MCX bases, eluate + filtrate; Fig. 2b $N = 7$ for eluate MCX bases + filtrate MCX bases and eluate MAX acids + filtrate MAX acid) to its mirror image, the stimulus was immediately injected in five 30 s pulses, separated by 30 s intervals, using a peristaltic pump at a flow rate of 40 ml.min^{-1}. After each set of experiments, the experimental aquaria were washed with warm water. The recorded videos were viewed with KMplayer 64X v. 2020,06.9.40 (www.kmplayer.com). The number of bites against the mirror image in the first five minutes to represented the most aggressive behaviour (Ashouri et al., 2024). Those focal males who did not react to their mirror image after 10 min were removed from the analysis.

Preparative high-performance liquid chromatography

The untreated urine pools from dominant and subordinate males (dominant male urine, 3 ml; weight: 87.5 ± 8.11 g L_S: 14.05 ± 0.54 cm; $N = 6$; subordinate male urine, 2.5 ml; weight: 74.16 ± 5.48 g; L_S: 13.53 ± 0.37 cm; $N = 6$) were fractionated using a preparative high performance liquid chromatography system equipped with a UV-Vis detector (Shimadzu Nexera series, Shimadzu Corporation, USA) with a C18 column (Amide column, 5µm, 4.6 × 150 mm, XBridge^{TM}). HPLC conditions were: samples (10 µl) were injected *via* an automatic injector in an isocratic mobile phase of 50:50 water/acetonitrile for 20 minutes. Fractions were collected every one minute. Due to the toxicity of acetonitrile, HPLC fractions were evaporated under nitrogen gas at room temperature, then were redissolved in 500 miliQ water.

Statistical analysis

A two-way analysis of variance (ANOVA) was used to compare the EOG amplitude in response to SPE and HPLC fractions using the square root transformation, with social rank and fraction as factors for males and ovulation status and fractions as factors for females, followed by the Holm-Sidak post hoc test. The significance level was 5%.

3. Results

The EOG responses to untreated urine and C18 SPE, MCX and MAX fractions, are shown in Figure. 1. The raw urine from dominant males evoked significantly higher responses compared to subordinate male urine. As expected, the C18 polar (filtrate) and non-polar (eluate) fractions from dominant male urine evoked higher EOG amplitude than those from subordinate male urine. Furthermore, the basic eluate fraction of dominant male urine obtained with the MCX cartridge evoked significantly higher EOG amplitude than the subordinate male urine. The olfactory epithelium of male Mozambique tilapia was also significantly more sensitive to the acidic eluate fraction of dominant male urine than subordinate male urine. The basic filtrate of dominant male urine obtained with the MCX cartridge, like the eluate, also evoked significantly higher EOG amplitude than the subordinate male urine.

The EOG experiment with C18 fractions obtained from preparative HPLC, showed higher amplitude of responses in fractions 2, 4, and 15 from dominant male urine compared to subordinate male urine (Fig.2).

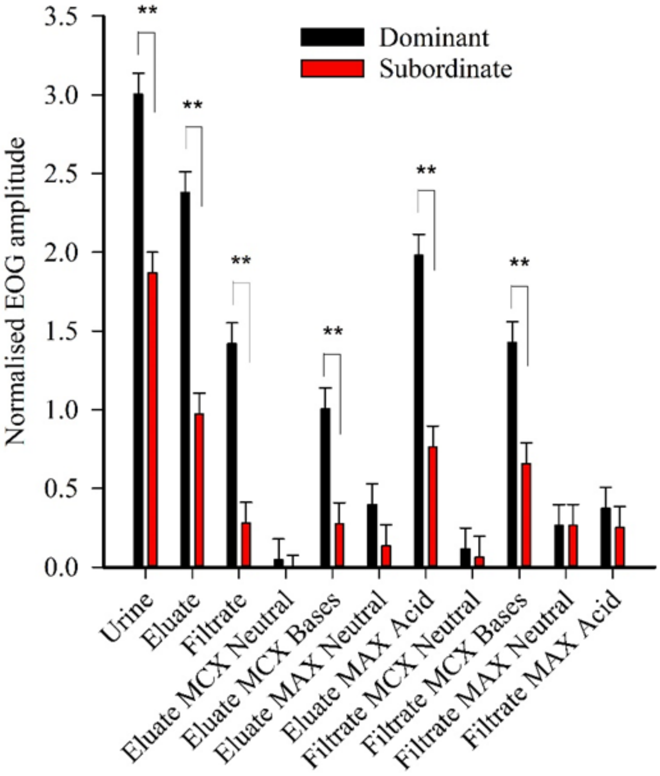

Fig. 1. Normalized EOG amplitude of males in response to untreated urine and respective C18-SPE, mixed-mode cation (MCX), and mixed-mode anion (MAX) fractions from dominant and subordinate males. The asterisk (**) indicates statistical difference ($p < 0.01$) between fractions of different social statuses.

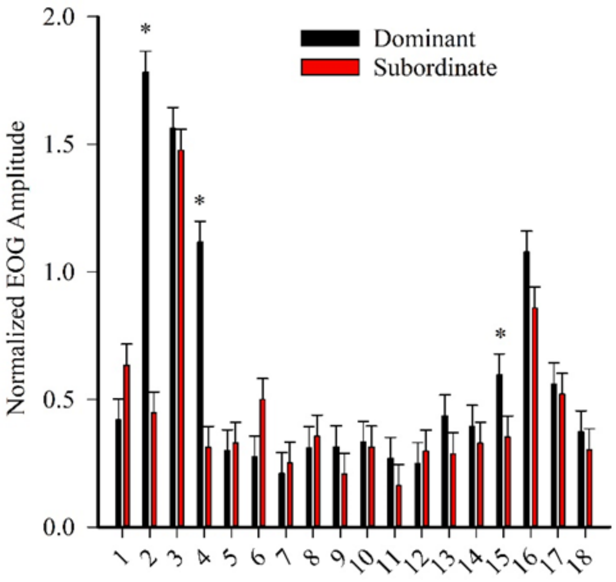

Fig. 2. Normalized electro-olfactogram (EOG) amplitude of males in response to high-performance liquid chromatography (HPLC) fractions of dominant and subordinate male urine, using a C18 column. The asterisk (*) indicates statistical difference ($p < 0.05$) between fractions of different social statuses.

The results of the mirror assays showed no reduction of aggression when dominant male urine eluate and filtrate fractions of C18 cartridges were used as stimuli (Fig. 3a). Furthermore, when eluate MCX bases, eluate MAX acids, filtrate MCX bases, eluate MCX bases + filtrate MCX bases, and eluate MAX acids + filtrate MAX acid were applied as stimuli to focal males, the number of bites were not reduced significantly in comparison to C18 eluate, C18 filtrate and control group (Fig. 3b). Only reconstitution of eluate and filtrate fractions reduced the number of bites compared to the control group; however, it was less effective than raw dominant male urine.

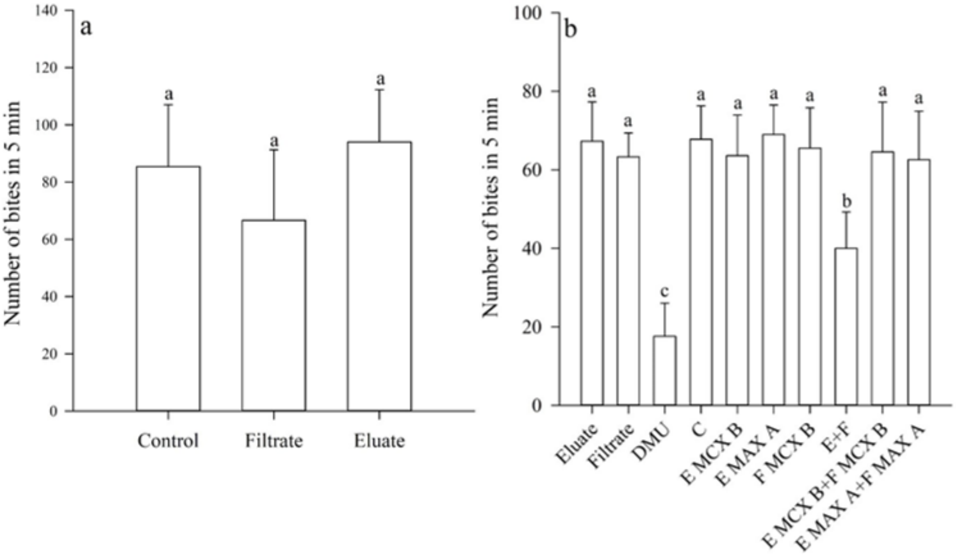

Fig. 3. Number of bites against the mirror image by the focal males exposed to (a) distilled water (control), filtrate C18 fraction, eluate C18 fraction; (b) distilled water (control), dominant male urine, eluate C18 fraction, filtrate C18 fraction, eluate mixed-mode cation (MCX) bases, eluate mixed-mode anion (MAX) acids, filtrate mixed-mode cation (MCX) bases, eluate + filtrate, eluate mixed-mode cation (MCX) bases + filtrate mixed-mode cation (MCX) bases, and eluate mixed-mode anion (MAX) acids + filtrate mixed-mode anion (MAX) acid. Different letters indicate significant differences between stimuli ($p < 0.05$).

4. Discussion

Dominant tilapia males store higher volumes of urine in comparison to subordinate males, due to their larger and more muscular urinary bladder (Keller-Costa et al., 2012) (Keller-Costa et al., 2012; Kutsyna et al., 2016). The urine can carry different chemicals such as nitrogenous waste products, electrolytes, and metabolites (Greenwell et al., 2003; Scott et al., 2008; Xu et al., 2007). Since the chemical structures of urinary odorants are quite different, different analytical chemical techniques, such as solid phase extraction, are needed to fractionate and isolate them based (Poole, 2003).

The present study used a bioassay-guided approach to identify the dominance pheromone in tilapia. Since the pheromone is likely multicomponent, we used various extraction methods directed at non-polar and polar compounds (acidic and basic). Testing the various fractions by EOG, we found that dominant male urine and its two C18 fractions (filtered and eluate) evoked higher responses than those from subordinate males. These results are consistent with previous studies that similar EOG responses were evoked to untreated and C18 fractions (Frade et al., 2002; Keller-Costa et al., 2014). The chemical composition of these two fractions has been partially identified; polar compounds like amino acids in filtrate fraction (Kutsyna et al., 2016) and hydrophobic compounds such as steroids in the eluate fraction (Keller-Costa et al., 2014).

The whole urine fractionation with HPLC C18 column showed similar EOG responses to the first two fractions and fractions containing the steroid pheromones (Keller-Costa et al., 2014). Our mirror assays with the C18 SPE fraction (filtrate and eluate) also confirmed that the C18 eluate and filtrate alone do not reduce aggression in males, consistent with a multicomponent pheromone (Keller-Costa et al., 2016); the pheromonal activity on the mirror assay can be evoked by recombining the C18 filtrate and eluate fractions (Keller-Costa et al., 2016), however, in the current study, the olfactory activity was lower than untreated urine. This difference in activity between untreated and urine and reconstituted C18 fractions might be due to the degradation of chemicals in the urine. However, the similarity between the reconstituted C18 fractions and untreated dominant male urine raises the question of whether further fractionation of the C18 fractions could help isolate and identify target compounds. To answer this question, we used MCX and MAX cartridges, and the three fractions obtained from MCX and MAX cartridges of C18 DMU had higher olfactory sensitivity than C18 SMU. Theoretically, the eluate MCX bases and filtrate MCX bases are composed mainly of strong basic amines (Cobo-Golpe et al., 2022); Although C18 SPE cartridges separate chemicals according to their polarity, they do not provide a sufficient sorbent area to completely separate complex mixtures like urine. Therefore, hydrophilic compounds might be retained in the eluate fractions (Thurman, 1998). This might explain the presence of amines, such as amino acids, in the Eluate MCX bases fraction and, accordingly, higher EOG responses to this fraction. It is evident that fish—including the Mozambique tilapia—have high olfactory sensitivity to amino acids (Hubbard et al., 2017; Kutsyna et al., 2016; Nikonov et al., 2017; Nikonov & Caprio, 2004; Yambe et al., 2006). On the other hand, higher EOG responses to Eluate MAX acid fraction may be attributed to the presence of negatively charged compounds, including steroid derivatives, bile acids, and other as yet unidentified chemical compounds (Domínguez-Romero et al., 2014; Wang et al., 2013). Furthermore, amino acids may also be present in this fraction because they are carried in high concentration in the dominant male urine (Kutsyna et al., 2016), and in their chemical structure, they have a carboxyl functional group that can behave as weak acids. However, it should also point out that the three EOG-positive MCX and MAX fractions were eluted and washed with NH_4OH, which can potentially increase the conductivity of water, reduce the resistance between electrodes and olfactory epithelium tissue, and therefore decrease the EOG amplitude.

The eluate MCX base, eluate MAX acid, and filtrate MCX base did not show any reduction of aggression in the mirror assay. For example, Kutsyna et al. (2016) suggested that the high urinary concentration of amino acids, particularly L-arginine and L-glutamate, may be involved in signalling dominance. However, this hypothesis can be rejected since the C18 filtrate fraction alone, presumably containing a high concentration of amino acids, did not reduce the aggressive behaviour of males. Furthermore, we have recently shown that faeces of dominant male tilapia, after fractionation with HPLC C18 column, had high concentrations of amino acids in the early eluting polar fractions, but the mirror experiment did not show any significant reduction of biting behaviour when focal males were exposed to those amino acid containing fractions (Ashouri et al., 2023). Furthermore, male tilapia did not show a preference for a mixture of amino acids (Ashouri et al., 2023). Nevertheless, since tilapia release large quantities of urinary amino acids and have strong olfactory sensitivity to them, there is the possibility that amino acids are part of a multi-component dominance signal and/or individual recognition (Kutsyana et al., (2016).

In contrast to the regaining of aggression/reduction activity upon urine reconstitution (C18 SPE eluate plus filtrate), combining the basic and acidic fractions of MCX and MAX cartridges did not modify the aggressive behaviours of focal males. Firstly, this could be due to the degradation of one component or the whole pheromone. Secondly, although we attempted to remove urine impurities (*i.e.*, faeces and semen) through centrifugation before loading onto the cartridges, there could still be some blockage of the cartridge, reducing the capacity of the stationary phase for the separation of analytes (Li et al., 2020). Thirdly, most pheromonal components are present at a relatively low concentrations (Stewart et al., 2013), and could undergo degradation when washed and with strong basic and acidic solutions. There could be other limitations that make the process of identification difficult; although collecting urine from dominant males is relatively easy due to the larger volume stored in their urinary bladders, obtaining urine from subordinate males is more difficult. Therefore, this makes the direct comparison of the effects of dominant and subordinate male urine, both chemically and behaviourally, more difficult.

Although the role of steroid glucuronates as a single-component pheromone on female reproductive physiology been clearly demonstrated, a multi-component signal better explains the aggression-modulating effects of dominant male urine.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Author Contributions

Samyar Ashouri, Peter C. Hubbard, and Adelino V.M. Canário conceived the study. Samyar Ashouri carried out experiments and data analysis. Samyar Ashouri wrote the first draft. Peter C. Hubbard, and Adelino V.M. Canário edited the manuscript.

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CHAPTER IV

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

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

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Abstract

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

Keywords: Bile acid; faeces; chemical communication; cichlid; behaviour; reproduction

1. Introduction

Teleost fish rely on conspecific chemical cues or pheromones released to the water for vital social functions such as kin recognition, parent-offspring interaction, dominance and sexual behaviours [1,2]. The compounds involved are generally small molecules such as amino acids, nucleotides, steroids, bile acids and prostaglandins, which include hormones or hormonal byproducts [3], [4], [5]. The pheromones may be a single compound, such 3keto petromyzonol sulphate released by sea lamprey (*Petromyzon marinus*) males or prostaglandin $F_{2\alpha}$ released by ovulating goldfish (*Carassius auratus*) females, or a mixture of compounds such as the panoply of bile acids released by sea lamprey larvae or the dominance pheromone in Mozambique tilapia, *Oreochromis mossambicus* [3,6,7]. Goldfish, which employ a promiscuous or polygynandrous mating system involving intense sperm competition, are a good example of a species using reproductive pheromones, steroids and prostaglandins for chemical communication. Female goldfish at the end of vitellogenesis release a pheromone mixture dominated by androstenedione (AD) that promotes agonistic behaviour among males. With the luteinizing hormone (LH) surge prior to oocyte final maturation, the ratio of 4-pregnen-17,20β-diol-3-one (17,20β-P) to AD increases and males start chasing conspecifics and spermiogenesis is stimulated mediated by LH. At ovulation, females produce $PGF_{2\alpha}$ which acts in the brain to trigger female sex behaviour and is released together with its metabolite 15keto-$PGF_{2\alpha}$ as a postovulatory pheromone stimulating both male spawning behaviours and additional LH increase [8,9]. The routes of release of these molecules depend mainly on the site of production and polarity of the compounds, some being released from special glands in the skin, or through the gills, urine, semen and, possibly, faeces [10], [11], [12], [13], [14], [15], [16], [17], [18], [19].

The first evidence of faeces as a source of chemical cues, comes from coho salmon, *Oncorhynchus kisutch*, being attracted by conspecific faeces [20]. Furthermore, ovulated and non-ovulated Mozambique tilapia females, have different olfactory sensitivity to faeces, [21] and chameleon cichlid, *Australoheros facetus*, had different sensitivity to intestinal fluid from dominant and subordinate males [15]. Intestinal fluid has been shown to contain, among other compounds, amino acids [22] and bile acids [23,24], both of which have been implicated in chemical communication [25].

The Mozambique tilapia, hereafter ‘tilapia’, is a maternal mouth-brooding African cichlid in which males congregate in “leks” where they establish a social hierarchy and dig and aggressively defend pits (“nests”). Ripe females then visit these leks and choose one or more males with which to spawn [26], [27], [28]. Territorial males release pulses of urine as a vehicle for pheromones when they encounter conspecifics [10,29]. The urine contains a pheromone composed of two epimeric steroids (20α- and 20β-pregnanetriol 3-glucuronate), which primes the female reproductive axis to produce the maturation-inducing steroid 17,20β-P [2]. The urine also contains a pheromone that signals dominance and lowers conspecific aggressiveness, although its chemical identity is still unknown [7,10,[29], [30], [31]]. Furthermore, it has been shown that other excretory products of tilapia, such as faeces, might be used as vehicle to carry putative pheromonal compounds that can be discerned by olfactory epithelium and convey information about the donor's physiological status [21]. As males seek to attract the best females to their nests, faeces could convey information about their feeding/nutritional and reproductive status. The nutritional status of females is important because they do not feed while mouth brooding [10].

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

2. Materials and methods

2.1 Establishment of social hierarchies and collection of samples

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

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

2.2 Sample fractionation

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

2.3 Chemical identification

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

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

Data were acquired under positive and negative polarity (separate runs) using the following parameters: spray voltage, 3.8 kV; sheath gas, 40 arbitrary units; auxiliary gas, five arbitrary units; heater temperature, 300 °C; capillary temperature, 350 °C; S-Lenses RF level, 64.9 %. The untargeted analysis was performed in data-dependent mode by selecting the three most intense ions under dynamic exclusion and collision-induced dissociation (CID) activation. The scan range was 100–1500 m/z. Targeted analysis of cholic acid (CA) and taurocholic acid (TCH) were performed under MRM using the following CID transitions: CA – MS2 (407→342–347); TCH – MS2 (514→352–354, 411–413, 495–497). Scan range was 100–600 m/z.

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

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

2.4 Electro-olfactogram

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

2.5 EOG cross-adaptation and binary-mixture tests

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

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

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

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

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

2.6 Mirror assay

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

2.7 Preference experiments

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

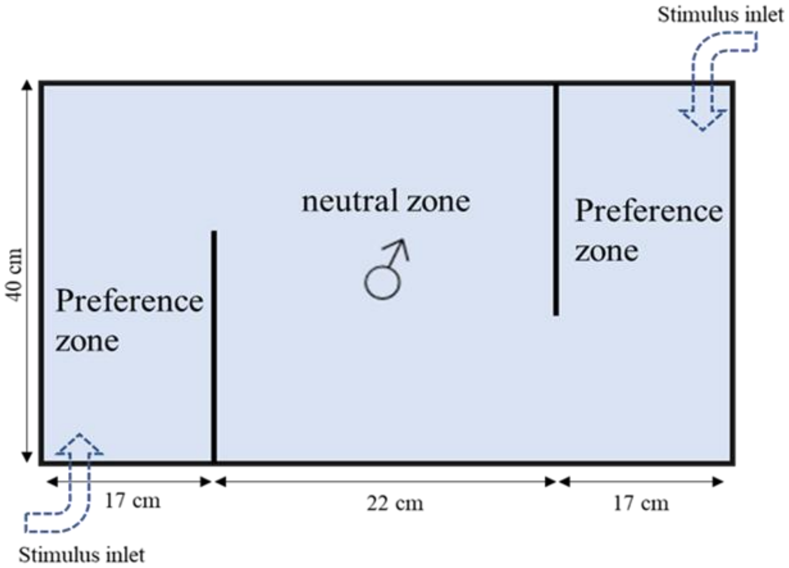

Fig. 1. Preference test aquarium design. Males were housed for 24 h in the test aquarium prior to testing. During testing, males were placed in the neutral zone and exposed to different stimuli that were injected simultaneously through the stimulus inlet into each preference zone. Each test consisted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

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

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

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

2.8 Statistical analysis

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

3. Results

3.1 Olfactory responses of males to faeces and their fractions

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

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

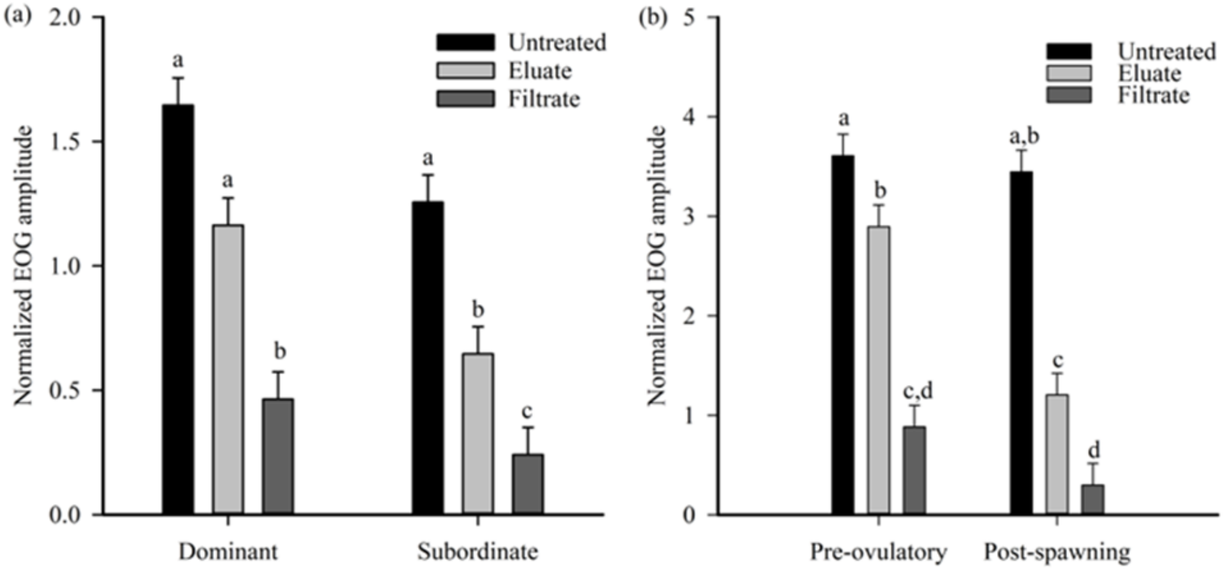

Fig. 2. Normalized EOG amplitude in response to untreated (raw) faeces and respective C18-SPE fractions from (a) males (n = 10) and (b) females (n = 7). EOG responses were obtained from males (n = 7; L_S: 11.8 ± 0.5 cm; weight: 46.4 ± 5.4 g) Different letters indicate significant differences ($p < 0.05$)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

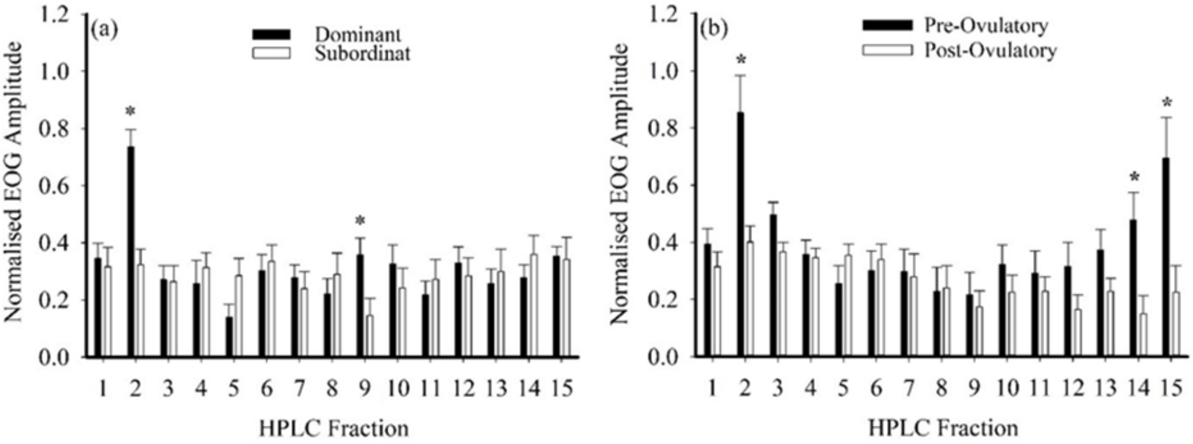

Fig. 3. Normalized EOG responses of tilapia olfactory epithelia to HPLC fractions of the C18-SPE eluate from (a) males (n = 10) and (b) females (n = 7). EOG responses were obtained were obtained from males (for male faeces n = 8; L_S: 10.9 ± 0.3; weight: 42.0 ± 2.4 g; for females faeces n = 6; L_S: 12.6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

3.2 Compounds identified in HPLC fractions

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

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

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

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

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

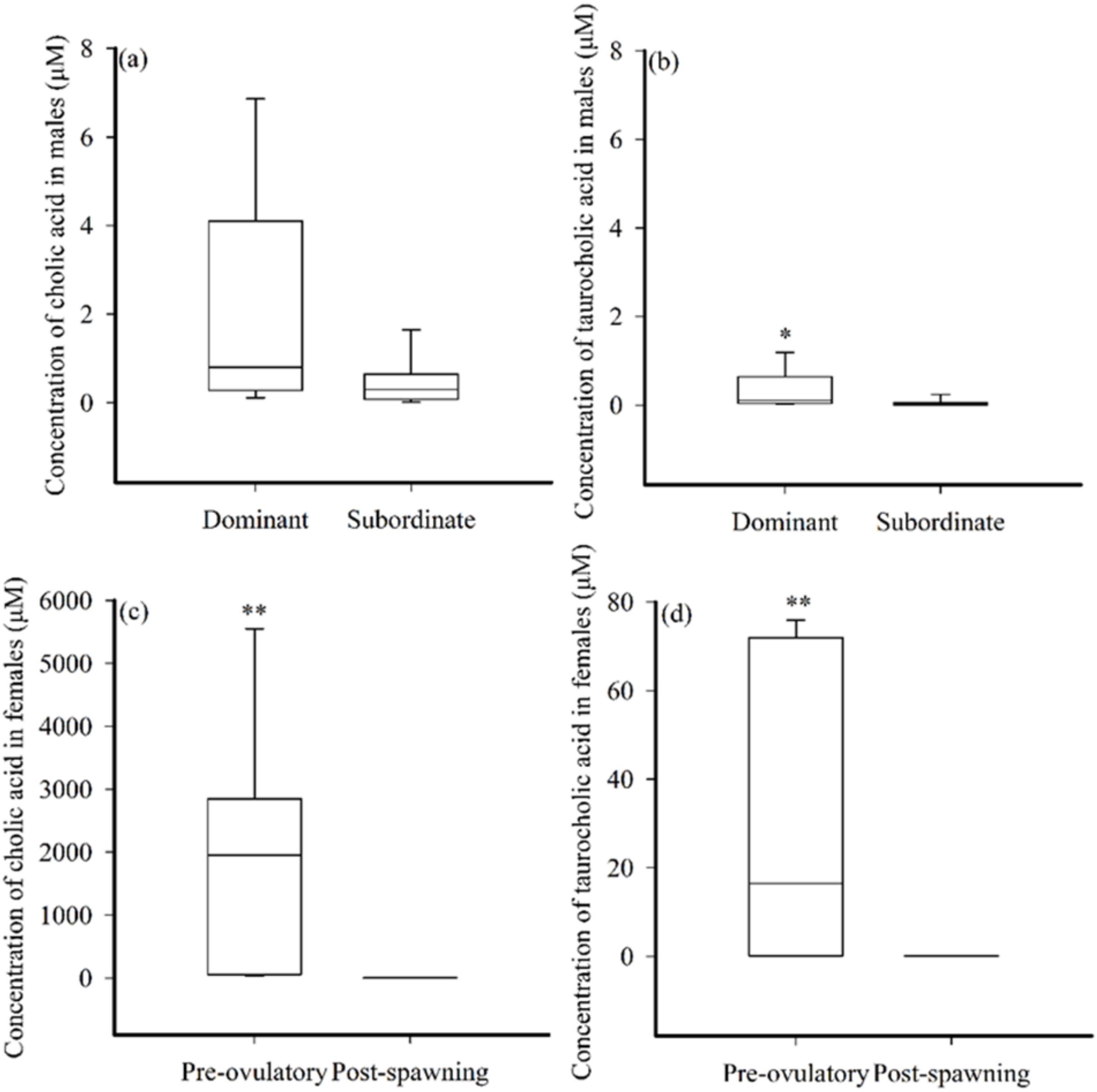

Fig. 4. Boxplot of the concentration of cholic acid and taurocholic acid (μM) in faeces from males (a and b, $n = 10$) and females (c and d, $n = 7$). The upper and lower edges of the box indicate the 25th and 75th percentile of the data set, the line in the box is the median. The asterisk (*) indicates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

3.3 Olfactory potency of identified compounds

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

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

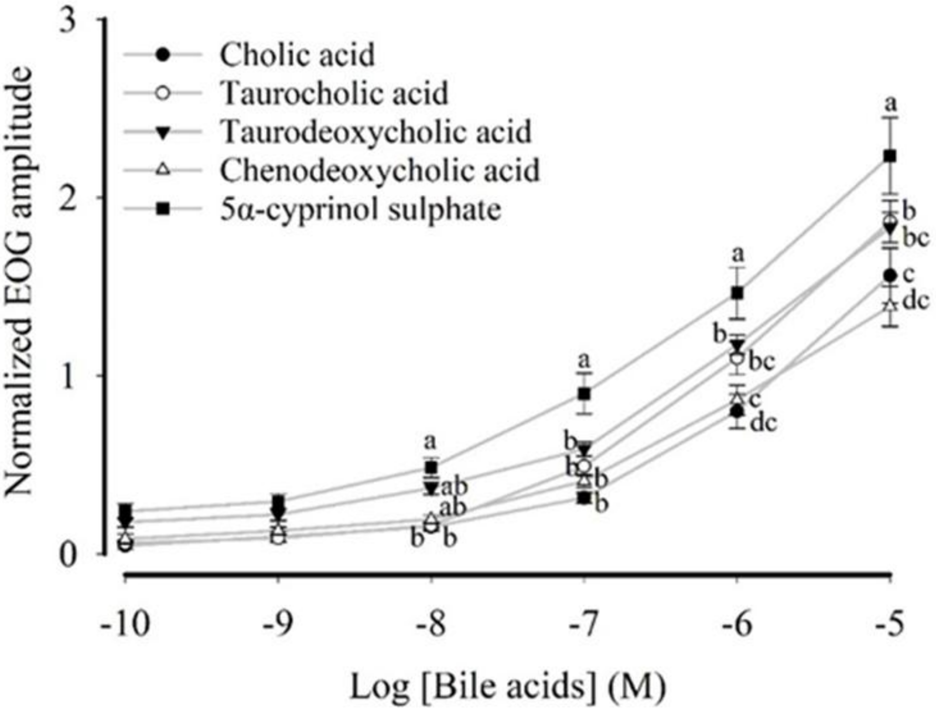

Fig. 5. Normalized EOG concentration–response curves to different concentrations of cholic acid, taurocholic acid, taurodeoxycholic acid, chenodeoxycholic acid, and 5α-cyprinol sulphate. Different letters indicate significant differences at each concentration ($p < 0.05$).

3.4 Cross-adaptation tests

Cross-adaptation tests showed that CYP-S and L-Ser act *via* independent receptor mechanisms (Fig. 6). Furthermore, adaptation to TCH did not reduce the EOG responses to CA, suggesting that these bile acids are acting through separate receptor mechanisms. However, the two taurine-conjugated bile acids, TDC and TCH, showed a strong reciprocity in their capacity to reduce each-others' EOG responses to a point that they were not significantly different from the self-adapted control (SAC). This suggests that they are acting *via* (a) common receptor(s).

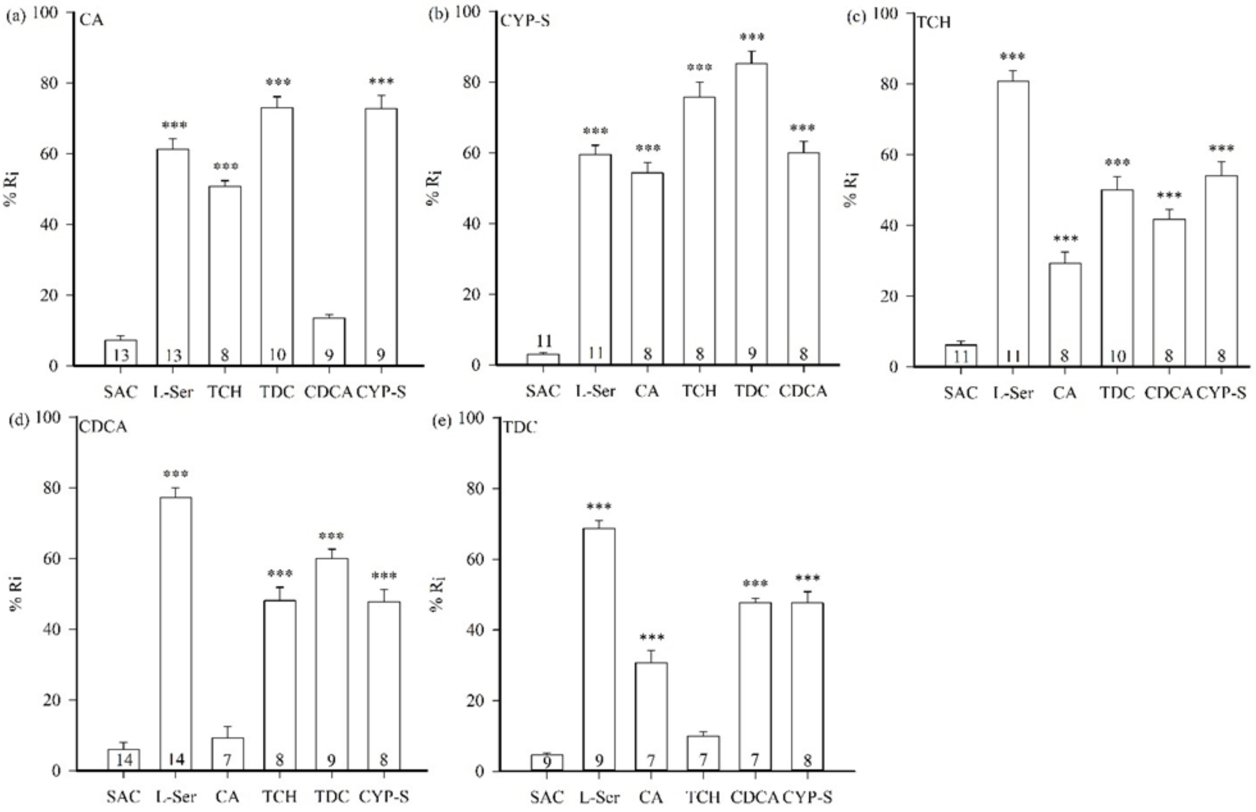

Fig. 6. Results of EOG cross-adaptation experiment expressed as a percentage of the initial unadapted response (% Ri) to the same bile acid delivered before cross-adaptation. Numbers in bars denote the number if independent replicates. The asterisks (***) indicate significant difference from the self-adapted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

3.5 Binary mixture tests

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

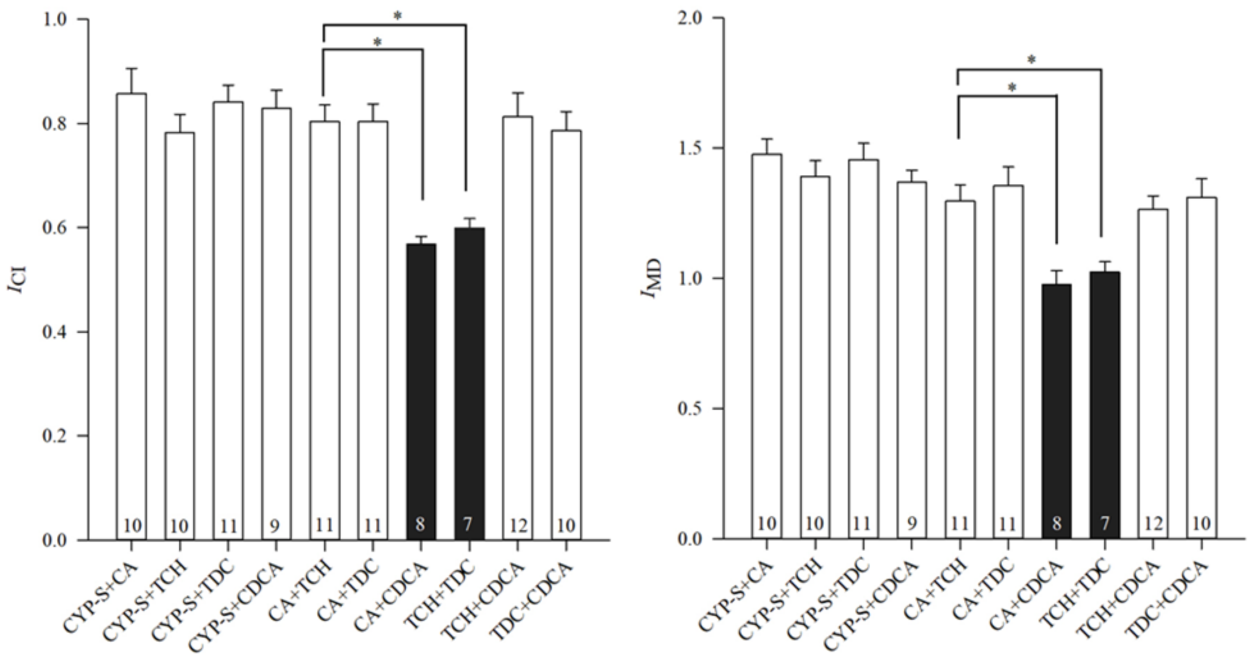

Fig. 7. Independent component and mixture discrimination indices from binary mixture test. Open bars indicate an interaction with a shared receptor mechanism. Black bars indicate interaction with an independent receptor mechanism. Numbers in bars denote sample size. The asterisk (*) indicates a significant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

3.6 Behavioural responses

Fractions 2 and 9 from male faeces had no significant effects on the biting behaviour of males towards the mirror image in comparison to the water control (one-way ANOVA, F 2,15 = 0.536, $p = 0.596$). Similarly, fractions 2, 14, and 15 from female faeces had no significant aggression-reduction effects on males (one-way ANOVA, F 3,20 = 0.394, $p = 0.759$).

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

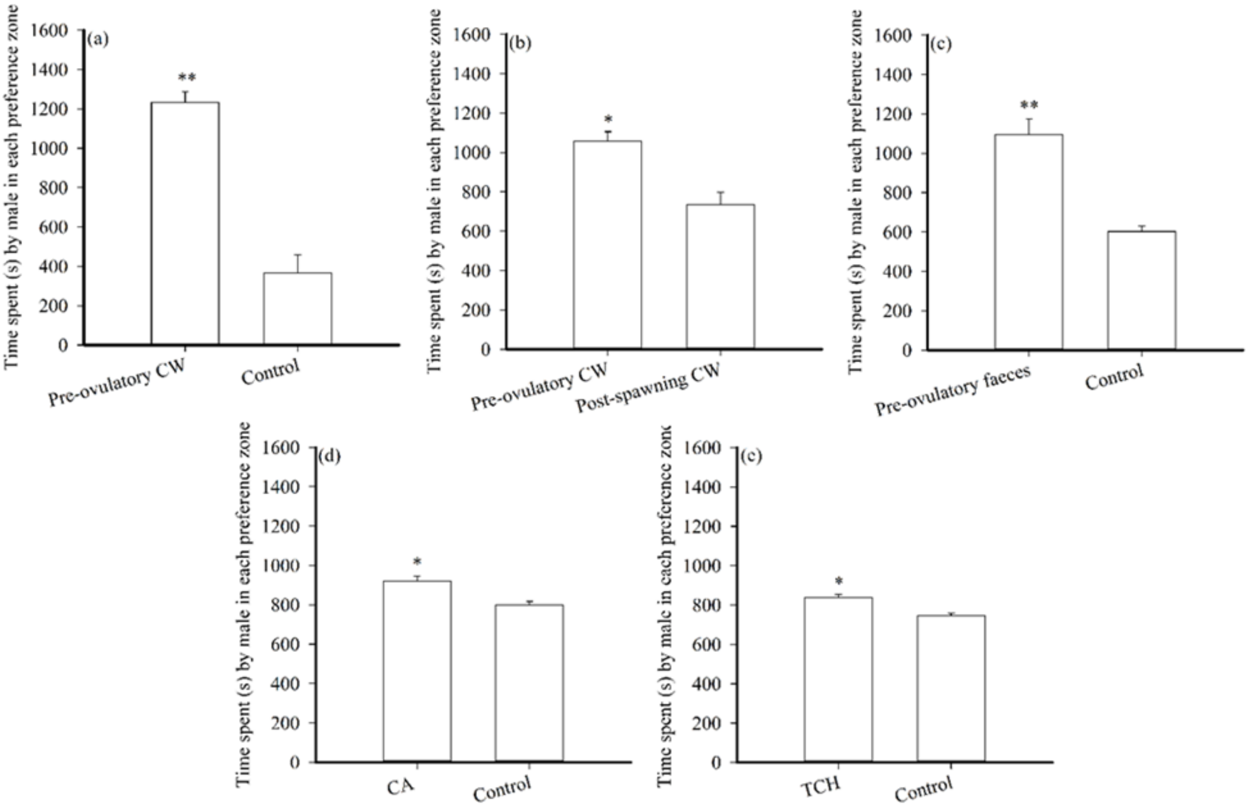

Fig. 8. The preference responses of focal males to: (a) conditioned water of pre-ovulatory female versus control; (b) conditioned water of post-spawning female versus control; (c) faeces extract of pre-ovulatory females versus control; (d) 10^{-3} M CA versus control; (e) 10^{-5} M TCH versus control.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

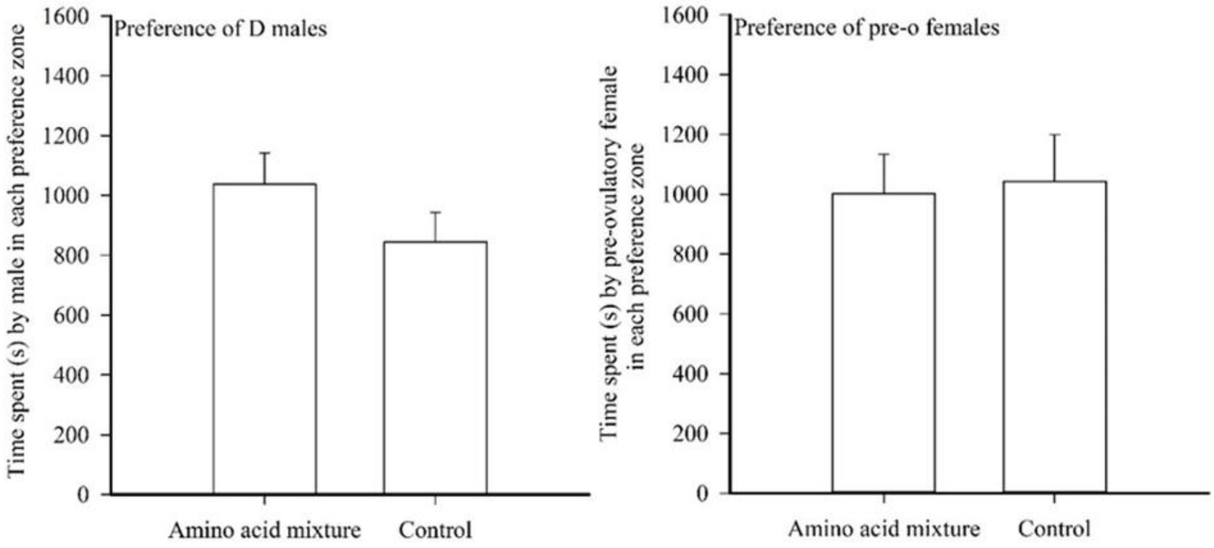

Fig. 9. The preference responses of dominant males (n = 9) and pre-ovulatory females (n = 9) to the mixture of amino acids.

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

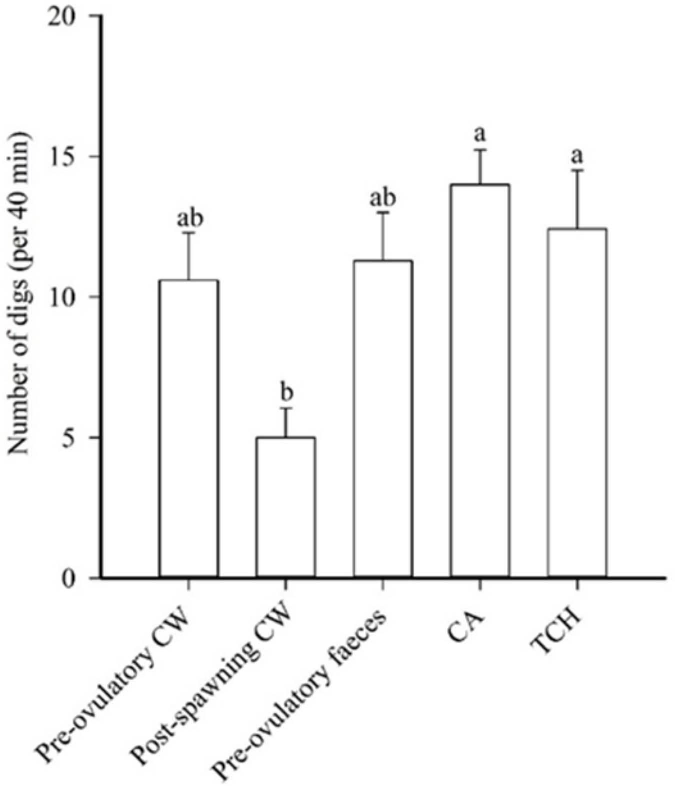

Fig. 10. The number of digging behaviours in focal males exposed to pre-ovulatory female-conditioned water (n = 5), post-spawning conditioned water (n = 5), pre-ovulatory faeces extract (n = 7), 10^{-1} M CA (n = 7), and 10^{-3} M TCH (n = 7) over 40 min of preference experiment. Different letters indicate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

4. Discussion

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

Dominant males and pre-ovulatory females release faeces with higher olfactory potency than subordinates and post-spawned females, respectively, because of the higher content of bile acids and amino acids. Based on the mirror assay, none of the faeces fractions from males and females, nor the amino acids or bile acids tested had aggression-reduction effect on focal males. This supports the hypothesis that the urine of dominant males is solely responsible for reducing aggression during interactions between rival males [7,42]. In contrast, the fact that pre-ovulatory female faeces, and in particular the bile acids CA and TCH identified in the faeces, attract males to a similar extent as pre-ovulatory female conditioned water and significantly more than post-spawning female conditioned water, suggest these bile acids may be part of a chemical bouquet related to reproductive behaviour prior to spawning. During mouthbrooding, Mozambique tilapia females do not feed and consequently produce less faeces than pre-ovulatory females [43]. Since the concentration of bile acids in the intestinal fluid is affected by diet, the higher levels of bile acids in the faeces of pre-ovulatory females may be perceived by males as indicative of female health status and reproductive potential. However, the higher concentrations of bile acids in the faeces of pre-ovulatory females cannot be solely linked to feeding, as males have lower levels of bile acids in their faeces and typically have higher food consumption rates [44]. Studies in mammals reported that the concentration of faecal bile acids among males and females can differ [45,46] and physiological factors such as hormonal changes can contribute to variation in excreted levels of bile acids between the sexes [47]. How bile acid release would be regulated is not yet clear; some evidence suggests that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) may restrict bile acid release [48], but whether there is a specific interaction with the reproductive system is not known. Interestingly, it has been shown recently that despite lacking sensitivity to $PGF_{2\alpha}$, *A. burtoni* males are attracted by females injected with $PGF_{2\alpha}$ and by fertile females, and this preference could be abolished by injection of PGF synthesis inhibitor [49]. Since the mechanism does not involve the hormonal receptor Ptgfr [49], it is tempting to speculate that bile acids, in particular CA and TCH, released by females under the regulation of $PGF_{2\alpha}$, are the chemical cues responsible for the preference of males by pre-ovulatory (fertile) females in Mozambique tilapia and perhaps other cichlids. Further studies are required to confirm this hypothesis.

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

Although olfactory sensitivity to bile acids is widespread in fish [reviewed by 25], only in the sea lamprey they have been established to have a pheromonal role, including as sex pheromones [*e.g.,* [53], [54], [55]]. In contrast, in teleost fishes, only two classes of reproductive pheromones, steroids and prostaglandins, have been demonstrated, who also play a fundamental role as hormones during the reproductive cycle, and readily produce polar metabolites highly soluble in water [9]. Thus, it appears that pheromone candidates tend to have high water solubility and are a product or by-product of a physiological state of social relevance open the possibility of chemical communication [9]. However, it is still poorly understood how fish have evolved to use bile acids as chemical cues, but probably involved externalization of bile acid receptors to the olfactory epithelium [25].

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

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

Author Contributions

Samyar Ashouri, José P. Da Silva, Adelino V.M. Canário, and Peter C. Hubbard conceived the study. Samyar Ashouri carried out experiments and data analysis. José P. Da Silva carried out MS-based structure elucidation. Samyar Ashouri wrote the first draft. Peter C. Hubbard, and Adelino V.M. Canário edited the manuscript.

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Declaration of Competing Interest

The authors declare no competing interests.

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Data availability

Data will be made available on request.

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CHAPTER V

A putative pheromonal role for 17β-estradiol 3-glucuronate in Mozambique tilapia (*Oreochromis mossambicus*)

Samyar Ashouri, Adelino V.M. Canário and Peter C. Hubbard

A putative pheromonal role for 17β-estradiol 3-glucuronate in Mozambique tilapia (*Oreochromis mossambicus*)

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Abstract

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

Keywords: Social hierarchy; Cichlid; Chemical cues; Visual cues; Conjugation; Behavior

1. Introduction

Among teleost fishes, the largest and the most diverse group of vertebrates, several establish social hierarchies and use multiple sensory channels, including visual, acoustic, and chemical, to transfer information about their social status (Escobar-Camacho & Carleton, 2015). Fishes live in an aquatic environment, often in the dark, and have developed a sensitive olfactory organ to receive and process chemosensory cues released by conspecifics and heterospecifics, which can elicit a variety of physiological and behavioural responses (Bowers et al., 2023; Kasumyan, 2004). If these compounds benefit both the sender and the receiver, they are called pheromones (Sorensen & Stacey, 2004). Various chemical compounds with pheromonal activity have been identified in fish, including steroids, bile acids, prostaglandins, and amino acids (Keller-Costa et al., 2014b; Kobayashi et al., 2002; Li et al., 2023; Li et al., 2002; Loranger & Bertram, 2016; Yambe et al., 2006).

Steroids are vital regulators of several biological processes, such as sex determination, embryonic development, and sexual maturation. Steroid hormones are derived from the common precursor cholesterol and are classified into C18, C19, C21, and C24 based on the number of carbon atoms in their chemical structure (Miller, 2013; Penning et al., 2019). In teleost fishes, steroid hormones and their metabolites, free or conjugated with glucuronide or sulphate, have been identified as sex pheromones (Keller-Costa et al., 2014b; Scott & Sorensen, 1994). These hormonally derived steroids have been selected to have a pheromonal function in fishes for several reasons. As hormones, they are biologically active at very low concentrations. Furthermore, they provide information about the physiological state of the senders, and when released, they provide a correlate of reproductive readiness. Furthermore, their conjugates (i.e., sulphates or glucuronides), have increased water solubility. The hormonal-pheromone system of goldfish (*Carassius auratus*) is a good example of the pheromonal role of free steroids and their conjugates in a teleost fish, where the maturation-inducing steroid (4-pregnen-17,20β dihydroxy-3-one) is released into the water by ovulated females both in its free form and conjugated with glucuronic acids or sulphate. The preovulatory pheromonal complex promotes spawning synchrony and stimulates milt production in males (Sorensen & Stacey, 2004; Stacey & Sorensen, 2002). In Mozambique tilapia males release 5β-pregnane-3α,17α,20β-triol 3-glucuronate and its 20α-epimer (20α- and 20β-P-3-G) as a primer sex pheromone through the urine pulses that affect the female endocrine system and stimulate the production of 17,20β-P (Keller-Costa et al., 2014b).

Previous experiments on Mozambique tilapia have shown that pre-ovulatory females release urine and faeces with higher olfactory potency in comparison to post-spawning females (Almeida et al., 2005; Ashouri et al., 2023). Moreover, male tilapia can distinguish the ovulation state of females on the basis of chemical odours, and increase their urine pulses in the presence of pre-ovulatory females (Miranda et al., 2005). Huertas et al. (2014) showed that pre-ovulatory female tilapia releases a higher concentration of 17β-estradiol (E_2) and its 3-glucuronidated form through the urine (100–150 ng ml^{-1}) than post-spawn females. E_2 is produced by the granulosa cells of secondary oocytes and its production decreases at the end of vitellogenesis prior to oocyte final maturation and ovulation (Babin et al., 2007; Servili et al., 2020; Zohar et al., 2010). Based on the reproductive role of E_2 during vitellogenesis and oocyte growth and studies showing fish olfactory sensitivity to E_2, it could be hypothesized E_2 could act as a chemical cue released by pre-ovulatory females, signalling their reproductive development.

For example, round gobby (*Neogobius melanostomus*) and Mozambique tilapia show olfactory sensitivity to free and conjugated E_2 (Keller-Costa et al., 2014a; Murphy et al., 2001). In the goldfish, plasma E_2 during vitellogenesis can stimulate the urinary release of an unidentified chemical cue that acts as a male attractant to initiate spawning synchrony (Kobayashi et al., 2002). Furthermore, it has been proposed that E_2 released by female zebrafish, may act as sex pheromone and attract the male conspecifics (Van den Hurk & Lambert, 1983; Van den Hurk & Resink, 1992).

However, fish communicate through multiple sensory channels, and in tilapia, visual and chemical signals together yield a stronger behavioural response than each signal separately (Barata et al., 2007).

The present study was designed to explore (1) the preference of focal males for visual and/or chemical stimuli in relation to the female tilapia reproductive stage and (2) whether E_2 glucoronate can evoke a preference response in focal males.

2. Materials and methods

2.1 Ethics

Fish maintenance and the experimental procedures were carried out in accordance with Directive 2010/63/EU on protecting animals used for scientific purposes under license number 0421/000/000/2020 granted by the Directorate-General for Food and Veterinary of Portugal.

2.2 Mozambique tilapia

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

2.3 Preference experiment

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

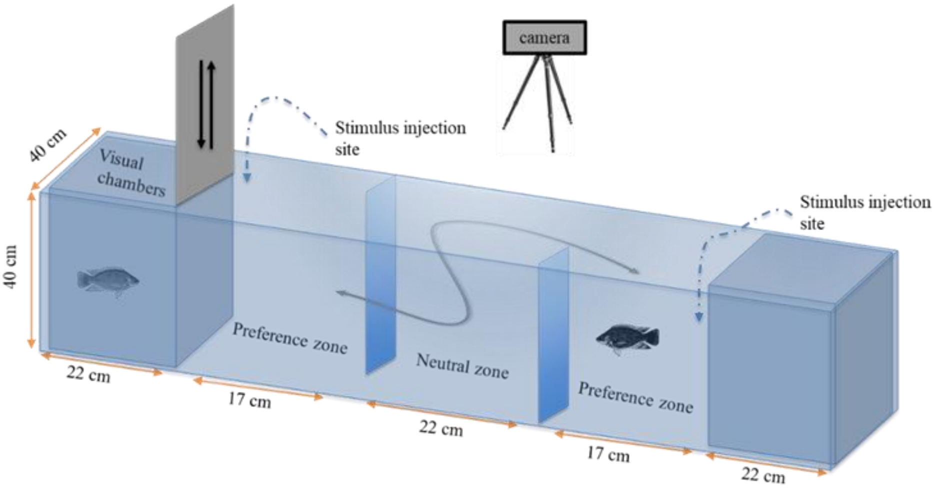

Fig. 1. Preference aquarium. Focal males were housed the preference aquarium for 24 h in prior to recording the behaviours. In each trial, focal males were exposed to different stimuli provided through a peristaltic pump (for chemical stimuli) and/or visual chambers (for visual stimuli) in each preference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

2.4 Experimental protocol

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

The control refers to one side of the preference tank receiving neither visual nor chemical stimuli. In the trials with only visual stimuli, the females of different ovulation statuses were taken from a different family tank of focal males and randomly isolated in the visual chambers with the opaque plate in place. In the trials with only chemical stimuli, the visual chambers remained empty, and only conditioned water obtained from females of different ovulation statuses was injected into the preference zones. For visual and chemical stimuli, the females that produced the conditioned water being tested were used. A total of 92 focal males were used, of which only 73 (weight: 240.1±5.3 g; L_s: 20.2±0.2 cm) reacted to visual and chemical stimuli. Almost 21% of focal males remained inactive and immobile without swimming in the experimental tank for more than 20 minutes. These focal males were removed from the experiment.

2.5 Preparation of visual and chemical stimuli

The visual stimuli were provided directly by housing pre-ovulatory and post-spawning females individually in the visual chambers. The chemical stimuli (pre-ovulatory and post-spawning females' conditioned waters) were obtained according to Miranda et al. (2005). Briefly, the females with different ovulation statuses were isolated in buckets filled with 20 L aerated, dechlorinated tap water for 2 hours; the water was collected and used immediately. EOG experiments have shown that the Mozambique tilapia threshold of detection for 17β-estradiol 3-glucuronate was about 10^{-10} M (Keller-Costa et al., 2014a). The concentration of 17β-estradiol 3-glucuronate (Sigma Aldrich Co. Ltd., St. Louis, USA) delivered to the preference zones was estimated to be 10^{-9} M from a stock solution of 10^{-5} M in distilled water.

2.6 Statistical analysis

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

3. Results

There was a statistically significant three-way interaction between the reproductive state of females, stimulus type and preference zone ($F_{(2,98)}$=54.746, $p < 0.001$). There were significant two-way interactions between stimulus type and preference zone ($F_{(2,98)}$=69.836, $p < 0.001$) and reproductive state of females and preference zone (F(1,98)= 28.409, $p < 0.001$) but not between stimulus type and reproductive state of females ($F_{(2,98)}$=0.624, $p = 0.538$) (Figure 2). The time spent by males in the stimulus preference zone, irrespective of stimulus or female type, was approximately 5 to 10 times the time spent in the preference zone of the control ($F_{(1,98)}$=1486.904, $p < 0.001$). The exception was males spending similar time in the preference zone of post-ovulatory females conditioned water and control water. Males were more attracted by pre-ovulatory females than by post-ovulatory females, both visually ($p < 0.05$) and chemically ($p < 0.05$). However, while males were equally visually and chemically attracted to pre-ovulatory females, they were less attracted by the chemical stimulus compared to the visual stimulus of post-spawning females ($p < 0.05$).

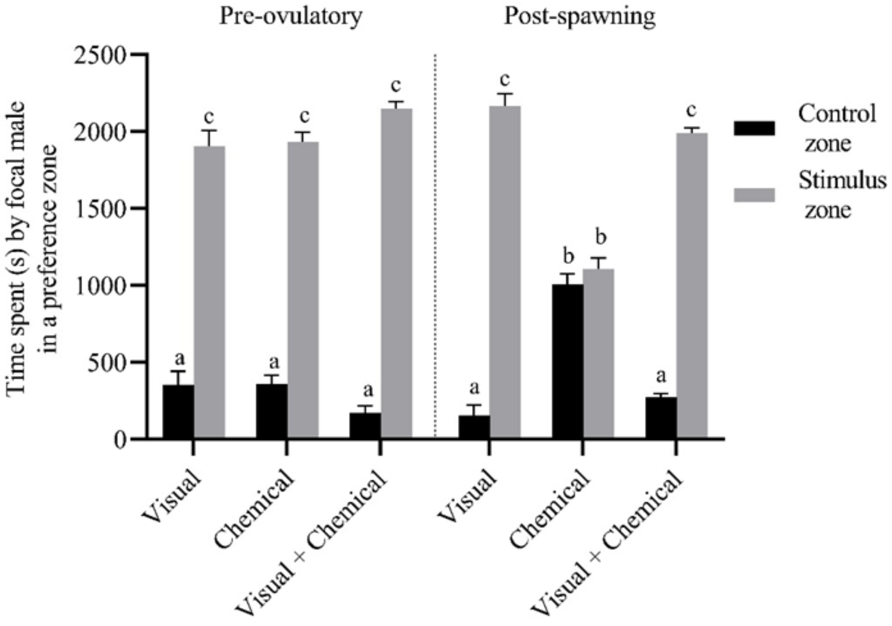

Fig. 2. Time (s) spent by focal males in a preference zone – control side (black bar) or stimulus side (grey bar) - in response to visual, chemical, or visual + chemical stimulus from pre-ovulatory and post-spawning females. Bars are mean ± S.E.M. Different letters represent statistically significant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

No digging was observed in the control zone, indicating that a visual or chemical stimulus is necessary. There was a significant interaction between the reproductive stage of females and stimulus type ($F_{(2,49)}$=3.631, $p = 0.034$). The amount of digging by focal males was higher when the stimulus was visual than when it was chemical ($p < 0.05$), with no additional effect of adding the chemical stimulus to the visual stimulus (Figure 3. left). Digging was similar for pre- and post-spawning females if the stimulus was visual or visual + chemical but higher for pre-ovulatory females when the stimulus was chemical ($p < 0.05$).

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

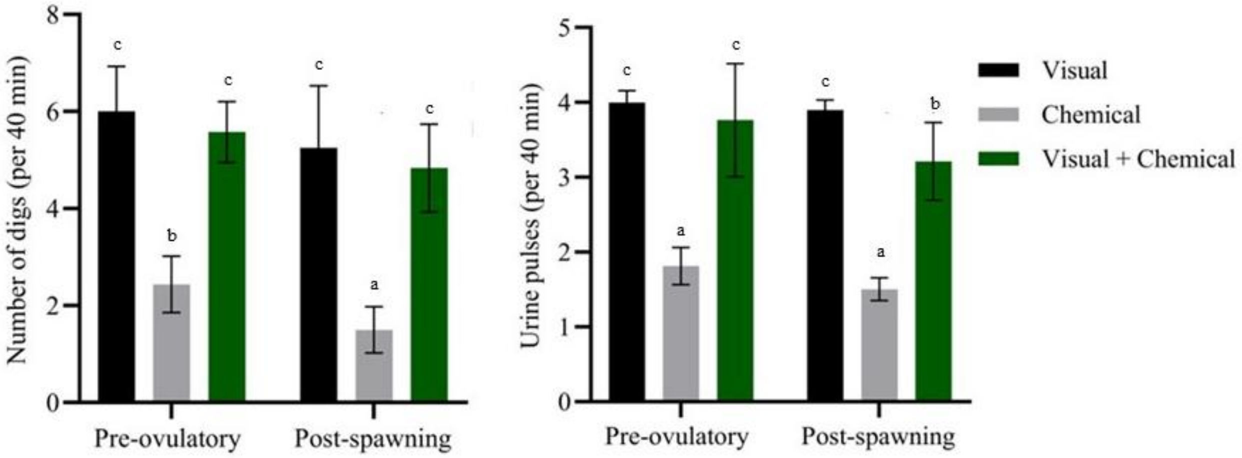

Fig. 3. Number of digs (left) and urine pulses (right) in response to visual, chemical, or visual + chemical stimulus from pre-ovulatory and post-spawning females. Bars are mean ± S.E.M. Different letters represent statistically significant differences ($p < 0.05$).

There was a significant effect of chemical treatments compared to the control visual cue only ($F_{(3, 32)} = 35.07$; $p < 0.001$). Focal males spent significantly more time near a chemical + visual stimulus, in particular, if the stimuli were from pre-ovulatory females, than near a visual stimulus only as control ($p < 0.001$, Figure 4). Furthermore, estradiol-17β glucuronate had the same effect on the time spent by males in the preference zone as conditioned water from pre-ovulatory females ($p < 0.05$).

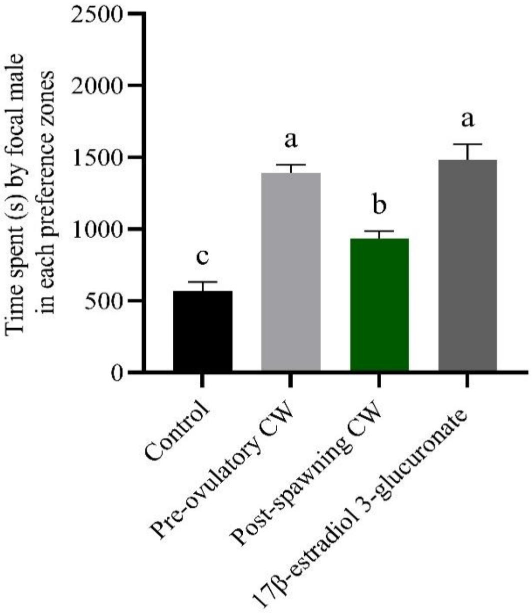

Fig. 4. Time (s) spent by focal males in preference zones receiving visual stimulus only (control) and a combination of visual and chemical stimuli (pre-ovulatory conditioned water [CW], post-

spawning conditioned water [CW], and 17β-estradiol 3-glucuronate). Bars are mean ± S.E.M. Different letters represent statistically significant differences ($p < 0.05$).

There was also a significant effect of chemical treatments on digging ($F_{(3, 32)} = 17.30$; $p < 0.001$) (Figure 5. left). However, only focal males exposed to pre-ovulatory conditioned water showed significantly higher digging activity than the control visual treatment ($p < 0.001$).

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

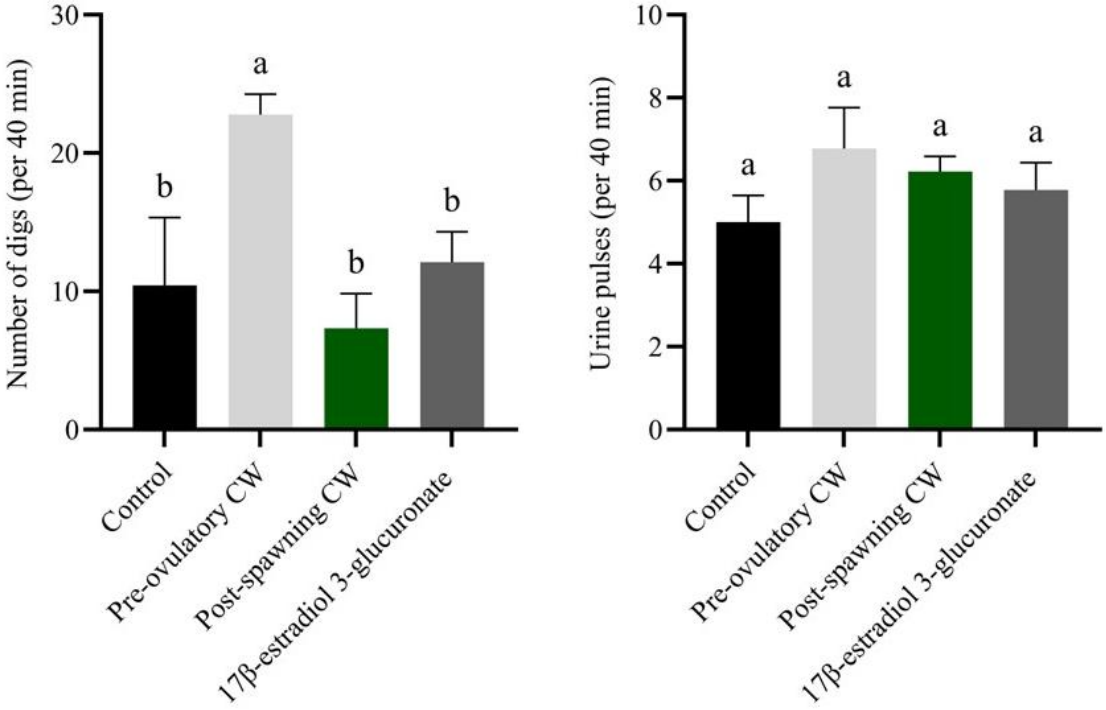

Fig. 5. Number of digs (left) and urine pulses (right) by focal males in preference zones receiving visual stimulus only (control) and a combination of visual and chemical stimuli (pre-ovulatory conditioned water [CW], post-spawning conditioned water [CW], and 17β-estradiol 3-glucuronate). Bars are mean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

4. Discussion

The present study showed that male tilapia discriminates visual and chemical stimuli from pre-ovulatory and post-spawning females. While focal males spent a similar time in a preference zone filled with post-spawning conditioned water or with no stimulus, their preference response to visual stimulus from post-spawning females was higher than pre-ovulatory females. These observations suggest that males are not only attracted to olfactory cues from pre-ovulatory females, but visual cues are sufficient to attract focal males to the preference zones, as shown by the preference for post-spawning females' visual cues rather than to control without stimulus. Previous studies have shown that conditioned water from pre-ovulatory female tilapia provide stronger olfactory cues to males than post-spawning females, indicating that potent odorants contained in the conditioned water pre-ovulatory females are responsible for the higher attraction of focal males in comparison to post-spawning females' conditioned water (Miranda et al., 2005). Our study arrived at a similar conclusion as pre-ovulatory conditioned water was more attractive for focal males in comparison to post-spawning conditioned water

It seems that tilapia males rely on chemical and visual stimuli from pre-ovulatory females, which indicates that they play a crucial role in mate choice and recognition of ovulation status. Visual assessment could use characters such as female colouration, body shape, body posture, and behaviour (Escobar-Camacho & Carleton, 2015; Rowland, 1999). In some cases, at least in changing colouration, it might not be possible to recognise the ovulation status of females by a human observer. Several studies have shown how visual stimuli can influence the effect of olfactory stimuli. In round gobies (*Neogobius melanostomus*) an imitation of a blank-coloured model of a reproductive male (visual stimulus) attracted reproductive females more easily than olfactory cues (urine) (Yavno & Corkum, 2010). In Nile tilapia (*Oreochromis niloticus*), which possess a highly sensitive visual system, female visual cues alone are sufficient to evoke reproductive behaviours and affect gonadal development (Castro et al., 2009). However, recognising a potential mate in a social hierarchy may not rely only on visual or chemical cues alone. Swordtail (*Xiphophorus nigrensis*) females use both visual and chemical cues to distinguish conspecifics from heterospecifics (Crapon de Caprona & Ryan, 1990). Exposure of male guppies to both visual and chemical stimuli of virgin females has confirmed that chemical cues are primarily used by males to assess and discriminate the reproductive status of females (Guevara-Fiore et al., 2009). In a social hierarchy, different sensory modalities are likely to be used by conspecifics to guide them towards the potential reproductive mate. Since chemical cues are dissolved in the water, they may orient the fish from longer distances followed by visual cues for further and detailed assessment of mate in closer vicinity (Crapon de Caprona & Ryan, 1990).

Recently, we have shown that focal males increase their nest-digging behaviour in response to conditioned water and faeces extract from pre-ovulatory females compared to post-spawning females' conditioned water (Ashouri et al., 2023). In the present study, the number of digs and urine pulses, both as courtship behaviour in male tilapia (Baerends & Baerends-van Roon, 1950; Barata et al., 2007) were quantified and focal males showed higher number of digs in response to conditioned water from pre-ovulatory in comparison to post-spawning females. This might be explained by a higher concentration of odours contained in the pre-ovulatory female-conditioned water, which could include bile acids, amino acids and/or steroids (Ashouri et al., 2023). Body fluids such as urine may be responsible for evoking higher nest-digging behaviour by focal males since urine from pre-ovulatory has higher olfactory potency than post-spawning females and elicits specific courtship behaviour in male tilapia (Miranda et al., 2005). However, the amount of male digging behaviour was not distinguishable in the presence of chemical and visual stimuli sent by pre-ovulatory and post-spawning females. One possible explanation is that the experimental design did not allow focal males to assess the females' reproductive status through courtship as they would if allowed to interact. Tilapia males typically show a wide range of sexual behaviours to attract and orient the pre-ovulatory females to the digging spot, including chasing and nipping, together with urinary sex pheromones (Keller-Costa et al., 2014b). In the absence of chemical stimuli, when focal males cannot physically examine the females, it can be expected that similar reproductive behaviour is evoked by focal males in response to pre-ovulatory and post-spawning females. Physical assessment, also known as tactile interaction, has been seen in other species to mediate anti-predator responses within a group-based hierarchy (Riley et al., 2019). Other species such as the Mexican molly (*Poecilia sphenops*) have a mustache-like structure used by males to attract females and provide tactile information during the nipping (Schlupp et al., 2010).

Focal males were more strongly attracted to a visual stimulus plus pre-ovulatory conditioned water than to a visual stimulus only or a visual stimulus plus post-spawning conditioned water. These results are different from when the control preference zone does not contain a stimulus in which case focal males showed no differences in preference between visual plus chemical stimulus from pre-ovulatory and post-spawning females. Having a choice of stimuli may be more discriminatory and better highlight the differences between stimuli.

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

However, unlike the effect on attraction, digging behaviour was only increased in pre-ovulatory females in conditioned water, as previously shown (Ashouri et al., 2023), and no differential effect on urination was observed for any of the treatments. This indicates that pre-ovulatory females likely produce an additional component other than E_2 3-glucuronate that stimulates digging behaviour and that urination results only from a visual stimulus. The component that stimulates digging could be another sexual steroid perhaps linked to final oocyte maturation, such as 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) or its metabolites, whose concentration increases dramatically during that period (Tokarz et al., 2015).

Tilapia males release a 17,20β-P metabolite in the urine to attract pre-ovulatory females and prime their endocrine system (Keller-Costa et al., 2014b). However, the current study brings new elements to the tilapia pheromonal system by highlighting E_2 3-glucuronate as a possible signal females use to attract dominant males. Once the focal males were exposed to pre-ovulatory conditioned water or E_2 3-glucuronate, their decision to spend more time in their vicinity increased, acting in conjunction with the visual appearance of females and behavioural interactions.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Author Contributions

Samyar Ashouri, Peter C. Hubbard, and Adelino V.M. Canário conceived the study. Samyar Ashouri carried out experiments and data analysis. Samyar Ashouri wrote the first draft. Peter C. Hubbard, and Adelino V.M. Canário edited the manuscript.

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CHAPTER VI

General discussion and conclusions

The present PhD thesis explored four important aspects of chemical communication in the Mozambique tilapia from behavioural and chemical perspectives. In this chapter, we will conclude by addressing the following questions:

1. How does dominant male urine modulate aggressive behaviours in response to a mirror image or a real male intruder?
2. What chemical compound(s) is(are) responsible for such modulation?
3. Could conspecifics faeces evoke a similar aggression-reduction response as dominant male urine?
4. How do females influence the sexual and aggressive behaviours of male conspecifics?

For almost three decades, the chemical communication of Mozambique tilapia has been investigated using different experimental approaches such as behavioural experiments (mainly mirror assay), analytical chemistry, and electrophysiology. Briefly, the role of urine as a vehicle to carry sexual pheromone (pregnanetriol-3-glucuronates) and signal dominance (aggression-modulating) in the social hierarchies of tilapia has been well established. However, we have tried to clarify previous findings using new analytical methods and gain more profound knowledge and more complete answers to the above questions.

To answer the first question, the effect of dominant male urine in reducing aggressive behaviours was tested using two bioassays: mirror experiments and interaction with live opponents, wherein urination was prevented. There were three important outcomes: firstly, both setups confirmed the previous findings that dominant male urine possesses an aggression-modulating effect (Barata et al., 2007; Keller-Costa et al., 2016; Saraiva et al., 2017). Secondly, a mirror assay is a reliable behavioural paradigm - at least in Mozambique tilapia - since soon after exposure of focal males to their mirror image, the highest-ranked aggressive behaviour, biting, is displayed by focal males (Cattelan et al., 2017). Thirdly, we have seen subordinate males react toward their mirror image with lower latency than dominant males.

The formation of aggressive behaviours in dominant (territorial) males from low to high levels typically starts by increasing the number of urinary pulses (Fig. 1a) (Barata et al., 2007). Next, a sideways orientation to the intruder is performed by the focal male (Arnott & Elwood, 2009) while dorsal fins are erected, opercula open widely, and the fish appears larger (Fig. 1b). Tail beating is the subsequent behaviour before biting and mouth-to-mouth fighting (Fig. 1c,d).

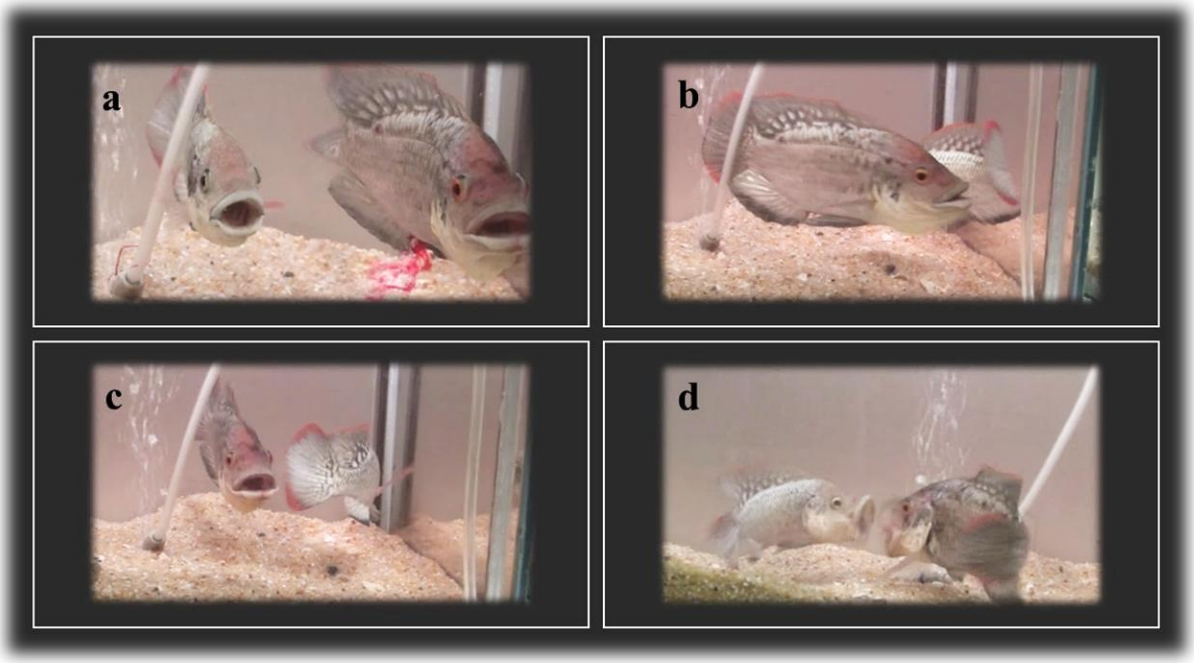

Figure 1 - Aggressive behaviours of dominant males during aggressive disputes with rivals escalate from increased urinary pulses (a) to sideways posturing and dorsal fin erection (b), tail beating (c), and eventually mouth-to-mouth (d).

In our study, we found that in the mirror assay focal males react faster to their mirror image than they do to a real opponent without any harmful effects. More importantly, the mirror assay is time-saving for testing the aggression-modulating effect of urine, its fractions and other chemicals that may influence aggression. We found that the duration of each set of mirror assays can be reduced from 15 minutes to 10 minutes without loss of information (Ashouri et al., 2024; Keller-Costa et al., 2016).

We have used the mirror assay to investigate the potential aggression-modulating effect of dominant male urines and their fractions. Also, we used the mirror assay to test whether HPLC faeces fractions from dominant males and pre-ovulatory females, which evoked higher EOG responses compared to subordinate males and post-spawning females, could have the same aggression-modulating effect in focal males (chapter 3 and 4).

The chemical fractionation of biological fluids, followed by electro-olfactogram (EOG) and isolation of the chemicals involved are common methods to identify pheromones in insects and fishes (Stewart et al., 2013). Most known fish pheromones have been identified through the use of this experimental approach (Appelt & Sorensen, 2007; Katare et al., 2011; Keller-Costa et al., 2014b; Li et al., 2002; Li & Sorensen, 1997; Poling et al., 2001; Sorensen et al., 2000;

Yambe et al., 2006). In the case of Mozambique tilapia male urine, isolation and fractionation have not only shown that high concentrations of steroidal sex pheromone (P3Gs) exist in the urine of dominant males, but amino acids also appear to be released through the urine of dominant males in higher concentration than in subordinate males (Kutsyna et al., 2016). The method that was used for the measurement of amino acids was SPE with C18 cartridges to separate aqueous and hydrophilic fractions of urine, followed by GC-MS. In the present study, we used SPE with C18 cartridges to separate hydrophilic and hydrophobic compounds from faeces. For urine samples, we used SPE with C18 cartridges plus mixed-mode anion exchange (MAX) and mixed-mode cation exchange (MCX) cartridges to obtain further insight into the chemical properties of the analytes from the aqueous and eluate fractions obtained from the C18 cartridge. This is an advance compared to previous studies in which only C18 cartridges were used (Keller-Costa et al., 2014b). MAX and MCX cartridges were chosen since filtrate and eluate fractions of C18 cartridges alone had no aggression-reduction effect on focal males in mirror assays. Interestingly, the reconstitution of C18 fractions restored the aggression-reduction effect of DMU (Keller-Costa et al., 2016). Our mirror assays for testing the MAX and MCX fractions of DMU failed to support our hypothesis that basic or acidic compounds could be responsible for the aggression-reduction effect of DMU. The absence of this effect when MAX and MCX cartridges were used might be due to the loss of retention, or decomposition of target molecules after fractionation (Dittmar et al., 2008). The other explanation would be the use of weak acid (formic acid) and weak base (ammonium hydroxide) for washing and retaining urine fractions in the MAX and MCX cartridges, which could have hydrolysed bioactive compounds. Some fish pheromones are composed of conjugated metabolites of amino acids and steroids, that may undergo hydrolysis or similar degradation in contact with acidic or basic conditions (Brown et al., 2002; Porteus et al., 2018). EOG of the chemical compounds identified in urine and faeces of tilapia indicated that the olfactory epithelium of tilapia was sensitive to steroids (17,20βP and estradiol glucuronides), amino acids and bile acids (Huertas et al., 2010; Keller-Costa et al., 2014a; Kutsyna et al., 2016). Four classes of chemical compounds have been identified with pheromonal priming and releasing effects in fishes including steroids, bile acids, amino acids, and prostaglandins (Appelt & Sorensen, 2007; Keller-Costa et al., 2014b; Li et al., 2002; Poling et al., 2001; Sorensen et al., 2000; Yambe et al., 2006). Therefore, to address our second and third questions about the biological functions of amino acids and bile acids identified in the urine and faeces, we conducted two behavioural bioassays: mirror assays and preference tests. Briefly, both experiments did not support the hypothesis that amino acids alone may signal dominance or convey mate-choice information in the Mozambique tilapia (Kutsyna et al., 2016); females also release amino acids *via* the faeces (Ashouri et al., 2023). Similarly, we have used two bioassays (mirror assay and preference test) to investigate the possible role of bile acids identified in the faeces of dominant males and pre-ovulatory females in carrying dominance signals. The outcome of the mirror assays with fractions containing bile acids showed no significant reduction of aggression in males. This might be due to the nature of bile acids that are released after foraging and remain in the water to transfer delayed and long-distance information (Buchinger et al., 2014). Another explanation to is the decision of territorial males to start releasing high-frequency urine pulses soon after the entrance of an unwelcomed intruder (Barata et al., 2008). These urine pulses theoretically should convey dominance signals that relay quickly to the intruder in order to prevent further energy-consuming and high-risk battles (Keller-Costa et al., 2012; Maruska & Fernald, 2012). However, our preference experiments with cholic acid and taurocholic acid showed that males were responsive to these two bile acids, suggesting an attractant role for pre-ovulatory female faeces (containing the bile acids). Consistent with our results, Hubbard et al. (2017) reported that intestinal fluid from dominant males of chameleon cichlid *Australoheros facetus* evoked higher EOG apmlitude in comparison to subordinates. Furthermore, it has been shown that in Senegalese sole (*Solea senegalensis*) and large yellow croaker (*Larimichthys crocea*), bile acids such as cholic acid, taurocholic acid, taurolithocholic acid, taurochenodeoxycholic acid, chenodeoxycholic acid, and taurodeoxycholic acid are released through the intestinal fluid (Velez et al., 2009; Zhu et al., 2023).

As stated earlier (General Introduction, Chapter 1), a social hierarchy consists of males and females, each playing distinct roles to stabilise the hierarchy to find mates and produce offspring (Milewski et al., 2022; Taborsky, 1994). In Chapter 5 of the present thesis, we designed a preference experiment to investigate the relative role of visual cues compared to chemical cues (female conditioned water) and whether 17β-estradiol 3-glucuronate contributed to the chemical signal of pre-ovulatory females. Consistent with earlier experiments, post-spawning conditioned water was less potent in attracting focal males to the preference zones than pre-ovulatory conditioned water (Miranda et al., 2005). Furthermore, we have shown that visual stimuli of females of different reproductive statuses alone did not cause a change in the preference of focal males. Our results indicate the need for both visual *and* chemical stimuli (Crapon de Caprona & Ryan, 1990) to induce an appropriate response. Furthermore, we showed that 17β-estradiol 3-glucuronate can evoke an attraction response to focal males, similar to pre-ovulatory female conditioned water. If we consider this, we can claim that the Mozambique tilapia hierarchies require both chemical and visual cues (multimodal communication) to establish dominance and convey appropriate reproductive signalling. For example, dominant males in both contexts (sexual and aggressive behaviours) are coloured black while they release urine pulses (Oliveira, 1995). If we consider changing color as a mating behaviour, why do focal males often turn black when first exposed to their mirror images in the mirror assay? In our preference experiments, the changing colour of focal males was also recorded but was not quantified for reasons such as the lack of accuracy of a human observer in distinguishing the differences in the colour pattern of focal males over the experiment. These questions and similar inquiries must be clarified to facilitate the identification process of dominance pheromone(s) in Mozambique tilapia.

In conclusion, consistent with the multimodal communication signals hypothesis (Partan & Marler, 1999; Partan & Marler, 2005), social behaviours of Mozambique tilapia can be modulated simultaneously by different sensory channels, including olfaction, visual and auditory (Van Staaden & Smith, 2011). This provides valuable insights into the design of behavioural bioassays, which are important to help identify fish pheromones. Furthermore, the release of bile acids, as potent odorants in pre-ovulatory female faeces, sheds light on the role of new excretory products in the chemical communication of tilapia and possibly other teleosts. It seems appropriate to conclude this thesis with some open questions:

1. Is there an alternative behavioural measure to the dominance index suggested by Barata et al. (2007) that offer a better measure of the social rank of males in a highly social species like Mozambique tilapia?
2. What is the role of individual recognition including olfactory cues in the social dynamics of the species?
3. Can we expect the aggressive interactions of younger tilapia males to be visually interrupted by adult (bigger) conspecific males? If so, what is the role of dominant male urine?
4. Does the dark colouration of dominant males convey dominance information to rivals, thereby reducing the harmful cost of agonistic interactions? Can we test this hypothesis by switching from patent blue to phenol red to visualise the urine pulses and prevent the dark colouration of focal males in our mirror assays?

5. Does urine from guarding females that protect larvae aggressively after hatching have the same effect as dominant male urine?
6. What about female-female chemical communication?

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