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Endocrine Responses of Tilapia (*Oreochromis mossambicus*) females to male pheromone(s)

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Resumo

A comunicação química desempenha um papel fundamental na vida dos peixes, pois o ambiente que estes habitam é muitas vezes desprovido de luz, o que proporcionou a que os peixes evoluíssem de forma a detetar e responder a sinais químicos libertados por conspecificos (membros da mesma espécies). Esses sinais químicos, feromonas, são libertados para o meio, e têm a capacidade de transmitir informação entre conspecíficos, desencadeando assim uma resposta fisiológica ou comportamental. Com base na sua função, as feromonas podem-se dividir em três categorias: estímulos sociais, reprodutivos e anti-predatórios. As feromonas podem induzir alterações endócrinas e de desenvolvimento (efeito "primer") e/ou alterações a nível comportamental (efeito "releaser"). Esteróides sexuais, ácidos biliares, prostaglandinas assim como os seus precursores e metabolitos são normalmente utilizados pelos peixes como feromonas. De forma geral, as feromonas são libertadas na água, principalmente através da urina, guelras e pela bílis. Os esteróides sexuais (androgénios, estrogénios e progestogénios) desempenham um papel ativo na reprodução. O androgénio mais comum nos teleósteos é 11-cetotestosterona, enquanto que o estradiol e a progesterona são importantes estrogénios. 17,20β-dihidróxi-4-pregnen-3-ona (17,20β-P) é um progestogénio, identificado em mais de 35 espécies de teleósteos.

A tilápia, *Oreochromis mossambicus,* teleósteo endémico dos rios e lagos de África oriental, devido às suas propriedades físicas e biológicas, tornou-se numa das espécies mais estudadas em laboratório. Esta espécie exibe um evidente dimorfismo sexual, no qual os machos se destacam principalmente, pelas proporções das maxilas, por apresentarem tamanhos superiores em relação às fêmeas, e pelas cores que exibem durante a época de acasalamento. Esta espécie apresenta um comportamento sexual característico, no qual, os machos formam arenas de reprodução, nas quais constroem e defendem ninhos, sendo que os machos dominantes assumem uma coloração escura. Quando estão prontas para acasalar, as fêmeas entram nas arenas, onde ocorre a desova e escolhem o macho para acasalar. Quando o ritual de acasalamento termina, a fêmea transporta o esperma e os ovos na sua boca, onde ocorre a fertilização. *O.mossambicus* são designados como incubadores bucais, uma vez que a fertilização e o desenvolvimento dos embriões e da fase larvar ocorre na cavidade bucal da fêmea.

De forma geral, sabe-se que a urina transporta feromonas que causam respostas endócrinas e comportamentais nas fêmeas. Uma dessas respostas é o aumento dos níveis de uma hormona esteróide responsável pela maturação dos oócitos, 17, 20β-P. A partir de estudos realizados anteriormente, sabe-se que existe um composto na urina dos machos que é detetado pelas fêmeas e que se encontra em concentrações superiores na urina de macho dominante quando comparado com a urina de machos subordinados. Este composto foi recentemente identificado como sendo um esteróide glucurinado e, acredita-se que é um dos componentes ativos responsável pelo aumento dos níveis da hormona esteróide responsável pela maturação dos oócitos, 17, 20β-P, nas fêmeas.

Este estudo teve como principal objetivo identificar qual a fração da urina que desempenha um papel feromonal, ou seja, qual parte da urina é responsável por causar um aumento nos níveis de 17, 20β-P e determinar se o esteróide glucurinado é capaz, por si só, um aumento nos níveis de 17, 20β-P.

Para tal, primeiramente procedeu-se à recolha de urina de macho, obtendo-se posteriormente um pool de urina. Este pool foi fracionado através de um sistema de extracção "*Solid-phase extraction*", que consiste na passagem da amostra por um cartucho de extração, onde os esteróides ficam retidos na matriz de sílica, sendo seguidamente obtidos por ação de um solvente, neste caso, o metanol. Assim, após extração, obteve-se o filtrado e eluato. Em laboratório, procedeu-se também à preparação do esteróide glucurinado, com concentração semelhante à presente na urina.

Fêmeas de *O.mossambicus* foram expostas a estes estímulos, tendo-se procedido à recolha de 1l de água, uma hora antes da adição do estímulo (tempo 0h) e uma hora após a adição do estímulo (tempo 1h). A mesma experiência foi realizada em machos da mesma espécie, porém foram expostos a um único estímulo: urina de macho dominante.

Após extração, as amostras foram submetidas a radioimunoensaios, para determinar a concentração de hormonas esteróides presentes. No caso das fêmeas, foram quantificadas as concentrações de 17, 20β-P e cortisol, enquanto que para as amostras dos machos, se quantificou 11-cetotestosterona e testosterona.

Uma hora após a exposição à urina, eluato, esteróide glucurinado assim como após exposição ao esteróide combinado com o filtrado e eluato juntamente com o filtrado, observou-se um aumento significativo nos níveis de 17, 20β-P. Contrariamente, uma hora após a exposição ao filtrado e metanol (usado como controlo), não se observou nenhuma mudança na taxa de libertação de 17, 20β-P. As taxas de libertação de cortisol não sofreram aumentos significativos, com exceção do cortisol glucurinado.

Relativamente à experiência realizada nos machos, após exposição à urina de macho dominante, de forma geral, observou-se um aumento nos níveis de 11-cetotestosterona mas sem alterações nas taxas de libertação de testosterona.

Estes resultados suportam a hipótese de que, de facto, a urina desempenha um papel feromonal, e que a fração responsável pelo aumento dos níveis de 17, 20β-P, é o eluato. O facto de o filtrado não causar alterações nas taxas de libertação, ao contrário do eluato, sugere que o método de extração foi altamente eficaz, visto que o filtrado não continha esteróides. O esteróide glucurinado, é capaz, por si só de causar, um aumento significativo nos níveis da hormona esteróide responsável pela maturação dos oócitos. Os reduzidos níveis de cortisol sugerem que o método de amostragem foi o mais indicado que, apesar da recolha de amostras repetidas, causou níveis reduzidos de stress nos indivíduos. Os níveis elevados de 17, 20β-P parecem interferir com o metabolismo do cortisol, causando aumentos nos níveis de cortisol glucurinado.

Relativamente à experiência conduzida nos machos, não se pôde retirar qualquer conclusão, uma vez que foi realizada com objetivo de praticar o método de amostragem a usar posteriormente nas fêmeas, e como tal, não se obteve controlos com os quais comparar os resultados obtidos. Contudo, seria interessante repetir esta experiência, explorando também outros estímulos, à semelhança da experiência realizada nas fêmeas.

Palavras-chave:

Feromona; Esteróide glucurinado; *Oreochromis mossambicus*; Respostas endócrinas; 17, 20β-P.

Abstract

Several lines of evidence suggest that male Mozambique tilapia (*Oreochromis mossambicus*) release a reproductive pheromone *via* their urine. A recently identified steroid glucuronide, present in male urine, is probably one of the active components that increase levels of a steroid hormone responsible for oocyte maturation, 17,20β-P, in females. The aims of this study were to identify which fraction(s) of male urine is responsible for this increase and whether the steroid glucuronide is sufficient, on its own, to cause a similar increase in 17,20β-P metabolism.

Pooled male urine was passed through C18 extraction cartridges, thus obtaining the filtrate (aqueous/polar) and eluate (hydrophobic/non-polar) fractions. Females were exposed to urine, its respective fractions, and the steroid glucuronide (and in combination with each-other). One hour after exposure, water samples were collected and steroids extracted. The same experiment was conducted in males, using a urine pool from dominant males. Steroid levels (17,20β-P, cortisol, 11-ketotestosterone, and testosterone) were measured by radioimmunoassay.

Exposure to male urine, its eluate, and the steroid glucuronide (and combinations containing the eluate or steroid) evoked a dramatic increase in release rates of 17,20β-P by females. The filtrate alone had no such effect. In males, release rates of 11-ketotestosterone, but not testosterone, increased after exposure to male urine.

These results suggest that the pheromonal activity of the urine is contained wholly in the C18 eluate. Furthermore, the steroid glucuronide (originally isolated from the urine eluate) is sufficient, on its own, to cause an increase in levels of 17,20β-P metabolism. Thus, the steroid glucuronide is responsible for the pheromonal activity of male urine, at least in this endocrine effect in females. The endocrine response of males to this urinary pheromone requires further investigation.

Key-words:

Endocrine responses; *Oreochromis mossambicus;* pheromones; steroid glucuronide; 17, 20β-P.

Contents

1. Introduction

Chemical communication is important in several aspects of fish biology, such as migration, the alarm response and – particularly - in reproduction.
Previous studies have shown that females and males release pheromones that affect the sexual behaviour and reproductive physiology of conspecifics. However, few fish pheromones have been chemically identified and fully characterized.

The Mozambique tilapia (*Oreochromis mossambicus*) is an African cichlid of immense scientific interest and economic value; it is also a prolific invasive species in countries far from its normal geographical range (e.g. South America and Australasia). Therefore, knowledge of its reproductive pheromones may not only increase scientific knowledge, but may also help in the management of farmed and invasive stocks.

Several studies suggest the importance of male urine during courtship and spawning in this species; male urination dramatically increases in the presence of ripe females, the urine contains potent odorants (the concentration of some depends on social status), and the urinary bladder is larger and more muscular in dominant males (Barata *et al.*, 2007, 2008; Keller-Costa *et al.*, 2012). Recent studies have shown that exposure to male urine dramatically increases the metabolism of 17,20β-P, the maturation-inducing steroid, in females (Huertas *et al.*, unpublished) and at least one of the active compounds is a steroid glucuronide (Keller-Costa *et al.*, unpublished). The current study was therefore designed to test the pheromonal activity of this steroid glucuronide on female 17,20β-P metabolism; does it act on its own, or are other urinary components necessary?

The following literature review intends to introduce and clarify some concepts required to understand the world of chemical communication, as well as the objective of the current study.

1.1. Hormones

The classical definition of a hormone is a specific messenger molecule synthesized and secreted by a group of specialized cells (endocrine gland) which is carried by the circulatory system to a distant target organ or tissue, where it evokes its effect(s). In vertebrates, hormones can be classified, according to chemical structure, as steroids (including sex steroids) which are formed in the adrenal glands and gonads;

amino acid derivatives, peptide hormones and prostaglandins (Crisp *et al.*, 1998; Cornish, 1998).

Steroids constitute a vast group of natural and synthetic organic compounds with a characteristic chemical structure consisting of a four-ring system (figure 1.1), with functional groups attached (Stacey & Sorensen, 2011; Gomes *et al.*, 2009). The important roles played by steroids include regulating body functions, such as growth, digestion, development, reproduction and functioning of the sexual organs (Gomes *et al.*, 2009; Crisp *et al.*, 1998).

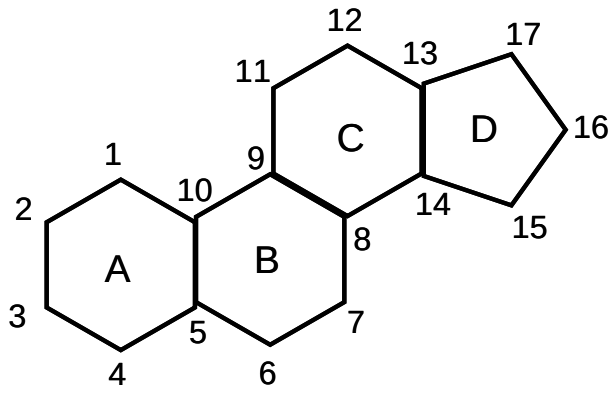


Figure 1.1 – Basic structure of a steroid, showing the four ring structure (A, B, C and D) and the numbering of the carbons (Based on Kime, 1993).

This group is composed of sterols, corticosteroids (mineralcorticoids and glucocorticoids), bile acids and sex steroids (Gomes *et al.*, 2009). Some steroids can act as hormones, such as 11-ketotestosterone and 17,20β-P (Stacey & Sorensen, 2011).

1.1.1. Sex Steroids

Sex steroids refer to a group of hormones which perform an active role in reproduction and in controlling secondary sexual characters (Sunny *et al.*, 2002). These hormones are classified as estrogens, androgens and progestogens (Gomes *et al.*, 2009). According to Kime (1993), the definition of androgens is associated with C19 steroids, estrogens with C18 steroids and progestogens have a C21 structure (figure 1.2).

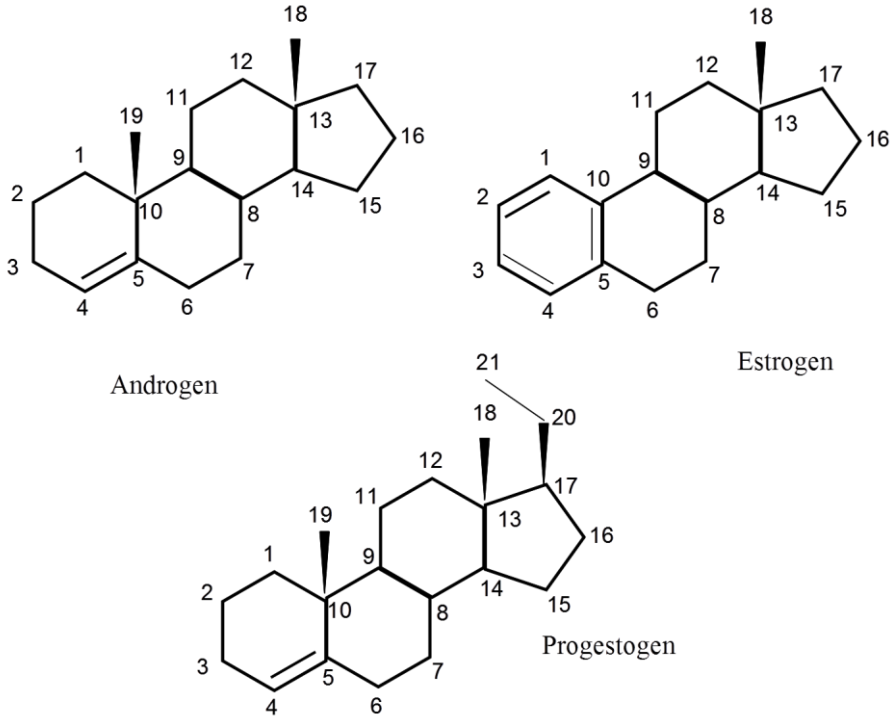


Figure 1.2- Structure of the androgens (C19), estrogens (C18) and progestogens (C21), showing the numbering of carbons.

Androgens can be characterized as "any natural steroid hormone", involved mainly in growth, development, maintenance of the reproductive system (spermiation, spermatogenesis, gonadal differentiation) of the male and are also responsible for secondary sexual characteristics and regulation of sexual behaviour (Stacey & Sorensen, 2011; Rocha & Henriques, 1996; Oliveira, 1995). In teleost males, testosterone and 11-ketotestosterone are the androgens more commonly found in plasma (Oliveira, 1995); 11-ketotestosterone is the more physiologically active (Kime, 1993). However, testosterone is also an important androgen, not only in males, but also in females' reproductive cycle, since this is the only androgen synthesized by the ovary (Kime, 1993).

Stacey and Sorensen (2011) have defined estrogens as "any natural steroid hormone that controls female sexual development, secondary sexual characteristics, and stimulates egg production". Estradiol and progesterone are the major estrogens regulating female reproductive cycle (Kime, 1993; Stacey & Sorensen, 2011). Progesterone is designated as the mammalian pregnancy hormone, thus and according to Kime (1993), does not play a role in the majority of teleost fishes. A well-studied progestogen is 17,20β-dihydroxypregn-4-en-3-one (17,20β-P), was first isolated in the plasma of Pacific salmon (*Oncorhynchus nerka*) (Kime, 1993; Nagahama, 1987), and

since then it was measured in females of more than 35 teleost species (Kime, 1993). However, progestogens are not exclusive to females; they also play a role during final gamete maturation in reproductive cycle of males in at least some species (Kime, 1993).

1.1.2. Corticosteroids

In teleost fish, corticosteroids are mainly synthesized in the interrenal tissue (Milla *et al.*, 2009; Galhardo, 2010). Cortisol and cortisone are the main corticosteroids isolated from fish blood (Milla *et al.*, 2009). Stress hormones, such as glucocorticoids, are released when the individual is exposed to a stress factor, such as threat of predation, confinement, social conflicts or pollution (Gabor & Contreras, 2012).

Cortisol is the major glucocorticoid in fishes and, as such, is often used as indicator of stress (Gabor & Contreras, 2012; Huang *et al.*, 2007; Galhardo, 2010). According to Fox *et al* (1997), its plasma concentration depends on reproductive and social status of an individual and on social situation stability. Cortisol is also an important osmoregulatory hormone in fish (Dharmamba, 1979).

An increase in glucocorticoid release can decrease rates of the sex steroids production, since energetic costs of stress affect reproductive processes by altering the production of other hormones (Gabor & Contreras, 2012; Scott *et al.*, 2008).

1.2. Chemical Communication

1.2.1. What is a Pheromone?

Although the environment surrounding fishes – water – is an excellent solvent, it is often turbid or devoid of light. Thus, fish have evolved systems to detect and respond to chemical cues released by individuals of the same species, rather than rely solely on visual and/or auditory cues (Sorensen & Stacey, 2004). To coordinate various aspects of their reproductive biology and non-reproductive functions (Cole & Stacey, 2006), and to mediate social behaviours (Sorensen & Stacey, 2004), fish use pheromones, chemical signals that pass between members of the same species (Stacey & Sorensen, 2006).

Pheromones were first described by Karlson & Luscher (1959) as "substances that are excreted to the outside by an individual and received by a second individual in which they release, for example, a definite behaviour or development process" (Sorensen & Stacey, 2004). According to Stacey and Sorensen (2006, 2011), a pheromone is a "chemical or a mixture of chemicals released into the environment by an

individual (the donor), capable of evoking a specific and adaptive response in conspecifics (the receivers), the expression of which does not require learning" (Stacey & Sorensen, 2006). Although pheromone detection involves specialisation within the chemo-sensory system(s) of the receiver, no specialization of the donor is required (Sorensen & Stacey, 2004). A pheromone might consist of a single compound, which does not need to be a specialized compound; however, pheromones are usually mixtures of chemicals (Sorensen & Stacey, 2004). Some fish employ reproductive hormones and their precursors and metabolites as "hormonal pheromones", to induce important physiological and behavioural effects in conspecifics (Stacey, 2010).

Stacey and Sorensen (2006) characterize a reproductive pheromone as a "pheromone that induces any behavioural or physiological response associated with reproductive activity", while a hormonal pheromone is any reproductive pheromone containing at least one derived compound from a chemical pathway that produces hormones, i.e., internal chemical signals. According to the same authors, hormonal pheromones can contain unmodified hormones, hormonal precursors and/or their metabolites (Stacey & Sorensen, 2006).

Evidence that hormones can function as pheromones is provided by 17,20β-P, an oocyte maturation inducing steroid that is released by female goldfish (*Carassius auratus*) and generates strong endocrinological and behavioural effects in conspecific males. Thus, conspecific behaviour and/ or physiology in several species are influenced by hormones and related compounds, which act as potent odorants (Stacey & Sorensen, 2006).

In 1963, Wilson and Bossert proposed that rapid behavioural and slower physiological effects induced by pheromones could be termed "releaser" and "primer" respectively (Sorensen & Stacey, 2004). However, these terms led to some confusion since the same pheromone can be both a primer and a releaser, and have since fallen out of favour (Stacey & Sorensen, 2006).

1.2.1.1 .The Goldfish (Carassius auratus)

A well-known sex pheromonal system in teleosts is that of the goldfish, in which hormonal pheromones from females primarily function to synchronize both male and female spawning physiology and behaviour (Kobayashi *et al.*, 2002). Male goldfish also respond to the presence of male conspecifics, by increasing sperm stores either in

response to nearby males with greater levels of sex steroids "or in response to isolation from a group of conspecifics males in a basal endocrine condition", whilst in females, there is some evidence that the released steroids are likewise detected and used as a priming pheromones, suggesting that female goldfish synchronize their ovulations (Kobayashi *et al.*, 2002; Sorensen & Stacey, 2004; Stacey & Sorensen, 2006). In summary, male goldfish enhance reproductive success through a set of physiological and behavioural strategies in response to the changing odour of peri-ovulatory females (Stacey & Sorensen, 2006).

1.2.2. Functions of Pheromones in Fish Biology

Chemical communication is important in several aspects of fish biology, such as migration, the alarm response and reproduction (Sorensen & Caprio, 1998; Smith, 1992; Selset & Døving, 1980; Bjerselius *et al.*, 2000 cited in Frade *et al.*, 2002), particularly during reproduction (Miranda *et al.*, 2005). Based on their function, pheromones can be divided into three categories: social, anti-predator and reproductive cues. Each of these categories includes 'primer' pheromones (that induce endocrine, physiological and developmental changes) and/or 'releaser' pheromones (strong behavioural changes) (Sorensen & Stacey, 2004). For example, several teleosts respond to pheromones, immediately before and during spawning, by increasing gonadal development and hormonal changes, capable of inducing final gamete maturation (priming response before spawning) (Sorensen & Stacey, 2004).

Pheromones include anti-predation and alarm cues: fishes display several chemically-mediated responses in order to reduce predation risk; kin and individual recognition in dominance hierarchies. Some fishes show a complex social system that helps to determine relatedness of conspecifics (including the establishment of dominance hierarchies and kin recognition) through the evolution of chemosensory mechanisms (Sorensen & Stacey, 2004).

Species recognition and aggregation, as well as migration, can be also mediated by pheromone release. Concerning migratory attraction, some species of migratory fishes seem to find spawning and feeding habitats through conspecific odours (Sorensen & Stacey, 2004).

1.2.3. Chemical nature of pheromones

Pheromones play an important role in the life histories of many fish species (Sorensen & Stacey, 2004), particularly in reproduction. However, few have been chemically identified and characterized to date: bile acids, sex steroids and F prostaglandins (Stacey & Sorensen, 2006).

Several electrophysiological studies, mostly using the electro-olfactogram (EOG), have shown that many species have high olfactory sensitivity to these substances (Stacey & Sorensen, 2006).Steroid and prostaglandin hormones, and metabolites, are capable of transmitting information to conspecifics, since their synthesis is predictably linked with reproductive events (Stacey & Sorensen, 2006). Therefore, such steroids and prostaglandins or their metabolites, once released into the water, have the potential to be used as hormonal pheromones (Barata *et al.*, 2007).

Pheromones may be released into the water through several routes, namely *via* the urine, gills, skin, faeces or gonadal fluids (Barata *et al.*, 2007; Stacey & Sorensen, 2006). Routes and rates of steroid release are both associated with conjugation of the steroids; free steroids are rapidly released across the gills, while sulphated and glucuronidated steroids are released in the urine and bile, respectively, although over a longer time-scale (Stacey & Sorensen, 2006; Vermeirssen & Scott, 1996).

1.2.3.1. Bile Acids

Bile acids are sterols produced by the liver and released in the bile and play a well know role in digestive system. Many fishes produce, release and detect bile acids: in the sea lamprey (*Petromyzon marinus*) a mixture of bile acids act as attractants for migratory adults and as male sex pheromone (Stacey & Sorensen, 2006); while in the European eel (*Anguilla anguilla*), Huertas *et al* (2010) suggested that bile acids could act as sex pheromones since the odour of bile depends on gender and changes with sexual maturity (Huertas *et al*., 2007).

1.2.3.2. Prostaglandins

Prostaglandins belong to a group of lipid compounds, derivatives of 20-carbon fatty acid, structurally characterized by a five-carbon ring. This class of compounds mediates several functions in fishes, such as female sexual behaviour, ovulation

(follicular rupture); they also perform pheromonal functions in some species (Stacey & Sorensen, 2011). In preovulatory female goldfish, levels of circulating prostaglandins $F_{2\alpha}$ increase considerably at ovulation (Sorensen *et al.*, 1995 cited in Sorensen & Stacey, 2006); thus being released as a postovulatory pheromone, that attracts male and it can increases milt volume (Kobayashi *et al.,* 2002; Fraser & Stacey, 2002). According to Appelt and Sorensen (2007), goldfish control the release of urinary prostaglandin pheromones to advertise about their location and physiological condition.

As demonstrated by Moore and Waring (1996) in Atlantic salmon parr (*Salmo salar*), F-type prostaglandins may function as priming pheromones, released by ovulated females, *via* the urine (Moore & Waring, 1996).

1.2.3.3. Sex Steroids

The oocyte maturation-inducing steroid (17,20β-P) released by female goldfish acts as a pheromone inducing strong behavioural and endocrinological changes in males, such as increase in concentration of male plasma gonadotropin II (GtH II), in volume of sperm and seminal fluid, as well as in sexual activity (Zheng *et al*., 1997; Stacey & Sorensen, 2006).

In tench (*Tinca tinca*), males demonstrated high olfactory sensitivity to some classic teleost sex steroids, in particular, highly sensitive to glucuronidated 17,20β-P while 100 times less sensitive to the sulphated form (Pinillos *et al*, 2002). Nevertheless, tilapia are neither sensitive to androgens found in male urine (testosterone and 11-ketotestosterone) nor to steroids that act as pheromones in other species (17,20β-P and its conjugated forms; Frade *et al*., 2002).

1.3. Conjugated Steroids

In endocrine studies, it is important to consider that the initial "steroid will be excreted in a modified and inactivated form" (Gomes *et al.,* 2009). Therefore, steroid metabolism needs to be taken into account. Catabolic pathways are divided into phase I and phase II reactions, being the principal goal to transform steroid substrate into a more polar and less active form (Kuuranne, 2010).

During phase I reactions, polarity increases by reduction, oxidation or hydrolysis reactions, which introduce new functional groups for the following phase II reactions, also designated as conjugation, where the biological activity of the free steroid is

reduced, thus converting the non-polar compounds to a more easily excreted form, to be subsequently released in the urine (Gomes *et al.,* 2009; Kuuranne, 2010).

Thus, conjugation implies the addition of sulphate or glucuronide groups to the free steroid, thereby promoting its excretion from the body (Gomes *et al.*, 2009). Glucurone and sulphate conjugation are catalyzed by enzymes: uridine-5'-diphosphoglucuronic acid (UDPGA), leading to the addition of a polar glucuronic acid to the structure of the steroid (glucuronidation) (figure 1.3); sulphotransferase enzymes which transfer sulphate group (SO_3), from a co-substrate, 3'-phosphoadenosine-5'-phosphosulphate, to the steroid (sulphation) (figure 1.4) (Kuuranne, 2010).

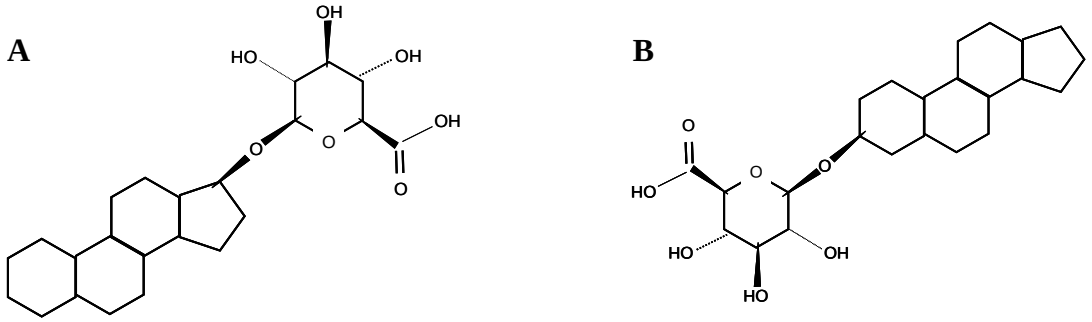


Figure 1.3- Basic structure of a steroid with a glucuronic acid attached at carbon 17 (A), however this acid can also be attached at carbon 3 in B.

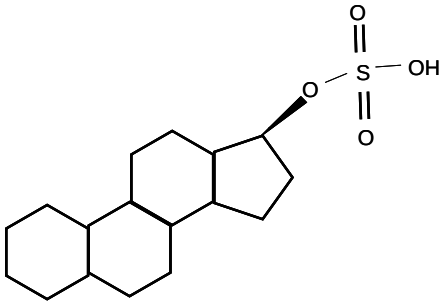


Figure 1.4- Basic structure of a steroid, with a sulphate group attached.

1.4. Steroid Measurement

Usually, the endocrine status of an individual is assessed by measurement of the concentration of hormones and their metabolites in the blood (Scott *et al.,* 2008). Nevertheless, blood sampling may be disadvantageous; in order to bleed the fish, it needs to be caught, handled and/or anaesthetised, all of which can modify behaviour and physiological condition, and therefore plasma concentrations of the hormones under

study. Furthermore, some fish may be too small, rare or valuable to be bled (Scott *et al.*, 2008). Measuring fish steroids in water enables the study of reproductive physiology of the fish through a non-invasive technique, an alternative to measurement in samples of blood (Scott & Ellis, 2007; Scott *et al.*, 2008). This technique offers minimal intervention (no anaesthetic, bleeding or handling stress), repeated measurements on the same individual and given that it involves measurements over time, this integration may reduce the short-term fluctuations in hormone levels that may occur in plasma (Scott & Ellis, 2007).

To understand the hormonal condition of the fish, it is necessary to know how much steroid has been released by the fish, over a given time, i.e. steroid release rate (Scott *et al.,* 2008). There are two ways of measuring steroid release rate: "static sampling procedure" and "dynamic sampling procedure". The first method has been used essentially for behavioural studies and consists of removing the fish from its tank and temporarily placing it in a tank with clean water for a given period. While the "dynamic sampling procedure" implies keeping the fish in controlled flow-through water conditions, and performing at least two water samples to estimate steroid release over time (Scott *et al.,* 2008). With only a single measurement, it is not possible to determine the amount of steroid that has been released over time and how much has been lost by degradation, water replacement or reabsorption by fish (Scott *et al.,* 2008).

In theory, since free steroids are released in the water mainly by passive diffusion across the gills, their release rate should be directly proportional to their concentration in plasma (Scott *et al.,* 2008). However, this correlation between plasma steroid and water steroid concentrations is not always direct and is dependent on a variety of factors, such as gill surface area, affinity for specific steroid binding proteins in plasma, fish size, salinity, water temperature, among others (Scott & Ellis, 2007; Scott *et al.,* 2008). Apart from these factors, steroid concentrations in water will also be affected by bacterial degradation, reabsorption by fish and adsorption to surfaces, as well as steroid instability during storage (Scott & Ellis, 2007).

1.4.1. Measurement of steroids

Glucuronidated and sulphated steroid concentrations are therefore heavily influenced by urination and defecation rates, and by the time that taken for the conjugation process (Scott & Ellis, 2007).

Nevertheless, the measurement of conjugated steroids is followed by advantages and disadvantages. It is advantageous to measure conjugated steroids when the goal is to reveal physiological and behavioural strategies that would not be seen by only measuring free steroids (Scott *et al.*, 2008). It is also advantageous because fishes are capable of using some conjugated steroids as pheromones and thus in order to understand both physiological and behavioural reactions of conspecifics and to study when and how they are released, conjugated steroids need to be measured (Scott *et al.*, 2008). However, measuring conjugated steroids can also be disadvantageous, since pathways from synthesis to release are more complicated than in free steroids and according to Scott et al (2008) "conjugated steroids can be temporarily "stored" in the bile and urine and their release is, thus, subject to factors such as glomerular filtration rate, urination frequency, feeding, gut passage time and defecation".

1.4.2. Procedures for steroid determination

A specific, relatively cheap and rapid method of steroid measurement is immunoassay, such as radio or enzyme-immunoassay (Scott *et al.*, 2008). Immunoassay methods, were first described by Yalow and Berson (1960), and are characterized by high sensitivity and precision, that allow the measurement of hormone levels at low concentrations in biological fluids (saliva, urine, blood) or, in this case, water (Wheeler, 2006).

Immunoassays rely on reaction of an antigen (ligand) with a specific antibody (binder). These methods are also referred to as "binding assays", since quantification of a substance depends on progressive saturation of the specific antibody by that substance and the subsequent determination of its distribution between free and "bound" phases, which is achieved by the incorporation of a "tracer" (Chard, 1990). The tracer consists of a small quantity of the ligand or the binder labelled with a material that can be precisely measured in very small amounts, as radioactive isotopes in radioimmunoassay (RIA), fluorescent compounds in fluoroimmunoassay (FIA) or enzymes in enzymoimmunoassay (EIA) (Chard, 1990).

To quantify the amount of a particular steroid present in a given sample, initially and before performing immunoassay procedures, steroids need to be extracted from the water and subsequently concentrated, since steroid concentrations in water are, in general, too low to allow direct determination (Scott & Ellis, 2007).

For steroid extraction from aqueous solutions, Solid-phase Extraction (SPE) is the preferred approach. This well-established technique allows the isolation of organic compounds present in aqueous samples (Junk *et al.*, 1988; Gomes *et al.*, 2009).

The water sample is pumped through a solid-phase cartridge, where steroid molecules are retained in a silica matrix; when using C18 cartridges, for example, the silica matrix consists of 18 carbons. Since steroids are non-polar compounds, they are retained by the silica matrix, whereas polar compounds pass through. In order to release the retained steroids, they eluted by using an organic solvent such as ethanol or methanol.

1.4.2.1. Radioimmunoassay

Radioimmunoassay (RIA) is one of the most sensitive methods for quantitative analysis of antigen-antibody reactions, in which radioisotopes are used as tracers and are attached to antibodies or antigens (Voller et al., 1976). It involves competition between a radioactive labelled antigen (Ag*), an unlabeled antigen (Ag) with a specific antibody (Ab) with fixed and limiting concentration. After an incubation period, Ag* will be quantified in the free fraction or in binding one (Ag-Ab complex), once antigen distribution in both phases is positively related with the amount of antigen in the sample (Chard, 1990).

In competitive immunoassays, where the bound fraction is used to determine antigen concentration in the sample, the amount of antigen and the quantity of radioactive labelled antigen that bound to antibody are inversely proportional, i.e., the higher the antigen concentration in the sample, the lower the amount of Ag*-Ab (Andrade, 2006). The amount of antibody to be used in the assay is assessed through a dilution curve, which involves the incubation of a fixed amount of labelled antigen with different antibody concentrations. Generally, the concentration of antibody to be used is the one for which there is a 50% binding of the tracer ligand (Chard, 1990).

A standard curve is the basic requirement for quantification of the ligand in unknown samples, since it allows quantification of the antigen (hormones) present in the samples through incubation of fixed amounts of labelled hormone and specific antibody with different concentrations of unlabelled antigen (Chard, 1990).

Radioimmunoassay is advantageous since it can be applied to several types of compounds to which there are antibodies available, also because it is extremely

sensitive, stable, specific and precise. On the other hand, it can be also disadvantageous, because it needs purified antigens for Ag* preparation and it requires special attention regarding to handling due to radioactivity (Chard, 1990). The utility of the assay is also dependent on the affinity and specificity of the antibody.

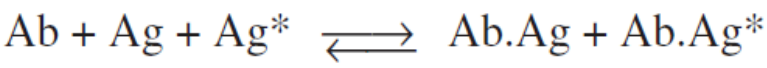


Figure 1.5 - Reaction between radiolabelled antigen, antigen to quantify and the antibody. Source: Wheeler, 2006

1.5. The Mozambique Tilapia, *Oreochromis mossambicus*

Tilapia are a large group of teleost fishes belonging to the family Cichlidae and order Perciformes, the most evolutionarily advanced and the largest order of teleosts (Cruz, 2006; Huang *et al*., 2007, Mulero *et al*., 2007). Due to the exhibition of complex social behaviour together with high reproductive rates and nutritional value, tilapia became one of the most studied fishes (Cruz, 2006). The Mozambique tilapia, a lek-breeding maternal mouth-brooding African cichlid, *Oreochromis mossambicus* (Amorim & Almada, 2005; Almeida *et al.*, 2005) was firstly described as *Chromis (Tilapia) mossambicus* by Peters in 1852, from specimens collected in the Mozambique region (Oliveira, 1995). The taxonomy of the species is shown below according to the Integrated Taxonomic Information System (ITIS):

Kingdom: Animalia
Phylum: Chordata
Subphylum: Vertebrata
Class: Actinopterygii
Order: Perciformes
Suborder: Labroidei
Family: Cichlidae
Genus: Oreochromis
Species: *Oreochromis mossambicus* (Peters, 1852)

The Mozambique tilapia is a euryhaline cichlid fish, endemic to the lakes and rivers of the east coast of Africa, though it has been introduced from its native habitat to tropical freshwater and marine environments around the world (Oliveira & Almada, 1998a; Barata *et al.*, 2007; Morgan *et al.*, 1997). Its physical robustness, combined with resistance to variation in physical and chemical factors, has allowed the occupation of a vast diversity of habitats in a wide geographical distribution that comprises equatorial rivers, tropical and subtropical lakes, estuaries and irrigation channels, for instance (Oliveira, 1995); tilapia have become a major invasive species in several countries, in all five continents (Russell *et al.*, 2012).

In its natural distribution, this eurythermal species can support temperatures ranging from 17ºC to 35ºC (Oliveira, 1995); its optimal temperature is around 27ºC (±1ºC). From the perspective of feeding, *O.mossambicus* is an opportunistic species, consuming phytoplankton, zooplankton, benthos and detritus (Caskey *et al.*, 2007; Oliveira & Almada, 1995). *O. mossambicus*, as well as other tilapine fishes, exhibits a clear sexual dimorphism, including the height of the dorsal and anal fins, the proportions of the jaw and in the shape and size of the genital papilla, which is emphasized during the breeding season (Oliveira & Canário, 2000) (figure 1.6). Male *O. mossambicus* exhibit characteristic breeding colours and generally they grow faster and reach larger sizes than females. Males also present one urogenital opening while females have two, and mature males have a thick lip in upper jaw (Oliveira & Almada, 1995). Females have a larger oral cavity due to a greater development of the pre-opercular and inter-opercular bones; although males have a larger and stronger mouth, they do not mouth-brood (Oliveira & Almada, 1995).

The highest expression of morphological (dorsal and anal fin height, mandible width and pre-maxilla length) and behavioural (nuptial coloration, time defending a territory, spawning pit volume and courtship rate) characters are showed by dominant fishes (Oliveira & Canário, 2000).

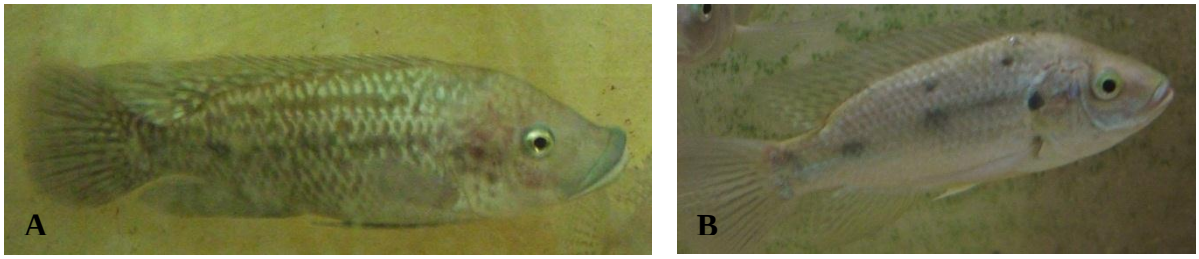


Figure 1.6 – Individuals of *Oreochromis mossambicus*: it is possible to observe the dimorphism in sexual characteristics of the species. Figure 5A shows a male while in Figure 5B shows a female.

This species displays a pronounced dichromatism, which is more evident during breeding season; breeding males exhibit an intense black coloration over the entire body, only with the lower jaw region displaying white and the edges of dorsal and anal fins showing a red coloration. Non-breeding males and females exhibit a grey coloration (Cruz, 2006) (figure 1.7).

Figure 1.7- Male tilapia exhibiting characteristic breeding colours.

In their classic study on cichlid behaviour, Baerends and Baerends van Roon (1950) distinguished three basic patterns of social relations: dominance hierarchies, territoriality and shoals. According to Oliveira (1995), males tend to be dominant over females, while large and medium sized fish tend to be dominant over smaller ones. Thus, females of *O.mossambicus* occupy the lowest places in the hierarchies (Oliveira, 1995).

As in many cichlids, in Mozambique tilapia, social status is revealed by the colour pattern of the skin, with dominant males having a darker pigmentation (Van der Salm *et al.,* 2005), larger gonadosomatic indices, longer dorsal fins, larger genital papilla and larger testes than subordinate males (Oliveira *et al.,* 1996; Oliveira & Canário, 2000). Social dominance allows dominant individuals to have priority in access to limited resources; either food or reproductive females (Oliveira, 1995). In teleosts, a correlation between hierarchical position of the males and reproductive success has been observed, where dominant individuals show a higher rate of mating and more access to females (Oliveira, 1995). From studies carried out in captivity, it has been shown that male tilapia form stable linear hierarchies, with the largest males (alpha males) receiving more visits of spawning females (Oliveira & Almada, 1996; Oliveira & Almada, 1998a).

In captivity, males are commonly divided into ‘territorial’ (dominant), ‘floater’ and ‘sneaker’. Dominant males usually dig nests, assume a dark coloration, defend a centred territory in the nest and dynamically court females. Floater males typically live

in the water column, display a light dark coloration and occupy territories for a short period (from seconds to minutes), when the owners are away to court females. On the other hand, sneaker males usually invade nests during spawning and try to remain near the female while exhibiting trembling behaviour, which is normally related to sperm release (Oliveira & Canário, 2000).

1.5.1. Sexual Behaviour and Reproduction

In this species, during the breeding season, males gather in breeding arenas or leks, forming dense aggregations over sandy or muddy substrates (Barata *et al.*, 2007; Oliveira & Almada, 1998b). After males' aggregation, dominance hierarchies are established, where pheromones may play an important role in modulating aggressive interactions (Barata *et al.*, 2007, Keller-Costa *et al*., 2012).

Within the arenas, males establish individual territories where they dig spawning nests (pits) in the substratum, which they defend, and the associated area, from possible invaders, and assume a characteristic black colouration (Barata *et al.*, 2007; Barata *et al.*, 2008; Frade *et al.*, 2002; Miranda *et al.*, 2005; Oliveira *et al.*, 1996; Oliveira & Almada, 1998b; Galhardo *et al.*, 2008). From a few seconds to a few minutes after the formation of the group, some individuals begin to darken and participate in symmetrical fights (i.e. when an individual retaliates to an agonistic act with another aggressive action), essentially involving circle fights, mouth-to-mouth fighting and mutual displays (Oliveira & Almada, 1998c; Oliveira & Canário, 2000; Barata *et al.*, 2007).

Circle fights consists of two individuals in antiparallel position, exhibiting lateral display, moving around a central spot, and as they attempt to bite, both individuals attack alternately (figure 1.7) (Oliveira, 1995). In mouth-to-mouth fighting (figure 1.8), fishes display frontally to each other and attack with open mouths (Baerends & Baerends van Roon, 1950; Oliveira, 1995). Both of these behaviours, as well as mutual displays of individuals (fish remains motionless in front of its opponent), are considered as symmetrical agonistic interactions (Oliveira, 1995).

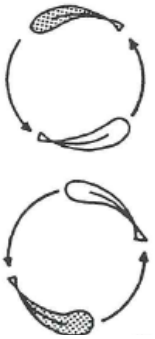


Figure 1.5 – Agonistic interactions between tilapia males: circle fights (from Oliveira, 1995).

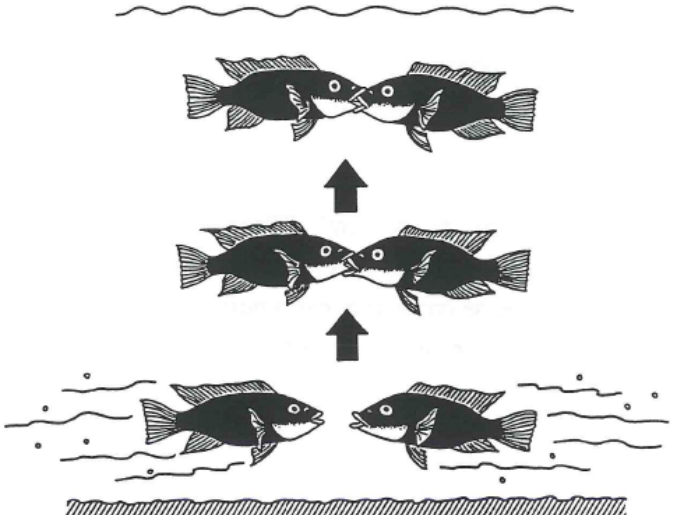


Figure 1.6- Agonistic behaviour exhibit by tilapia males: mouth-to-mouth fighting (from Oliveira, 1995).

Females are attracted to spawning pits, which are usually located in shallow waters, where males display to attract females for mating, exhibiting a series of behaviours, such as nest digging, trembling and circling the female (Amorim & Almada, 2005; Almeida *et al.,* 2005).

Although Mozambique tilapia can spawn repeatedly throughout the year, with females having a regular ovulatory cycle of 15–20 days, only when the females are ready to spawn do they visit the breeding areas (Barata *et al.*, 2008; Barata *et al.*, 2007).

In lekking cichlids, with a breeding system analogous to *O.mossambicus*, dominant fish occupy a central position in the nest, dig larger nests, are more effective at defending territories, also court at a higher ratio and have a higher breeding succession (Amorim & Almada, 2005; Oliveira *et al.,* 1996), which can be explained by the fact that females prefer males with larger nests (Amorim *et al.,* 2003).

Mature females enter the lekking area, where spawning takes place, and choose one or more males with which to mate (Frade *et al.*, 2002). When the mating sequence ends, males tremble while circling the nest followed by female, which in turn, takes both eggs and sperm into her mouth, where fertilization occurs (Oliveira & Almada, 1998b; Amorim *et al.*, 2003). After spawning, the female leaves the arena and during the next 20-22 days carries the embryos and the offspring in her mouth, to brood in a nursery area separate from males (Frade *et al.*, 2002). Hence, parental care is restricted to females, which delay their next ovulatory cycle until the brood is released (Oliveira *et al.*, 1996; Miranda *et al.*, 2005).

Throughout the mouth-brooding cycle, females become progressively more aggressive to other conspecifics. However, contrary to males, they defend mobile space around themselves, instead of defending territories on the substrate (Oliveira & Almada, 1998b; Oliveira, 1995).

In this species, maternal aggression is a well-developed phenomenon; however, female agonistic behaviour differs from that of territorial males in that aggressive acts are limited to charges, chases and butting (Oliveira & Almada, 1998b). Female pigmentation pattern also changes progressively during the mouth-brooding cycle; the body becomes light grey with an overlaid pattern of dark stripes, the eyes show horizontal bars in the irises, the lips get darken and display a characteristic mandibular spot (Oliveira & Almada, 1998b; Oliveira, 1995).

Maternal aggression seems to function principally to defend the brood against predators, conspecifics included. This is different from the aggression directed against the brood as a way to impose control over their behaviour (Oliveira & Almada, 1998b).

Throughout the oral incubation cycle, Oliveira (1995) observed that females suppressed almost totally their feeding activity; however, there was no decrease in the condition factor of the incubating females, whilst a decrease in the number of eggs/juveniles during the incubation cycle was observed. This suggests the existence of a partial cannibalism of the eggs as a reproductive strategy of the females (Oliveira, 1995).

1.6. Role of urine among conspecifics

Urine is used as a chemical signal, at least for conspecific females (Almeida *et al.*, 2005; Barata *et al.*, 2007). Several studies have shown that Mozambique tilapia males regulate urine release depending on the social context; territorial males (males

from a higher social rank) may signal their status and aggressiveness to other males, potential rivals, through the release of urine (Almeida *et al.,* 2005), which may contain male-male pheromones (Barata *et al.,* 2007), as well as by changing behaviour and coloration (Almeida *et al.,* 2005). The females' olfactory system is extremely sensitive to substances released by territorial males into the water as well as to male body fluids, demonstrating that urine has a special importance (Barata *et al.,* 2008; Almeida *et al.,* 2005).

O. mossambicus males are capable of storing urine: the bladder of a fish weighting 100g may contain up to 2 ml of urine (Almeida *et al.,* 2005). According to Barata *et al* (2007), dominant males dynamically release chemical information through increasing urination rate during aggression, and in the presence of females, dominant males dramatically increase their release of urine (Almeida *et al.,* 2005). Urination rate remains high during courtship, being considerably higher in the presence of pre-ovulatory females, and their urine has higher olfactory potency than that of subordinate males (Almeida *et al.,* 2005; Barata *et al.,* 2007). On the other hand, females release urine at higher frequency and smaller pulses, and this seems to be unaffected by the presence of dominant males (Almeida *et al*., 2005).

In a study carried out by Barata *et al.* (2008), using a liquid chromatography linked to mass spectrometry and recording the electroolfactogram (EOG) it was demonstrated that female tilapia detect a potent odorant in the non-polar fraction of male urine suggested to be a sulphated amino-sterol. This odorant is present at higher concentrations in urine from dominant males than in subordinate males. However, further work has shown that this compound is, in fact, a steroid glucuronide (Keller-Costa *et al.*, unpublished).

These authors suggested that the urine of dominant males has a higher concentration of this compound and it is related to a higher olfactory potency. The same authors also suggested that social dominance, instead of reproductive capability, is reflected by both urine volume stored in the bladder and concentration of the odorant (Barata *et al.,* 2008). However, according to the same study, the most active and important odorant found in male urine is non-polar. Consequently, this study suggested that this urinary odorant "is may be detected as part of mixture of odorants and that females may use the ratio, rather than the absolute amount, to discriminate between dominant and subordinate males" (Barata *et al.*, 2008). In turn, subordinate males are able to store less urine in their samller bladder, so they are less capable of increasing

their urination rate and are consequently less able to stimulate females (Barata *et al.*, 2008, Keller-Costa *et al.*, 2012).

As mentioned before, male urine may act as a pheromone signal, causing responses in female endocrine status and behaviour. One such response is the increasing the synthesis for of the oocyte maturation steroid. Frade *et al* (2002) suggested that males release a signal in their urine in way to attract females ("releaser" effect), but this signal may be also capable of inducing ovulation ("primer" effect). This possibility is supported by a recent study where females were exposed to male urine in way to explore "primer" effects on females' endocrine system. Less than one hour after exposure, an increase in the levels of 17,20β-P, responsible for oocyte maturation (Huertas *et al.*, unpublished).

1.7. Aims of the study

Pheromones present in male urine, once released, affect steroid levels in females. In the case of the Mozambique tilapia, exposure to male urine elevates the release rate of the maturation-inducing steroid hormone 17,20β-P by females.

The main objective of this study was to identify which constituent(s) of male urine (filtrate, eluate or both) contain(s) the pheromonal components. In other words, which fraction of urine is responsible for causing an increase in the oocyte maturation-inducing steroid (17,20β-P) in females.

Specifically, the study aimed to determine whether the steroid glucuronide recently identified as the main component in the eluate of male urine is sufficient - on its own - to cause the increase in 17,20β-P metabolism in females, or whether other urinary components are necessary.

2. Material and Methods

2.1. Experimental Animals

Mozambique tilapia (*Oreochromis mossambicus*) used in the current study were obtained from a stock population previously established at the University of the Algarve. This stock originated from wild specimens caught in Mozambique, from the River Incomati (early 1970s), kept and raised at Aquário Vasco da Gama (Lisbon) from which some individuals were bred at Instituto Superior de Psicologia Aplicada (Lisbon); subsequently, individuals were donated to the University of the Algarve (Frade *et al.*, 2002).

In order to form families, animals were grouped (one male and four females) in fibre-glass tanks (250 l) with sand substratum and kept at 27ºC (±1ºC), where they were maintained and fed once a day with an appropriate diet. Under these semi-natural conditions, individuals exhibited the normal mating behaviour and, as such, spawning occurred in each tank naturally. Nevertheless, after each spawning, eggs were removed from the mother's mouth, by applying a slight pressure in the posterior area of the opercula, to maintain the females' ovulatory cycle and to predict the next ovulation. The removal date of the eggs was recorded, so that a given female could be designated as 'pre-ovulatory' (predicted to ovulate in the next three days or 'post-ovulatory' (having ovulated during the past three days).

2.2. Urine Sample Collection

Urine samples were collected daily (except at weekends) by application of slight pressure at the terminal part of the abdomen, immediately above and anterior to the urogenital opening (Frade *et al.*, 2002), and avoiding any possible contamination with faeces, from each male (n=7) to 1.5 ml Eppendorf tubes. Males were then replaced into their tank of origin. Urine samples were clearly identified (number of the male and collection date) and frozen (-20ºC) until a given volume per male was collected. Then a pool was made, using an equal volume of urine (4.9 ml) from each male, and this pool was subsequently aliquoted, extracted and frozen until use (see below).

2.3. Experimental Design

2.3.1. Stimuli Preparation

Of the total volume of the urine pool (34.3 ml), 15 ml was aliquoted into 1.5 ml Eppendorf tubes, each with 0.5 ml of urine, designated as "urine pool". Another 15 ml of urine pool was extracted (see *Solid-Phase Extraction*), to obtain 15 ml of filtrate (aqueous fraction), which was aliquotted into Eppendorf tubes, each with 0.5 ml; and 5 ml of eluate (hydrophobic fraction), which was stored in glass vials containing 1 ml each.

Two glucuronidated steroids present at high concentration in dominant male urine and previously identified as the most potent olfactory stimuli in the eluate, [5β-pregnane-3α,17α,20β-triol-3α-glucuronide (20β-P-Gluc) (main compound); 5β-pregnane-3α,17α,20α-triol-3α-glucuronide (20α-P-Gluc) (minor compound)] (Keller-Costa *et al.*, unpublished) were mixed in a proportion of 4:1, respectively (figure 2.1).

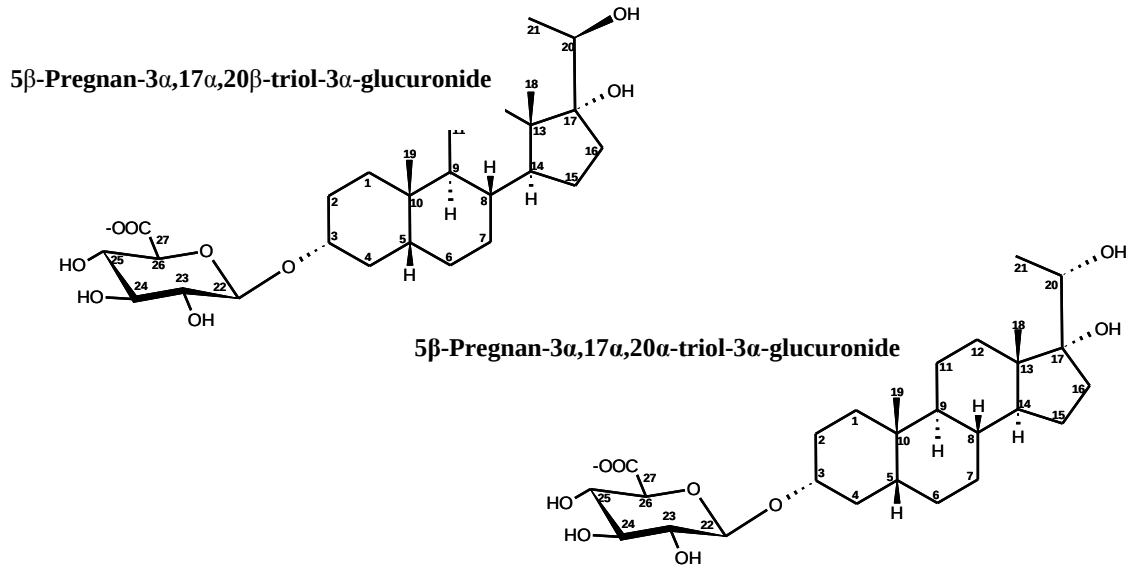


Figure 2.1- Structures of steroid present in high concentrations in urine of dominant male, identified by Keller-Costa *et al* (unpublished), consisting of two steroid glucuronides: 5β-Pregnan-3*α*, 17*α*, 20β-triol-3*α*-glucuronide (main compound); 5β-Pregnan-3*α*, 17*α*, 20*α*-triol-3*α*-glucuronide (minor compound).

Each steroid has a molecular weight of 534.6 g mol^{-1} and to achieve a steroid concentration of $5x10^{-3}$M (2.673 mg/ml, *see table 1*), it was added 1.407 ml and 0.449 ml of methanol to 3.76 mg of steroid 20β-P-Gluc and 1.2 mg of 20α-P-Gluc, respectively (*see table 1*). From these stock solutions, 1.2 ml of 20β-P-Gluc, corresponding to 4-fold of 20β-P-Gluc ($5x10^{-3}$M), and 0.3 ml of 20α-P-Gluc,

equivalent to 1-fold of 20α-P-Gluc ($5x10^{-3}$M) was then taken and mixed together in glass vial. This 1.5 ml of 4:1 20β-P-Gluc/20α-P-Gluc ($5x10^{-3}$M) mixture was diluted with 13.5 ml of ethanol to a total volume of 15 ml, thus achieving a total mixture concentration of $5x10^{-4}$M, approximately the same concentration as in urine. This stimulus was then divided into glass vials (with a capacity of 1.5 ml), each containing 1ml of the stimulus and stored at -20ºC.

Table 2.1: Preparation of the stimulus "Steroid" in order to achieve a concentration of $5x10^{-4}$ M

Preparation of the stimulus:			
20-β-P-Gluc-sodium salt (charge III) in mg	mg	3.76	Methanol
20-α-P-Gluc-sodium salt (charge III) in mg	mg	1.2	
molecular weight (each) in g /mol	g/mol	534.6	
concentration of the stock solution $5x10^{-3}$M: mmol/ml	mmol/ ml	0.005	
steroid concentration_ $5x10^{-3}$M: mg/ml	mg/ml	2.673	
volume (ml) to 20-β-Gluc to obtain stock solution	ml	1.407	
volume (ml) to 20-α-Gluc to obtain stock solution	ml	0.449	
4x 20-β-P-Gluc $5x10^{-3}$M	ml	1.2	Ethanol
1x 20-α-P-Gluc $5x10^{-3}$M	ml	0.3	
4:1 20-β/20-α MIX total: $5x10^{-3}$M	ml	1.5	
4:1 20-β/20-α MIX total: $5x10^{-4}$M	ml	15	

All the four stimuli obtained (urine pool, filtrate, eluate, "steroid") were stored at -20ºC until further use. Some of the stimuli were combined during the experiment: eluate plus filtrate and filtrate plus "steroid". Methanol (Sigma-Aldrich or VWR) was also used as control stimulus.

2.3.2. Exposure of Females to Male Urine and Derivatives

Firstly, females (n=8) were selected from the family tanks according to their ovulatory stage; pre-ovulatory females with a regular ovulatory cycles were preferred. The weight and length of each female were recorded, and the female was then isolated in a glass aquarium with 6l of de-chlorinated tap-water at 27ºC (±1ºC) and equipped with an air supply, and maintained over-night. The next day, females were transferred to an identical aquarium with a volume of clean de-chlorinated tap-water normalized to the weight of the fish (1 litre of water per 10 g of fish; Huertas *et al.*, unpublished). An hour after the transfer, one litre of water was collected (control sample, at time 0h), through siphoning with a tube previously placed in the tank. This sample was extracted (see *Solid-Phase Extraction*).

After collection of the first water sample, sufficient stimulus was added to the aquarium to obtain a final dilution of 1:10 000 (e.g. to 100 g fish in 10 l of water, 1 ml of stimulus would be added). The volume previously collected (1l) was then replaced with clean de-chlorinated tap-water. In this case, each female was exposed to each stimulus (n=7: urine pool, filtrate, eluate, "steroid", filtrate plus eluate, "steroid" plus eluate and methanol) in a randomized order, but no female was exposed to more than one stimulus during one ovulatory cycle. In the case of the eluate, since it was three times more concentrated, it a third of the volume was used; for example, in 10 l of water (100 g female) to achieve the desired dilution (1:10 000) 1 ml of stimulus (such as the urine pool) would be necessary; for the eluate, only 0.33 ml would be used.

One hour later, another water sample (1l) was collected (treatment sample, at time 1h), used the same sampling method as before. Each water sample collected was identified with the females' number, stimuli number and time of collection (0h or 1h). After the experiment, females were placed back into the family tank, to which they belonged.

2.3.3. Exposure of Males to Male Urine

Initially, males (n=8) from the main stock were randomly selected and their weight and length were recorded. Subsequently, each male was placed in a glass aquarium with 6 l de-chlorinated tap-water at 27ºC (±1ºC), equipped with an air supply, where individuals were isolated and maintained over-night. By using the same procedure described above for the female experiment, males were exposed to male urine from a pool previously obtained from males of the same species. Water samples were collected for further extraction (see *Solid-Phase Extraction*) in the same way as described for the females. Each water sample collected was identified with the males' number and the time of collection (0h or 1h), reaching in the end a total of 16 samples. After the experiment, males were placed back in the stock tank.

2.3.4. Hormone Analysis

2.3.4.1. Solid-Phase Extraction

In this assay, solid-phase extraction (SPE) was used to extract non-polar compounds present in urine of male tilapia and in water samples collected before and after the exposure to stimuli, both in males and females. In order to activate the 500 mg

C18 SPE cartridge ('Isolute'; International Sorbent Technology Ltd., Hengoed, UK), 5 ml of methanol was passed through (Sigma- Aldrich or VWR) followed by 5 ml distilled water.

The urine pool from males (15 ml) was passed through under vacuum (flow rate approximately 1 $ml.min^{-1}$) according to the instructions given by the manufacturer. The filtrate was collected in a glass extraction tube and then was divided into the Eppendorf tubes, as previously described (*see Stimuli' Preparation*). To elute, after passing the urine pool, 5 ml of methanol was passed through the cartridge, and this was collected and distributed into glass vials (1ml each).

The same technique was used to extract water samples derived from the experiments with females and males (1l of water before and after exposure to the stimuli). However, the volume used in these cases was 1l of water and the filtrate was discarded, only the eluate (5 ml) was collected and frozen at -20ºC until be processed for use in radioimmunoassay (see below).

2.3.4.2. Steroid Extraction

Methanol eluates from SPE were dried under nitrogen gas (2 bar), in a water bath at 40ºC. After the methanol had totally evaporated, 100 µl distilled water 3 ml of diethyl ether (Merck), were added to the tubes, being then stirred for 10 minutes and centrifuged at 500 rotations per minute (rpm) for 5 minutes, to separate the two phases (aqueous and diethyl ether).

The aqueous phase was frozen by submerging the extraction tube in liquid nitrogen and the ethyl ether phase was decanted into glass assay tubes (round bottomed tubes Normax [10x75x0, 6 mm]. The diethyl ether was evaporated in the water bath at (±) 40ºC. Since only approximately 70% of the steroids are extracted during the first extraction, this process was repeated twice in order to increase the efficiency.

Phosphate buffer (1 ml) with gelatin (Sigma G-9382) (0.05mM sodium-phosphate buffer, 0.1% gelatin and 0.01% sodium azide) was added to the assay tubes, thus obtaining the free steroid fraction, which was frozen at -20ºC.

Extraction of sulphates

The aqueous phase resulting from the previous procedure was evaporated under nitrogen gas, in a bath at 40ºC. One ml of tri-fluoroacetic acid (TFA) (Merck) in ethyl

acetate (VWR) (1:100) was added to the extraction tubes, containing the dried residues of each sample and the extraction tubes were maintained in a water bath at 40ºC stirring overnight and evaporated with nitrogen gas.

To these extraction tubes 500 µl acetate buffer (8.2% sodium acetate and 8.5 % HCl at 10:2.8) was added along with 3 ml of diethyl ether, stirred for 5 minutes and centrifuged at 500 rpm for 5 minutes. The remaining procedure was the same as for the extraction of free steroids (see above).

Thereafter, extraction tubes were submerged in liquid nitrogen, thus freezing the aqueous phase while ether remained liquid, which was poured into assay glass tubes. These tubes were then placed in a bath at 40ºC, to evaporate the ether. This process was done twice, so as to maximise recovery. Then 1 ml of phosphate buffer with gelatin was added and the samples were frozen at -20ºC.

Extraction of Glucuronides

The diethyl ether traces from the acetate buffer was removed by evaporation with nitrogen gas and 10µl of the enzyme β-glucuronidase (Sigma G-7019) was added to the aqueous phase and left to incubate at 37ºC, overnight, stirring in a water bath. Extraction followed with diethyl ether as previously described.

2.3.4.3. Radioimmunoassay

In the current study, radioimmunoassays were used to quantify the concentrations of sex steroids in eluates resulting from the previously described experiments with males and females: testosterone (T), 11-ketotestosterone (11KT), in the case of males' samples; and 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) and cortisol, as regards to females' samples (figure 2.3).

The concentrations of steroids in some samples were higher than the highest standard used in the construction of the standard curve (see below). It was therefore necessary perform some dilutions (see table 2). In these cases, samples were diluted in phosphate buffer with gelatin.

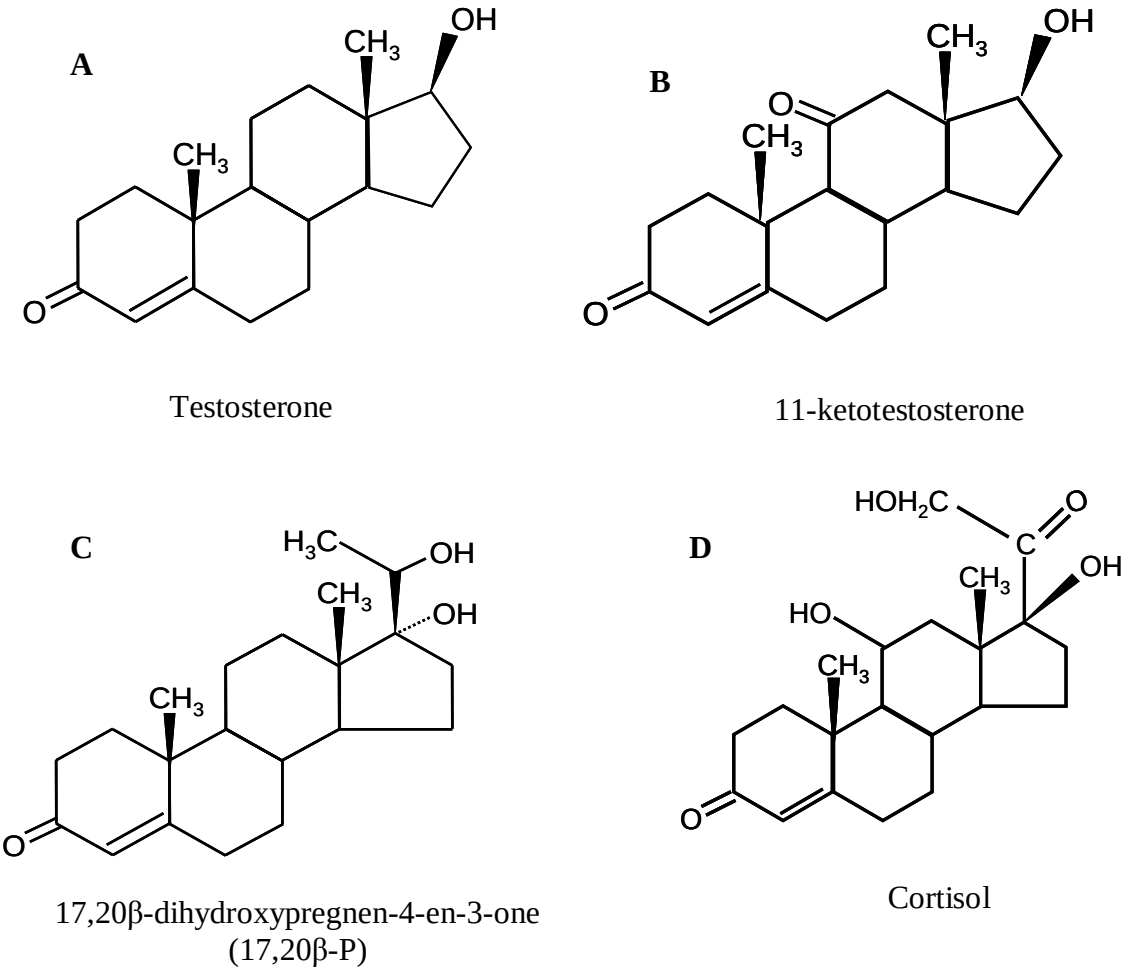


Figure 2.3 – Structures of steroids quantified by radioimmunoassay: testosterone (A); 11-ketotestosterone (B); 17,20β-dihydroxypregnen-4-en-3-one (17,20β-P) (C) and cortisol (D).

In each radioimmunoassay, 100 µl of each extracted sample (free fraction, sulphated and glucuronides) was placed in duplicate into polypropylene 0.5ml vials [RIA tubes (Sarstedt)], previously allocated on metal racks (Sarstedt).

Table 2.2 - Table of dilutions used in this experiment. Depending on the quantity of steroid present in the samples, it was necessary to repeat assays, diluting some of the samples, in accordance with the following table.

Dilution	Sample Volume	Volume of Phosphate Buffer with Gelatin
1x	100 µl	
2x	50 µl	50 µl
5x	20 µl	80 µl
10x	10 µl	90 µl
20x	5 µl	95 µl
25x	10 µl	240 µl
26x	10µl	250 µl

50x	10 µl	490 µl
50x	5 µl	245 µl
60x	5µl	295 µl
75x	5µl	370 µl
100x	10 µl	990 µl
200x	5 µl	995 µl
400x	5µl	1995 µl

Standard Curve

For each assay, a standard curve was performed, with successive dilutions of a standard solution of the steroid in question (see table 3), with known amounts of the steroid, ranging from 0.5 to 500 pg per tube. These standard solutions were then loaded into separate vials, designated as standard vials (from S1-S12); each one was loaded with a certain volume of the standard solution, using phosphate buffer with gelatin to make a total volume of 100 µl (see table 4).

In each assay, three tubes were also prepared, designated as Blank (B), Maximum (M) and Total (T); to these tubes 100 µl phosphate buffer with gelatin was added.

Table 2.3 - Dilutions of steroid stock in order to prepare Standard Solutions.

	Standard Solutions					
	#1	**#2**	**#3**	**#4**	**#5**	**#6**
Steroid Stock (0.5mg/ml of ethanol)	50µl	50µl **#1**	100µl **#2**	100µl **#2**	100µl **#3**	100µl **#4**
Phosphate Buffer with Gelatin	5ml	5ml	900 µl	4900µl	4900µl	4900µl

Table 2.4- Volume of each standard solution (#1 to #6) added to each standard RIA vial, in order to obtain known concentrations of the steroid.

	Standard RIA Vials											
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**	**S12**
	100µl **#3**	50µl **#3**	25µl **#3**	100µl **#4**	50µl **#4**	25µl **#4**	100µl **#5**	50µl **#5**	25µl **#5**	100µl **#6**	50µl **#6**	25µl **#6**
[Steroid] pg/tube	500	250	125	100	50	25	10	5	2,5	2	1	0,5

Antibody and Label Preparation

The antibody solution was prepared in a 20 ml polyethylene vial (Packard). For this, the total volume of phosphate buffer with gelatin for all samples and standard tubes was determined, considering that 100 µl was needed for each RIA tube.

Then the concentration of labelled steroid previously established was added to this flask, so that each tube contained about 1500 cpm (counts per minute) of [^{3}H], mixed and 100 µl added to the blank tube.

To obtain approximately 50% maximum binding, sufficient antibody was added to the former solution. The required quantity of antibody was established through a tritation. After mixing, 100 µl of the solution was added to each tube, with exception of the blank tube.

A suspension of dextran-charcoal was prepared according to the volume needed, usually 110 ml of phosphate buffer with gelatin to 0.165g of dextran (Sigma D-4751) and 1.65g of charcoal (or activated carbon) (Sigma), and stored in the fridge over-night. The "charcoal suspension" was added (250µl) to all 0.5ml tubes, with exception of "Total" tube, which received 250 µl of phosphate buffer with gelatin. After 12 minutes incubation on ice, the metal racks with the tubes were centrifuged at 2500 rpm for 12 minutes at 4ºC. The supernatant was transferred into plastic scintillation tubes (Sarstedt), which were filled with 3 ml scintillation liquid (EcoliteTM (+) ICN). The radioactivity in the vials was then counted by using a scintillation Microbeta Trilux counter; each tube was counted for 10 minutes.

Free molecules are adsorbed by charcoal, thus separating bound and free label; only the antigen-antibody complex remains in solution.

2.4. Steroid Release Rates

Data from radioimmunoassay were available in an excel spread sheet. From this, it was possible to calculate release rate at time 0h and at time 1h.

From steroid concentration in 100 µl (pg), it was necessary to calculate the concentration of steroid in 1l of water (ng):

Equation 1: Steroid Concentration in 1l of water

$$Steroid\ concentration\ in\ 1l\ (ng) = \frac{(pg \times dilution\ factor) \times 10}{1000}$$

Total steroid concentration in the total water volume was calculated through *equation 2*, where steroid concentration (ng) represents the steroid concentration in 1l of water and water volume (l) corresponds to the water volume normalized to the weight of the individual.

Equation 2: Total Steroid (ng)

$$Total\ Steroid\ (ng) = Steroid\ Concentration\ in\ 1l\ (ng) \times Water\ Volume\ (l)$$

After the total steroid, the steroid release rate at time 0h was calculated by the following equation:

Equation 3: Release Rate at time 0h ($ng.kg^{-1}.hr^{-1}$)

$$Release\ Rate\ at\ time\ 0h = \frac{Total\ steroid\ (ng)}{Weight\ (kg)}$$

where the total steroid (ng) represents the amount of steroid in total water volume normalized to the weight at time 0h, depending on the weight of the female (kg).

Through *equations 1* and *2* it was calculated the steroid concentration and total steroid in 1l of water at time 1h. To the total steroid (1h) was subtracted the steroid concentration in the total volume of water minus one litre, from time 0h, in order to obtain only the total steroid released at time 1h (*equation 4*). Then, release rate at time 1h was calculated through *equation 5:*

Equation 4: Total Steroid at time 1h (ng)

$$Total\ steroid\ (1h)(ng) = total\ steroid\ (1h) - (total\ steroid\ (0h) - steroid\ concentration\ in\ 1l\ (0h))$$

Equation 5: Steroid Release Rate at time 1h ($ng.kg^{-1}.hr^{-1}$)

$$Release\ rate\ at\ time\ 1h = \frac{total\ steroid\ 1h\ (ng)}{weight\ (kg)} - \frac{total\ steroid\ 0h\ (ng)}{weight\ (kg)}$$

For each stimulus, the mean and standard error of the mean (SEM) of the steroid release rates at time 0h and time 1h were calculated.

2.5. Statistical Analysis

Data for female steroid release rates were analyzed using two-way repeated-measures (TW-RM ANOVA). When TW-RM ANOVA was significant, the Holm-Sidak *post-hoc* test was performed to identify the differences between treatments and to compare each stimulus to a control group (urine). Data failed on normality test, but data were of equal variance (F-test).

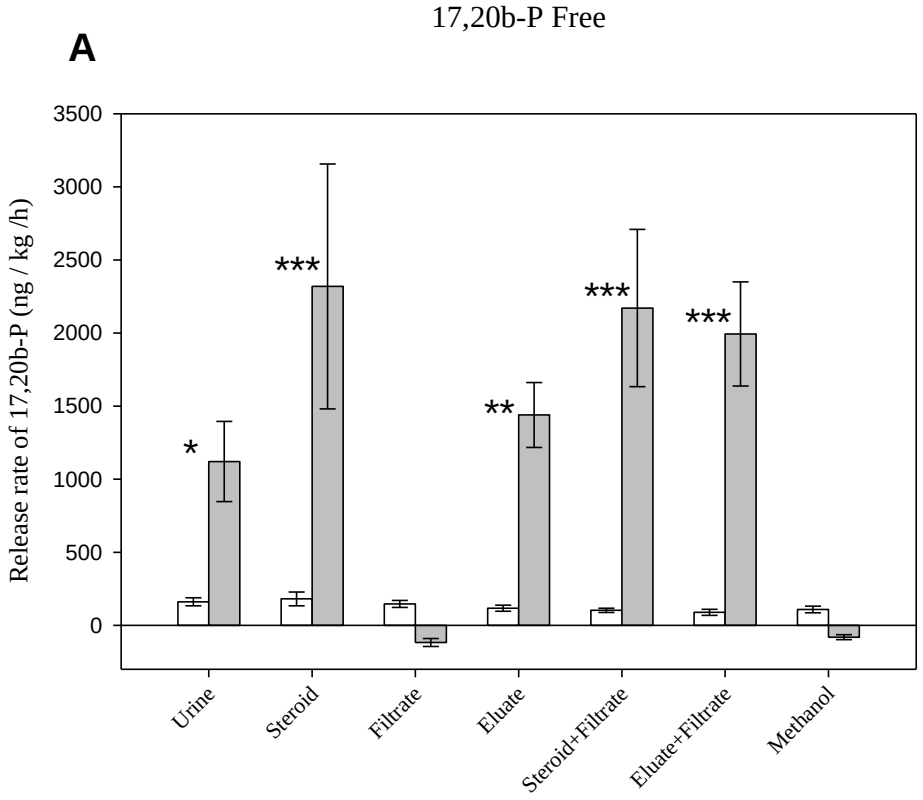
Male steroid release rates were analysed using paired Student's *t*-test; when data failed the normality test, Wilcoxon Signed Rank Test was used to compare release rates of androgens before and after exposure to stimulus. All data are shown as mean ± SEM and statistical significance was established at $p<0.05$.

3. Results

3.1. Exposure of Females to Male Urine and Derivatives

To evaluate the effect of the male urine-derived stimuli on the release of 17, 20β-dihydroxypregnen-4-en-3-one (17,20β-P) by females into the water, release rates of this steroid was calculated one hour before (time 0h) and one hour after (time 1h) exposure to a given stimulus: male Urine, Steroid 5β-pregnen-3α, 17α-20α-triol-3-glucuronide and 5β-pregnen-3α, 17α-20β -triol-3-glucuronide (from now on designated as "Steroid"), Filtrate, Eluate, "Steroid" plus Filtrate, Eluate plus Filtrate and Methanol (as vehicle control).

3.1.1. Release Rate of 17, 20β-dihydroxypregnen-4-en-3-one (17,20β-P)



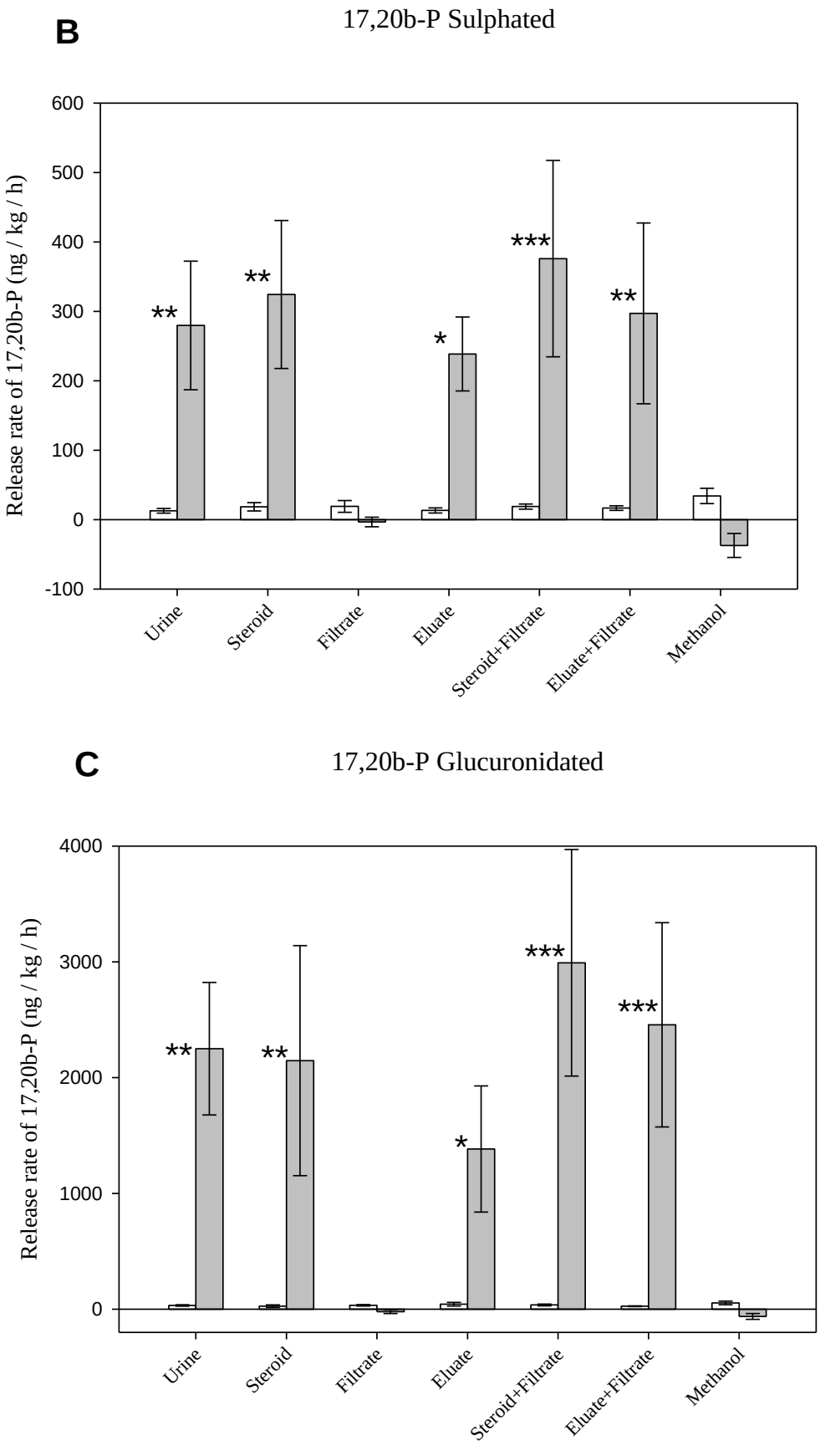


Figure 3.1- Comparisons of release rate of free (A), sulphated (B) and glucuronidated 17,20β-P (C) (mean ± SEM; ng/kg/h), before (white bars) and after exposure to stimulus (grey bars). The asterisks indicate the significant differences between release rates before and after exposure to stimulus: * *p*<0, 05; ** *p*<0, 01; * *p*<0,001.**

Exposure to male urine dramatically increased the release rate of free, sulphated and glucuronidated 17,20β-P. Higher increases were shown by glucuronidated 17,20β-P (32.1 ± 6.1 ng/kg/h to 2249.7 ± 572.5 ng/kg/h) and by free 17,20β-P (161.3 ±27.6 ng/kg/h to 1121.1 ± 273.9 ng/kg/h). The less pronounced increased was showed by sulphated 17,20β-P (12.7 ± 3.4 ng/kg/h to 279.7 ± 92.5 ng/kg/h).

Concerning the "Steroid" stimulus, a response similar to that evoked by urine was seen, with free 17,20β-P (181.2 ± 47.1 ng/kg/h to 2319.2 ± 837.6 ng/kg/h) and glucuronidated 17,20β-P (26.2 ± 11.2 ng/kg/h to 2146.9 ± 993.6 ng/kg/h) showing a more accentuated response than sulphated 17,20β-P (18.5 ± 5.9 ng/kg/h to 324.3 ± 106.6 ng/kg/h).

However, "Steroid" caused higher release of free 17,20β-P than urine. On the other hand, glucuronidated 17,20β-P showed similar release rates after exposure to urine and to "Steroid".

After addition of the filtrate into the holding water of females, the release rate of free 17,20β-P and its conjugated forms did not change.

Exposure to the eluate alone caused an increase in release rate of 17,20β-P, both free and conjugated forms, similar to that seen with untreated urine, except that the increase in 17,20β-P glucuronide was slightly less.

"Steroid" and filtrate, combined a single stimulus, caused a sharp increase in release rate of 17,20β-P. Again, the more pronounced effect was seen in free and glucuronide. However, the increase was slightly greater than that evoked by untreated urine or eluate.

Eluate and filtrate, combined as a single stimulus, induced an increase in release of 17,20β-P conjugated and free alike. In the case of free 17,20β-P, the observed increase was greater than that of untreated urine or eluate. However, the increase in sulphated 17,20β-P release by eluate plus filtrate was similar to that for untreated urine, and eluate. The effect on 17,20β-P glucuronide release was similar to that of untreated urine but slightly higher than that evoked by the eluate alone.

Exposure to the control carrier, methanol, had no effect on the release rates of free or conjugated 17,20β-P.

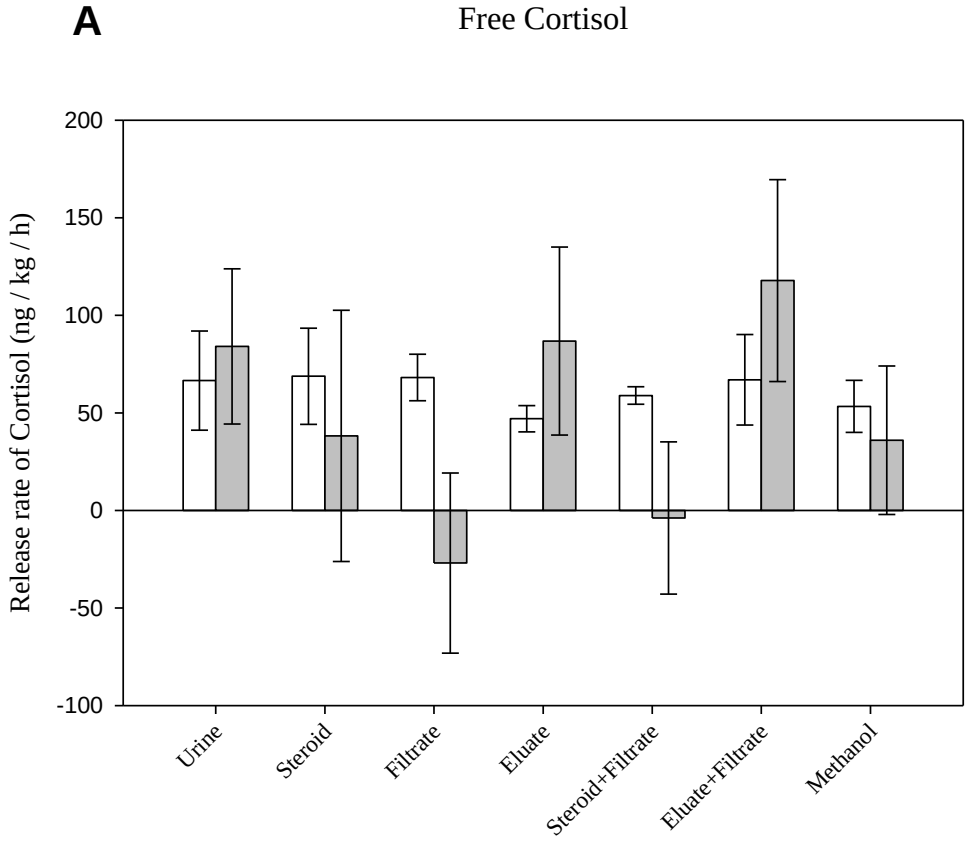
3.1.2. Comparisons of responses to different stimuli

Urine was assumed as control and all stimuli, one hour after the treatment, were then compared to it. Regarding to free 17,20β-P, the effect observed after exposure to urine was significant different from the effects observed after exposure to "Steroid" (t=3.007, p=0.003), were also different from those observed after exposure to "Filtrate" (t=3.108, p=0.003), those observed after exposure to "Steroid plus Filtrate" (t=2.635, p=0.010) and different from effects observed after exposure to methanol (t=3.017, p=0.003).

Regarding to sulphated and glucuronidated 17,20β-P, only the effects after exposure to "Filtrate" (t=3.539, p=<0.001; t=3.543, p<0.001, respectively) and "Methanol" (t=3.962, p<0.001; t=3.607 p<0.001, respectively) were significantly different from urine effect.

3.1.3. Release Rate of Cortisol

In general, release rates of cortisol were lower than 17,20β-P, both before and after stimulus addition.



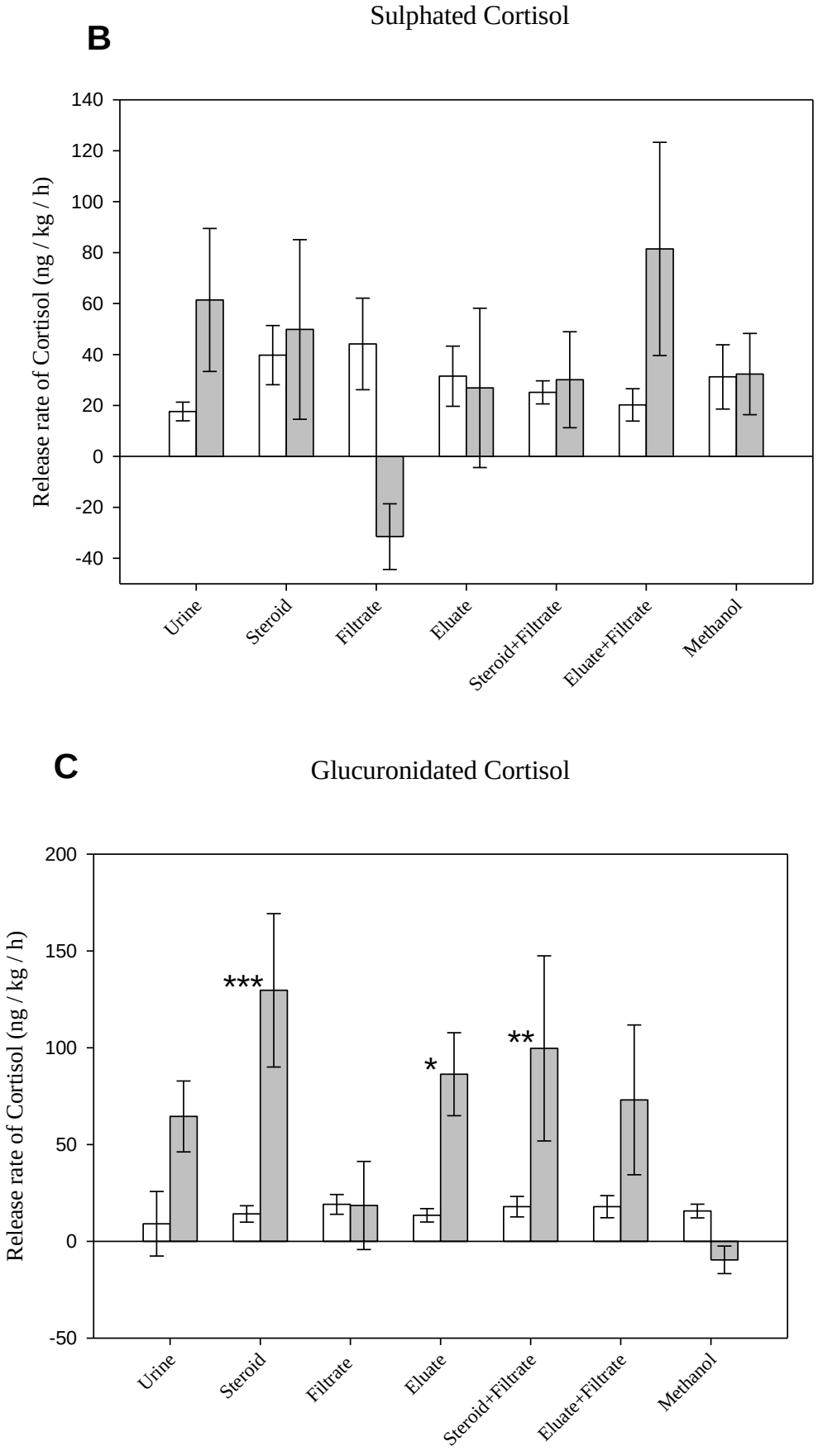
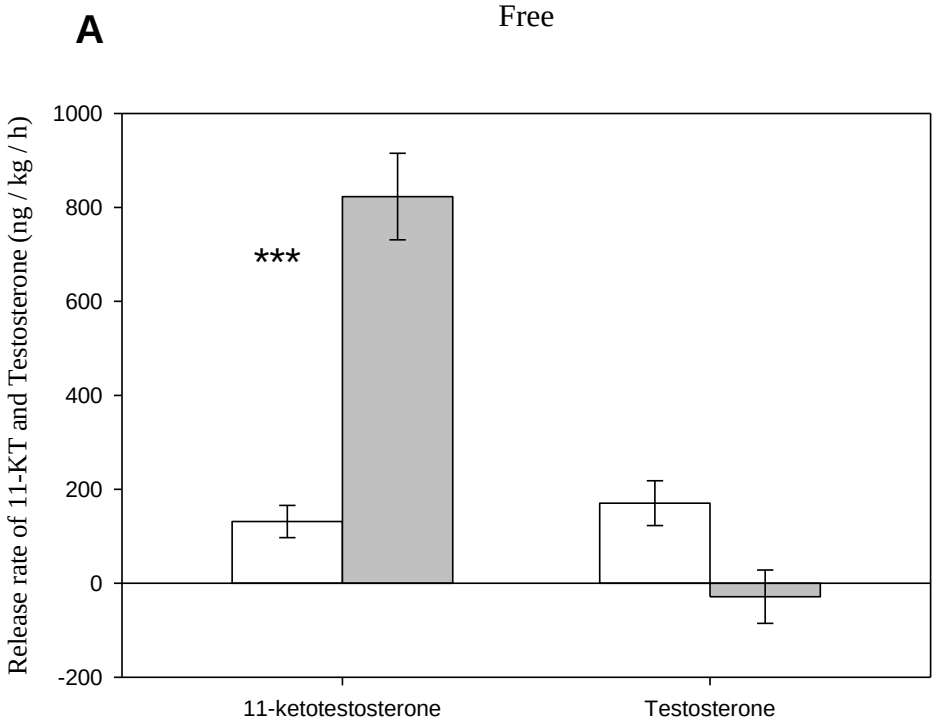


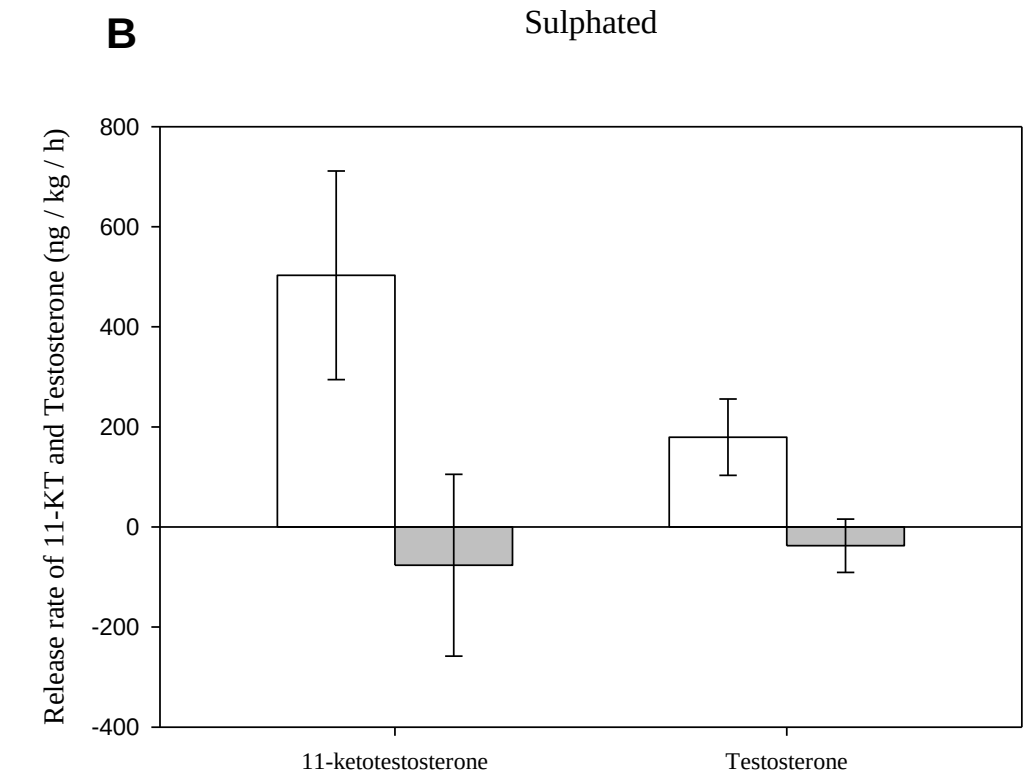
Figure 3.2- Release rates of free (A), sulphated (B) and glucuronidated Cortisol (C) (mean ± SEM; ng/kg/h) before addition of the stimulus (white bars) and after exposure to stimulus (grey bars). The asterisks indicate the significant difference between release rates before and after exposure to stimulus: *p*<0, 05; ** *p*<0, 01; * *p*<0, 001.**

Both in release rate of free and sulphated cortisol, the differences in the mean values among stimuli and time were not great enough to exclude the possibility that the observed difference was due to random sampling variability. Therefore, there was not a statistically significant difference between stimuli (p=0.202) and time (p=0.531), according to two way repeated measures ANOVA performed.

When individuals were exposed to "Steroid", the release rate of glucuronidated cortisol demonstrated a significantly increase: from 14.2 ± 4.3 ng /kg/h to 129.7 ± 39.6 ng /kg/h (t=3.965, p<0.001). The same effect could be observed after exposing females to eluate, where was observed an increase in the release rate of glucuronidated cortisol (13.4 ± 3.5 ng.kg^{-1} to 86.3 ± 21.4 ng/kg/h; t=2.502; p=0.016). The increase noticed in release rate of glucuronidated cortisol (17.9 ± 5.3 ng/kg/h to 99.7 ± 47.8 ng/kg/h), after exposure to "Steroid plus Filtrate", was also greater enough to be considered as statistically significant (t= 2.898; p= 0.007).

3.2. Exposure of Males to Male Urine





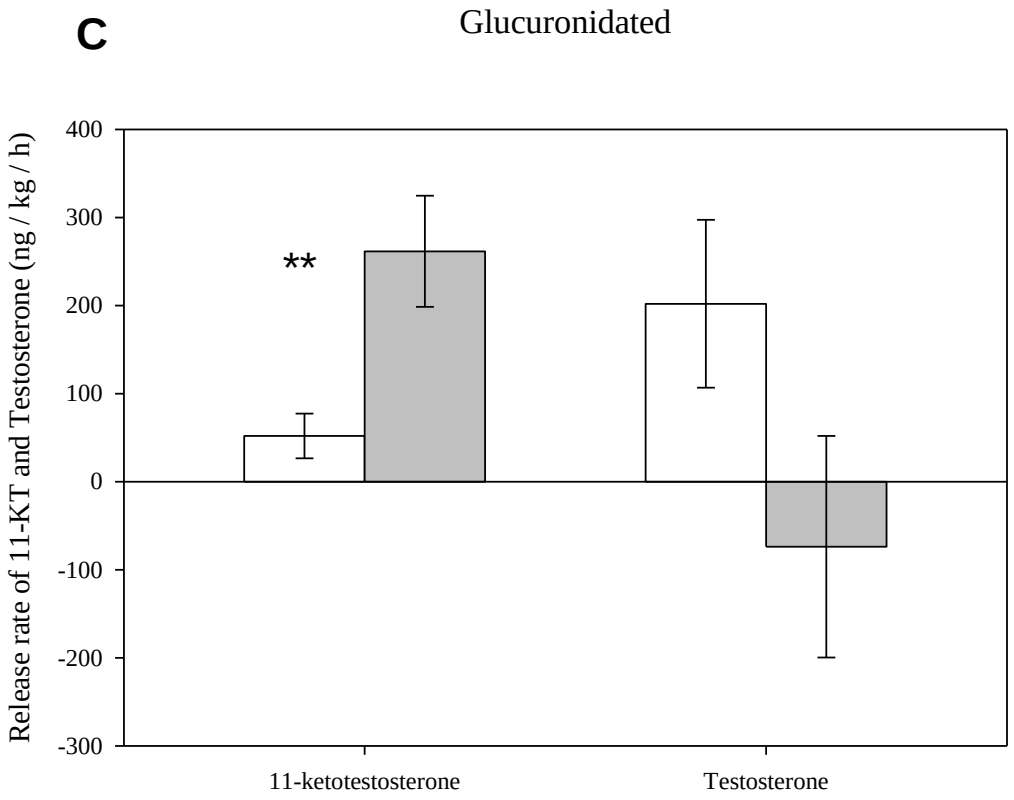


Figure 3.3- Comparisons between release rates (mean ± SEM; ng/kg/h) of 11-ketotestosterone and testosterone before (white bars) and after (grey bars) exposure to urine pool of dominant male. The asterisk indicate the significance of the treatment (before vs. after exposure to stimulus): ** *p*<0, 01; * *p*<0, 001.**

When males were exposed to urine pool of dominant males, the release rate of free and glucuronidated 11-ketotestosterone (11-KT) increased within an hour of treatment. The greatest increase was observed in free 11-KT, where there was a statistically significant increase from 131.4 ± 34.3 ng/kg/h to 823 ± 92.1 ng/kg/h (paired Student's *t*-test, N=8, t =7.647, $p<0.001$). Release rate of glucuronidated 11-KT also exhibited a statistically significant increase from the sample without stimulus to the sample after stimulus exposure (51.9 ± 25.4 ng/kg/h to 261.6 ± 63.2 ng/kg/h; paired Student's *t*-test, N=8, t =3.674, $p=0.008$).

There were no significant effects in the release rate of testosterone, free or conjugated, into the water after exposure to dominant male urine.

4. Discussion

The present study clearly demonstrates that the pheromonal components of male urine are contained wholly in the eluate. In other words, this is the fraction of male urine responsible for causing an increase in levels of the oocyte maturation-inducing steroid (17,20β-P) in females. Moreover, the steroid glucuronide is sufficient, on its own, to cause a similar increase in 17,20β-P metabolism, thus suggesting that this is the urinary pheromone used by male tilapia.

The "primer" effects on females' endocrine system suggested by Frade *et al* (2002) were observed in a recent study where females were exposed to male urine: less than one hour after exposure, it was observed an increase in the levels of 17, 20β-P, both in free and its conjugated forms (Huertas *et al*, unpublished). Thus, male urine may play a pheromonal role, since it is a mixture of compounds that are released into the environment by an individual and it is capable of evoking a specific response in conspecifics (Stacey & Sorensen, 2006); in this specific case, male urine synchronizes spawning through induction of final maturation of the eggs (Huertas *et al.*, unpublished).

The study carried out by Huertas *et al.* (unpublished) is similar to the present study and was conducted in the same lab. However, when compared release rates of both experiments, the responses observed by Huertas *et al* are about 10 times lower. Differences may be due to different labelled antigen, standard solution of the steroid and different antibody. Regarding to the antibody, the one used in the present study may be detecting not only the desired steroid (17,20β-P), but other components present in samples, in other words, the antibody may not be as specific, and thus it is reacting with other steroids.

Despite this, Huertas *et al* (unpublished) observed that release rate of glucuronidated 17,20β-P was notably higher than free or sulphated forms, also seen in the current study; after exposure to male urine,17,20β-P release dramatically increased, particularly free and glucuronidated 17,20β-P.

Exposure to eluate causes a dramatically increase in levels of 17,20β-P; whereas filtrate does not. Indeed, after exposure to filtrate, the release rate of 17,20β-P did not change. Therefore, this suggests that the solid-phase extraction was highly efficient, being supported by Huertas *et al* (unpublished), since a recovery rate between 80 and 95 % was calculated for all steroids, using the same extraction method.

Levels of 17,20β-P after exposure to urine eluate show a significant increase within an hour of treatment. Since this fraction should contain the steroid(s) present in

untreated urine, it would be expected to cause a similar increase in release rates of 17,20β-P. Thus, the eluate has a similar effect to urine and, therefore, contains the component(s) responsible for causing the increase in 17,20β-P metabolism.

Internal and external signals (hormone levels and social interactions, for instance) regulate reproduction in vertebrates. Environmental cues that influence reproduction, such temperature, photoperiod, food availability and social interactions, need to be integrated by the nervous system and, *via* the hypothalamus-pituitary-gonadal (HPG) axis, regulate the gonads (Soma *et al*, 1996). Therefore, all external cues that have an influence on reproduction, act at the level of the HPG axis (Soma *et al*, 1996).

Gonadotropin-Releasing Hormone (GnRH) is the "key reproductive regulatory peptide" (White *et al*, 2002). It is produced and secreted by a group of neurons, which are the central nucleus of the hypothalamus-pituitary-gonadal axis (Rissman, 1996).

Production of gonadal steroids and gonadal growth are stimulated by gonadotropin hormones released by the pituitary (follicle-stimulating hormone, FSH, and luteinising hormone, LH) which, in turn, are regulated by GnRH secretion (Soma *et al*, 1996).

According to Ramakrishnan and Wayne (2009), social cues modulate cell physiology of an "additional population of GnRH neurons", present in teleost fish, the terminal nerve, which is associated with the olfactory bulb. In turn, Rissman (1996) suggests that steroids regulate GnRH release, by acting through negative feedback on gonadotropin secretion, which may modulate behaviour directly or indirectly (Oliveira, 1995). According to Huertas *et al.* (unpublished), hypothalamic-pituitary-gland axis is modulated by male urine, inducing the production of 17,20β-P.

Hence, if there is no exposure to any social cue, such as pheromones, GnRH is not secreted, there is no release of gonadotropin hormones from the pituitary and, consequently, there is a reduction on gonadal steroid production.

Assuming that the pheromone in male urine is a steroid, the filtrate should not cause an increase in levels of 17,20β-P, since it does not contain any steroids. In the present study, exposure to filtrate did not cause any increase in release of 17,20β-P.

"Steroid" has a similar effect to the untreated urine, suggesting that the pheromone present in male urine is the "steroid" acting on its own. However, the differences observed between release rate after exposure to urine and "steroid", in particular, the higher release rate of free 17,20β-P after addition of "steroid" may be

explained due to a lower concentration of the "steroid" in the used urine pool when compared to the concentration of the synthetic steroid, since its concentration in urine was estimated to be around $5x10^{-4}$M (Keller-Costa, personal communication) and the exact steroid concentration in the used urine pool was unknown.

Interestingly, when filtrate was combined with eluate as a single stimulus, and with "steroid", the effect in release rate of free 17,20β-P was greater than urine, but only the effect after exposure to "steroid plus filtrate" was significantly different from the effect of urine, probably due to steroid concentration. However, when all responses were compared with that of urine, only increases in release rate of free 17,20β-P were evoked by "steroid" and "steroid plus filtrate" were greater.

Vermeirssen and Scott (1996) demonstrated that fish release steroids into the water by three main routes: gills, urine and bile. In this study, the authors injected rainbow trout (*Oncorhynchus mykiss*) with tritiated 17,20β-P, and then conclude that 40% of free17,20β-P was excreted unaltered *via* the gills, whereas 50% was converted, by the liver, to glucuronide and sulphate and stored in the bile, for consequent excretion through the faeces. The remaining 10% was converted to sulphate and released *via* the urine. Therefore, free steroids are released mainly *via* gills, sulphated steroids *via* urine while glucuronidated steroids are released through the bile (Vermeirssen & Scott, 1996). However, the tilapia urinary pheromone appears to be a steroid glucuronide and yet is released in the urine.

In the current study, it is possible to observe that free and glucuronidated 17,20β-P show higher release rates than 17,20β-P sulphated. Free steroids are released *via* gills due to passive diffusion, a result of the concentration gradient between plasma and water (Vermeirssen & Scott, 1996; Scott *et al*, 2008; Scott & Ellis, 2007). Since free fraction is released by passive diffusion through the gills in favour of concentration gradient, it is non-polar, poorly water-soluble and its degradation rate is lower, this may explain the observed release rates of free 17,20β-P after exposure to several stimuli.

On the other hand, the observed high release rate of free 17,20β-P can also be due to degradation of released sulphated and glucuronidated 17,20β-P, which were rapidly de-conjugated after release to the water (Scott *et al*, 2008; Scott & Ellis, 2007), thus exaggerating the release rate of free steroids.

Conjugation is the main method of deactivating steroids *in vivo*, making them more water-soluble (Scott *et al*, 2008), but also greatly reducing their affinity for their

receptors. In general, glucuronidated 17,20β-P exhibits high release rates, thus suggesting that glucuronidated form of 17,20β-P is more easily released.

Sulphated form of 17,20β-P is excreted mainly *via* the urine and, in the current study, it is possible to observe that sulphated 17,20β-P shows lower release rates when compared with free and glucuronidated 17,20β-P. This may be due to the fact that only 10% of 17,20β-P is converted to sulphate and excreted in urine (Vermeirssen & Scott, 1996). Moreover, female urination rates are unaffected by the presence of males. Furthermore, females release urine in higher frequency and shorter pulses than males (Almeida *et al*, 2005; Barata *et al*, 2008). Since it is not advantageous to females to advertise their reproductive status (Almeida *et al*, 2005) while in the presence of males, females may control their urination rate. This may explain why sulphated 17,20β-P is released at lower rates; given that sulphated steroids are released in the urine, the lower levels of sulphated 17,20β-P may be explained due to a reduction in urination frequency of females, after exposure to male urine, or the steroid glucuronide.

Cortisol is commonly used as an indicator of stress and for this reason is also known as the stress hormone (Galhardo, 2010; Gabor & Contreras, 2012). The low rates of cortisol release during the experiment suggest that the experimental design did not cause stress to the fish. During the experiment, cortisol release showed no significant increases, with exception of the glucuronidated form. The values obtained were within the range of values reported for undisturbed tilapia (Foo & Lam, 1993; Huertas, *et al*, unpublished).

Due to the temporal relationship between the increase in 17,20β-P and the death of some salmonids species after spawning, Barry *et al* (2010) hypothesized that excess in cortisol levels in Pacific salmon was regulated by the pre-spawning increase of 17,20β-P. Therefore, these authors suggested that "under normal physiological conditions, even in face of elevated stress-induced cortisol levels, peripheral targets are protected from cortisol excess by cortisol-metabolizing enzymes, which inactivate cortisol before it bind to cellular receptors and thus initiate a biological response" (Barry *et al.*, 2010). During the spawning season, when 17,20β-P levels are elevated, 17,20β-P inhibits the metabolism of peripheral cortisol, exposing cortisol receptors in various targets to high concentrations of cortisol (Barry *et al.*, 2010).

17,20β-P can be considered as a substrate for cortisol biosynthesis, therefore competing with cortisol for binding to corticosteroid binding protein, thus raising the

concentrations of biologically active cortisol (Barry *et al.*, 2010), and its subsequent release to the water.

The elevated levels of 17,20β-P demonstrated after exposure to "steroid", eluate and "steroid plus filtrate" may cause a competition with cortisol, thus explaining the increases observed in glucuronidated cortisol release. In other words, these stimuli caused an elevated release of 17,20β-P, which in turn competed with cortisol for attaching to corticosteroid binding protein, thereby causing an increase of biologically active free cortisol. Only glucuronidated cortisol exhibited a significant increase over time, because glucuronidation as well as sulphate conjugation act in a way to transform free form of a steroid into a more polar and less active form (Kuuranne, 2010). Hence, elevated levels of 17,20β-P probably compete with cortisol in its metabolic pathways, resulting in high levels of free cortisol, which in turn is transformed in its glucuronidated form to be more easily excreted.

Stress in fishes can be minimised by using non-invasive sampling as demonstrated by Gabor and Contreras (2012) in a study carried out in *Poecilia latipinna,* which suggested that to non-invasive water-borne hormones collection causes minimal stress to *P. latipinna*, and allows repeated measures without causing additional stress. In the current study, "dynamic sampling procedure" was used as a non-invasive technique to measure steroid concentration in fish-holding water. As suggested by Scott *et al* (2008), this technique seems to be the most suitable approach, since it allows taking several samples from the same fish, causing minimal disturbance in individual which is shown by low rates of cortisol, besides that it is also useful to estimate release rate of steroids by the fish over time after exposure to a given stimulus (Huertas *et al.*, unpublished; Scott *et al.*, 2008). Therefore, the used sampling technique does not cause severe stress to tilapia and it is the most suitable approach to determine endocrine responses, at the level of steroids, of the species.

Regarding the measurement of conjugated steroids, we can assume that is an advantageous when we want to know about physiological strategies that would not be noted only by measuring free steroids (Scott *et al.*, 2008). In the current study, measuring conjugated steroids enabled us to conclude that glucuronidated forms appear to be more easily excreted (possibly by the bile), thus suggesting that is more effective to release glucuronidated forms, instead releasing sulphated forms. Whereas, control of urination frequency by females and consequently the release of sulphated forms, seems to be a strategy used by females, in order to not advertise males about reproductive

status, when they are not ready to spawn. Therefore, measuring conjugated steroids may allow us to determine which the preferential routes of excretion of metabolites and conjugated steroids.

The experiment conducted in tilapia males had, as a primary goal, to practice the sampling technique prior to use in the female experiment. Furthermore, males were randomly selected from the main stock, thus we did not record their social status, dominant or subordinate.

11-ketotesterone (11-KT) is considered the most biologically active androgen in teleosts (Kime, 1993; Hirschenhauser *et al*, 2008), although testosterone is also important (Kime, 1993). As a result of exposure to the urine pool from, levels of 11-KT demonstrated a significant increase of free and glucuronidated form, while, levels of testosterone decrease within one hour of treatment.

According to Hirschenhauser *et al* (2008), free fraction of 11-KT reflects "systemic circulating" levels of 11-KT diffuse through the gills and conjugated fractions contain urinary and faecal components, and it seems that social interactions affect the release rate of conjugated fraction. Specific social stimuli seem to cause variations in urination behaviour. "Male intruder stimulus" reflected the levels of free 11-KT and also caused modification in conjugated 11-KT fraction. Thereby, if individuals used in this experiment were subordinate males, the fact that they were exposed to urine of dominant males, thus, in order not to advertise the "dominant male" about their social status, the experimented males, may have reduced their urination and defecation rate (principal routes of releasing conjugated steroids), therefore explaining why free 11-KT exhibits higher rates of release, since they are released through the gills, similar to Hirschenhauser *et al.* (2008); free 11-KT shows a higher release rate after exposure to "male intruder stimuli" than its conjugated forms, though glucuronidated form exhibits slightly higher levels than sulphated fraction. However, this hypothesis needs further investigation.

Control of sexual and agonistic behaviour is one role of androgens, in particular testosterone (Oliveira *et al*, 1996). According to the same authors, testosterone is probably involved in agonistic behaviour thus, and since males were on their own and therefore had no-one to be agonistic with, this may explain why levels of testosterone did not increase. Moreover, cytochrome-P450-11β-hydroxylase is the responsible for catalysing the conversion from testosterone to 11β- hydroxytestosterone, which is the

“immediate precursor of 11-KT” (Pfenning *et al*, 2012). For that reason, if 11-KT is a metabolite of testosterone, it is possible to assume that if levels of 11-KT increase, levels of testosterone should decrease.

Nevertheless, and as mentioned before, we did not expect to have significant responses and the experiment was not conducted in the most recommended way, thus, in order to better understand this results, it is necessary to repeat this experiment with males in order to clarify the obtained responses.

5. Conclusion

This study aimed to identify which fraction of male urine (filtrate, eluate or both) is responsible for causing an increase in the oocyte maturation-inducing steroid in females, 17,20β-P and determine whether the recently identified steroid glucuronide is sufficient - on its own - to cause the increase in 17,20b-P metabolism, or whether other urinary components are necessary. Indeed, urine plays a pheromonal role and the fraction responsible for causing an increase in levels of 17,20β-P is the eluate. The filtrate, on its own, does not cause any response. Furthermore, the steroid glucuronide is sufficient - on its own - to cause an increase in levels of 17,20β-P, thus it may be assumed as the pheromone present in male urine.

6. Future Research

In order to understand the physiological response of males to the urine of a dominant male, it would be necessary to repeat the experiment carried out in this study, however, this time with respective controls and knowing the social status of individuals used in the experiment.

It would also be interesting to perform the same experiment but with males being exposed to the same stimuli to which females were exposed, however, to analyse the release rate of 11-ketotestosterone, 17,20β-P and cortisol.

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