Tomás Geraldes dos Santos

Effect of kisspeptin treatment on reproductive traits and chemical communication in Senegalese sole, *Solea senegalensis*, both during puberty and breeding stages

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(Tomás Geraldes dos Santos)

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Abstract

This thesis aimed to understand the effects of kisspeptin administration on the onset of puberty and chemical communication, of juvenile and adult Senegalese sole (*Solea senegalensis*), respectively. Senegalese sole is a highly valuable species for European aquaculture, currently facing problems regarding F1 breeders (born and reared in captivity) not reproducing naturally in captivity. Kisspeptin, a crucial hormone to the normal functioning of the reproductive axis, has been successively used as a treatment in other fish species, especially as a puberty accelerator. In a first trial, prepubertal Senegalese sole (approximately 1.5 year old), were treated with either single kisspeptin injection or PBS (control). Blood plasma was used for sex steroids determination (T, E_2 and 11-KT) by ELISA, before, 4 hours, 2 and 4 days after treatment. Gonads were excised at 4 days post treatment, to determine gonadal maturation stage. Testosterone levels were found to be higher in both sexes with kisspeptin treatment, with treated females also having an estradiol increase trend, however without statistical significance. Histology analyses in males revealed increased spermatids and reduced spermatocyte numbers in the cortical region of treated fish indicating a more advanced stage of the gonad. These results evidence the positive effect of kisspeptin treatment on sole puberty, reinforced by studies on other species. A second experiment was conducted similarly to the first one, but with sole breeders, testing kisspeptin effect on their chemical communication. Urine samples from both groups were run to a LC-MS analysis to identify relevant compounds, and an EOG analysis, comparing their effect on sole olfactory epithelium. Two compounds were found in higher concentration in the treated sole's urine, one undocumented hypothesized to have a steroid structure, and 3-hydroxyphenylacetic acid, both theorized to be pheromones, given that treated urine had increased potency on olfactory responses for both sexes.

Keywords: *Kisspeptin*; *Senegalese sole*; *Hormones*; *Puberty*; *Chemical communication*

Resumo

Com esta tese pretendeu-se averiguar o efeito da kisspeptina usada como um tratamento hormonal, tanto no início da puberdade em juvenis, como na comunicação química em adultos de linguado senegalês (*Solea senegalensis*). Esta é uma espécie importante para a indústria da aquacultura na Europa, mas apresenta ainda constrangimentos relacionados com a ausência de reprodução natural por parte dos reprodutores F1 (nascidos e criados em cativeiro). A Kisspeptina é uma hormona essencial para o normal funcionamento do sistema reprodutivo, tendo sido usada com sucesso noutras espécies de peixes como um estimulador da puberdade. Numa primeira experiencia, um grupo de linguados com cerca de 1.5 anos de idade foram tratados com uma única injecção de kisspeptina e um segundo grupo com PBS, servindo de controlo. Foram recolhidas amostras de plasma sanguíneo com a finalidade de determinar os níveis de esteróides sexuais (T e E_2 em fêmeas e T e 11-KT em machos, analisados por ELISA) antes e 4 horas, 2 dias e 4 dias após do tratamento. As gónadas foram extraídas 4 dias depois to tratamento, de forma a determinar o seu estado de maturação nos peixes tratados com kisspeptina e nos do grupo controlo. Os níveis de testosterona aumentaram significativamente em ambos os sexos, nos peixes tratados com kisspeptina, 4 dias após o tratamento. No caso das fêmeas de linguado foi também observada uma tendência de aumento no estradiol, apesar de não ser um aumento estatisticamente significativo. As análises histológicas revelaram um aumento de espermatídios e uma diminuição de espermatócitos na zona cortical das gónadas dos machos tratados, indicando um estado de maturação mais avançado. Estes resultados evidenciam o efeito positivo que o tratamento com kisspeptina tem na puberdade do linguado senegalês, indo de acordo com estudos anteriores noutras espécies. Uma segunda experiência foi realizada à semelhança da primeira, mas e reprodutores de linguado senegalês, com a finalidade de testar o efeito do tratamento de kisspeptina na sua comunicação química. Amostras de urina, recolhidas de peixes tratados com kisspeptina e de peixes do grupo controle, foram analisadas através de LC-MS, numa tentativa de identificar compostos relevantes, e usadas num ensaio de EOG, para determinar o estimulo induzido epitélio olfactivo de linguados receptores. Foram encontrados dois compostos em maior concentração na urina dos peixes tratados, sendo um deles um composto não documentado previamente, com uma estrutura potencialmente de um esteróide, e um segundo composto identificado como o ácido 3-hydroxyphenylacetic. Ambos os compostos foram sugeridos como sendo potenciais feromonas, devido ao aumento de potência olfativa induzido pelas urinas de linguados de ambos os sexos, tratados com kisspeptina.

Palavras-chave: *Kisspeptina; linguado senegalês; Hormonas; Puberdade; Comunicação química*

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1. State of the art

The increase of industrialization and population over the past decades showed also a rise in global food demands, particularly seafood (Bostock et al., 2010; Fukase and Martin, 2020). Mainly due to the growing awareness of its cheaper prices and health benefits (Supartini et al., 2018). This increase helped to boost aquaculture as a more sustainable alternative to fisheries. One of the most crucial aspects in this industry is the control and understanding of the reproductive system in fish, in turn leading to an optimized production.

1.1. Fish reproductive axis

Among the circa existing 24,000 fish species, the diversity of reproductive strategies is enormous. Depending on the species, one fish species may only reproduce once or twice a year, while others may be able to reproduce throughout the year (Chemineau et al., 2007). Generally, spawning occurs in a determined time of the year, in order to optimize the survival of the offspring, with environmental cues (such as water temperature and photoperiod) being crucial to synchronize gonadal maturation (Baroiller et al., 2009; Chemineau et al., 2007; García et al., 2019; Quintana et al., 2004). These cues are able to influence the brain-pituitary-gonad axis (BPG axis) through the action of melatonin, a hormone produced mainly during the night (Falcón et al., 2010; Pazarci et al., 2020). This indoleamine is derived from the essential amino acid tryptophan that is transformed into serotonin, through two enzymatic reactions. Two further reactions once again transform serotonin into melatonin. These two hormones have opposite peaks in the bloodstream, with serotonin levels being higher during the day and melatonin during the night (Falcón et al., 2010). One of the mediator enzymes of this process, the aryllkylmine N -acetyltransferase (AANAT), has its activity inhibited during the night, thus interrupting melatonin production. One of the several functions that melatonin possesses is to stimulate the endocrine system via the hypothalamus and the pituitary gland through the action of the kisspeptin neurons (Kim and Cho, 2017; Kitahashi and Parhar, 2013). These organs, present in the brain, have crucial roles in the endocrine system, regulating the release of multiple hormones required for the normal functioning of the body. Among these hormones, the gonadotropin-releasing hormone (GnRH) is considered the most important. It is produced by the hypothalamus and stimulates the release of follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) in the pituitary gland. These hormones in turn, act

mainly on the gonads, stimulating additional hormones, release, including sexual steroids. In the case of males, the Fsh stimulates the secretion of several growth hormones that maintain and initiate spermatogenesis, and sexual steroid hormones like testosterone (T) and 11-ketotestoesterone (11-KT) in the testis. In females, Fsh stimulates the production of 17β-estradiol (E_2) in the ovaries and also T. Regarding male Lh, it has important roles in spermatogenesis and spermiation while in females this hormone plays a part in the meiotic maturation and ovulation (Cahoreau et al., 2015). One common aspect in the function of these hormones is on the benefit of gametogenesis. This process generates gametes through cell divisions and differentiations.

1.2. Kisspeptin system

From 1995 to early 2000, an important discovery in neuroendocrinology was made; the discovery of kisspeptin, a peptide capable of inhibiting metastasis in melanoma cells and breast cancer, later found as having an important role in reproductive signalling (Lee et al., 1996). The gene encoding for this peptide was named *kiss* and the later described kisspeptin receptor, *kissr*. De Roux and colleagues described that inactivating mutations on *kissr* caused idiopathic hypothalamic hypogonadism (IHH) syndrome in mammals (de Roux et al., 2003). This condition is characterized by delayed/absent puberty and a deficiency in gonadotropin release (Clarke et al., 2015; Felip et al., 2009; Mechaly et al., 2013, 2012). The connection of the kisspeptin system with reproduction and its subsequent up-regulation of the BPG axis was found (Clarke et al., 2015; Felip et al., 2009), representing a major breakthrough in the reproductive biology field. Kisspeptin, acting centrally via the *kissr*, stimulates GnRH neurons in the hypothalamus to release GnRH (Clarke et al., 2015; Roseweir and Millar, 2009), in turn stimulating the anterior pituitary into releasing LH and FSH into the gonads (Ohga et al., 2015).

A few years later, a significant discovery was made while studying this system on non-mammalian vertebrates. In some species, the region that was previously thought to be the *kiss* gene, was actually an isoform. At this point, both genes were renamed: *kiss1* to the original gene described in mammals and *kiss2* to its newly discovered isoform from non-mammalian vertebrates (Felip et al., 2009; Lee et al., 2009). By performing phylogenetic and genomic synteny analyses, both the isoform and the original gene were found in several additional non-mammal species (Felip et al., 2009; Lee et al., 2009). Their paralogous nature became evident, with the possibly originated from an ancestral gene. In all mammal studies, only the platypus (*Ornithorhynchus anatinus*) was

found to have both *kiss1* and *kiss2* genes (Lee et al., 2009; Mechaly et al., 2009a). For the majority of fish species, both *kiss* genes were found, with some exceptions having only *kiss2*, which is the case of Senegalese Sole (*Solea senegalensis*) (Mechaly et al., 2009b), puffer fish (*Takifugu rubripes*) (Mechaly et al., 2009b) and Atlantic halibut (*Hippoglossus hippoglossus*) (Mechaly et al., 2009a). This fact point towards a loss of genes during evolution, since more "ancient species" belonging to *Agnatha* and *Gnathostoma* also possessed both genes (Felip et al., 2009; Mechaly et al., 2009a).

When comparing both sequences, the KISS1 peptide is also called the Y-Y form, due to its core amino-acid (aa) sequence KISS-10 (YNLNSFGLRY), while in the KISS2, the KISS2-10 peptide has an F-F form (FNYNPFGLRF) (Kitahashi et al., 2009). In general, when they coexist, they have low aa sequence identity, which is case for medaka (*Oryzias latipes*), 20 %, and zebrafish (*Danio rerio*), 25 % (Kitahashi et al., 2009). In these cases, a difference of expression could be observed, like in the case of European seabass (*Dicentrarchus labrax*), where more potent gonadotropin-releasing activity was observed for KISS2 when compared to KISS1, suggesting a more dominant role in the control of the BPG axis in fish (Felip et al., 2009). One crucial role of this peptide is its role in the control of the onset of puberty as evidenced by previous studies. For example, in the chub mackerel (*Scomber japonicus*) it was found that the levels of expression of *kiss* were higher just before the onset of puberty (Ohga et al., 2015). Additionally, this hormone has been suggested as having a multitude of other functions related with reproduction in mammals, such as metabolic control of fertility, regulation of the reproductive capacity by environmental cues, control of placental formation and pregnancy, while also possessing other pleiotropic functions, like regulation of the cardiovascular system, adipocyte biology and pancreatic secretions (Brown et al., 2008; Greives et al., 2007; Hauge-Evans et al., 2006; Selvaraj et al., 2013a). However, in fish, due to the focus of kisspeptin research efforts in reproduction and the variability within kisspeptin paralogous genes, limited information is available about its potential pleotropic effects (Akazome et al., 2010; Mechaly et al., 2013).

1.3. Reproduction control in aquaculture

Several approaches have been developed to counteract fish reproductive dysfunctions occurring in aquaculture. Environmental conditioning is a very important aspect to improve broodstock management and fish reproduction, always taking into consideration each species'

biology when optimizing rearing conditions. Also, by artificially regulating the environmental cues that trigger puberty, it is possible to control its onset in some species to increase growth rates, by suppressing gonad maturation, a laborious and energy consuming process (García et al., 2019; Taranger et al., 2010). In Nile tilapia (*Oreochromis niloticus*), continuous exposure to light inhibited gonadal maturation and increased somatic growth with a similar result in several other species like the Atlantic cod (*Gadus morhua*) (Ginés et al., 2004; Imsland et al., 2007; Navarro et al., 2015; Rindorf et al., 2008; Unwin et al., 2005). In broodstock management, in the absence of natural spawning, more invasive approaches, like hormonal treatments have been used. This is a widely employed technique in the aquaculture industry, to increase the efficiency of eggs or sperm production and enhance reproduction in captive conditions (Mylonas et al., 2010). There are several different hormonal treatments that can be administered, which will act at distinct levels of the BPG axis. While GnRH acts directly at the pituitary level, stimulating endogenous gonadotropins and other pituitary hormones, steroids like E_2 act directly at the gonadal level, stimulating maturation and secondary sexual characteristics (Cahoreau et al., 2015; Hoga et al., 2018). Some examples of treatments used are carp pituitary in Amazon catfish (*Leiarius marmoratus*) (Araújo et al., 2014); GnRH agonists in Senegalese sole (Rasines et al., 2013); GnRH agonists coupled with dopamine antagonists in carp (*Cyprinus carpio carpio*) (Vazirzadeh et al., 2011) and with E_2 in Atlantic cod (Lin et al., 2012). The administration methods are varied, with many possibilities to treat fish, like injections, microspheres, slow releasing implants, food supplements or even through immersion. Injections can be administered individually or periodically, intramuscular or intraperitoneal (Hoga et al., 2018). These kinds of treatments can put the fish at high stress due to manipulation. Both, implants and microspheres, which the usage is in the same manner, an injectable pellet/sphere made of specific materials that progressively releases hormones require much less manipulation (Nocillado et al., 2013). However, these types of administration methods are more expensive in comparison to the injections, which are considered the most accessible method by its lower price and easier implementation (Harvey and Carolsfeld, 1993).

1.4. Hormonal treatment with kisspeptin

In humans, the use of kisspeptin as a treatment has been applied with success in several infertility cases, both in women and in men (Welt et al., 2004; Young et al., 2013). In the case of fish, kisspeptin treatment has shown successful results both in inducing puberty in juveniles and

gonadal maturation in adults, by acting at an upper levels of the BPG axis, translating in a more natural physiologic response (Beck et al., 2012; Felip et al., 2009; Mechaly et al., 2013; Selvaraj et al., 2013b). One of the first studies to produce promising results in fish was in the European seabass. By administrating both prepubertal and adult fish with a single injection of 250 ng/g of both KISS1 and KISS2, the KISS2 had a larger effect on Lh and Fsh secretion, at both 60 and 120 minutes post injection (Felip et al., 2009). In the case of zebrafish, mature females were treated with 2 nmol/g of both kiss2 and kiss1, also significantly increased Fsh and Lh mRNA levels in the pituitary 12 hours post treatment (Kitahashi et al., 2009); In white (*Morone chrysops)* and striped bass (*Morone saxatilis*), adult and juveniles, using multiple injection protocol of 250 ng/g of both kisspeptins twice a week for 8 weeks, had a successfully stimulation of puberty and gonadal maturation, increasing the gonadosomatic index (GSI) and the quantity of more developed germ cells in the gonads (Beck et al., 2012); In the chub mackerel, in juveniles and immature adults using either a slow releasing medium or a single injection of KISS1-15 and KISS2-12, increased sex steroids circulating levels and pituitary Fsh and Lh in addition to stimulating spermiation (Ohga et al., 2015; Selvaraj et al., 2015, 2013b, 2013a). These studies evidenced the promising prospect of using kisspeptin as an exogenous hormonal treatment with the possibility to enhance the stimulation in the reproductive axis.

1.5. Chemical communication and pheromones

Chemical communication is very important to regulate several physiological processes, like reproduction, where chemical cues help to search potential partners, evoking behaviour, endocrine responses, synchronizing gametogenesis and spawning (Burnard et al., 2008; Huertas et al., 2014; Stacey et al., 2003). Chemical communication can also play a part in processes such as, schooling, territorial marking (dominance), species recognition, parent-young interactions (imprinting), among others (Liley, 1982).

In terms of reproduction, the chemical communication for the recognition of conspecific odorants and pheromones is essential to aquatic animals, like fish (Hubbard et al., 2014; Liley, 1982; Velez et al., 2013). One of the best examples is the Nile tilapia, used as a model species for electrophysiology. This species has a characteristic courtship behaviour, where dominant males acquire a specific zone which they control and protect from other males, mainly using pheromones in their urine to signal their dominance (Hubbard et al., 2014; Keller-Costa et al., 2012). These

alpha males urinate more often and possess a more potent urine than subordinate males (Hubbard et al., 2014). Dominant tilapia male urine evokes an increase in 17, 20β-P, an oocyte maturation inducing steroid, in females which stimulates a synchronization of reproduction between males and females (Hubbard et al., 2014). In addition, urine from pre ovulated females evokes a stronger response in males, when compared to post ovulatory ones (Huertas et al., 2014). Altogether, these are strong evidence of the use of urinary pheromones in tilapia. The discovery of the steroids glucuronides 5b-pregnane-3a,17a,20b-triol-3a-glucuronide (20b-P-3-G), and 5b-pregnane3a,17a,20atriol-3a-glucuronide (20a-P-3-G), which are important compounds in fish reproduction, reinforce this hypothesis (Hubbard et al., 2014). Another model species, the goldfish (*Carassius auratus*) was a well-documented case of fish pheromones with female urine containing 17,20β-P sulphate and prostaglandin F2α that also increase male aggressiveness and creates a surge in male Lh and spermiation (Sorensen et al., 1995; Van Der Kraak et al., 1989). Similar results have also been observed in other fish species. For instance, the urine of the rainbow trout (*Oncorhynchus mykiss*) female induces Lh and steroidogenesis in males; Atlantic salmon (*Salmo salar*) female urine and ovarian fluid increase plasma sex steroids concentration in males and the female masou salmon (*Oncorhynchus masou*) urine contains amino acids that attract mature males (Yambe et al., 2006, 1999).

1.6. Senegalese sole biology and production

Senegalese sole is a common demersal flatfish in the south European waters (Morais et al., 2016) which is naturally distributed from Senegal (Africa) to La Rochelle (France) and in the Western Mediterranean Sea. This species belongs to:

Type: *Vertebrata*;
Subtype: *Gnathostomata*;
Superclass: *Pisces*;
Class: *Osteichthyes*;
Order: *Heterosomata* (*Pleuronectiforms*);
Suborder: *Soleoidei*;
Family: *Soleidae*;
Subfamily: *Soleinae*;
Genre: *Solea*;
Species: *Senegalensis*.

This species is highly valuable for Mediterranean aquaculture, gaining interest since the 1980s, due to its prospects in commercial aquaculture (Morais et al., 2016). Consequently, Senegalese sole cultivation became increasingly widespread, with production methods better optimized. In general, the knowledge of the biology of this species has been improved in countries like Portugal and Spain, which are leading research efforts (Morais et al., 2016). Basic culture conditions are already well optimized, with temperatures ranging around 17 – 20 ºC, since disease outbreaks are known to occur at high temperatures (Morais et al., 2016). Regarding reproduction, one main spawning period exists during spring, from February/March to May/June, and a secondary one during autumn (Anguis and Cañavate, 2005).

The reproductive efforts of this species' aquaculture are primarily made with wild caught broodstook. In the wild, these fish achieve maturation around 3 year old, and are able to acclimatize well to captive environments. With the manipulation of some external factors like temperature conditions, some studies managed to regulate the spawning season (Morais et al., 2016). The need to use wild individuals emerged from the lack of success when using captive reared and born fish, that, after several studies, it was concluded the problem derived from the males (F1 males). Spawns using these individuals were infrequent, comprised mostly with non-viable eggs (Agulleiro et al., 2007; Carazo et al., 2013; Guzmán et al., 2008; Morais et al., 2016). Additionally, F1 males were demonstrated to lack the courtship behaviour needed to fertilize the eggs, specifically the paired synchronized swimming and the pre-spawning chasing (Carazo et al., 2017, 2013, 2011). In terms of endocrinology, two to three times lower Lh levels were observed in F1 males in comparison with wild, with other hormones (androgens and Fsh) showing similar or higher concentrations (Chauvigné et al., 2016). These hormonal differences, coupled with the courtship behaviour absence, lead to wild male dependency, generating pressure on wild populations, which is not sustainable at long term period.

Some studies demonstrated that sole males isolated from females presented lower androgen levels than males cohabiting with females. Furthermore, sole males in presence of females treated with MIS (maturation inducing steroids, 17, 20β-dihydroxy-4-pregnen-3-one) obtained better sperm viability, velocity and motility,, showing the male-to-female chemical communication in this species (Cabrita et al., 2011). In addition, recent studies demonstrated that the olfactory sensitivity in Senegalese sole depends on the maturity and sex of the donor and receiver (Fatsini et al., 2017). This study remarked that distinct sensitivity was observed when Senegalese sole

juveniles (receiver) smelled urine from mature males and females, and at the same time, that potency also changed if the receiver was a male or female. Moreover, urine from mature sole females increased circulating Lh levels in mature males, surprisingly, more potently on cultured fish than wild caught ones. Therefore, another explanation was proposed for the lack of reproductive success of F1 males, related to the chemical communication system.

This species is born with bilateral symmetry, starting as pelagic active predators, relying heavily on their visual sense. A few days after hatching, sole goes through a complex metamorphosis, and their characteristic flatfish asymmetry starts to develop (Fernández-Díaz et al., 2001). This shift also increases their reliance on the olfactory system and decreases their need for visual cues, since they start being benthonic (Padrós et al., 2011). This also creates an asymmetry on sole olfactory epithelia (Velez et al., 2013, 2009). The lower olfactory epithelium (blind side) evolved to smell the interstitial waters where their preys, benthic invertebrates and small crustaceans, live, so they have adapted to detect odours associated to them. The upper epithelia (eyed side) evolved to be more sensitive to conspecific communication, and certain conspecific odorants, like the bile acid and taurocholic acid (Velez et al., 2013, 2009).

In terms of the kiss system, the *kiss2* gene has been found in this species, present in two variants, the *kiss2A* and *B*. While the B peptide is more present in the gonads, coding for a truncated non-functional protein, the A is more expressed in the brain and produces the functional peptide protein (Mechaly et al., 2009b; Mechaly et al., 2011). Seasonally, endocrine levels of Fsh, Lh, T, 11KT, E_2, as well as *kiss2* gene expression, tend to peak concomitantly at the end of winter, just before the spawning season in spring (Mechaly et al., 2012).

Several exogenous hormonal treatments have been tested in Senegalese sole to enhance reproduction and mitigate dysfunctions. In females, the best outcome was observed when using slow-release implants of GnRHa, which stimulated oocyte maturation and steroids secretion (Agulleiro et al., 2006; Guzmán et al., 2009). In the case of males, GnRHa injections and implants resulted only in a slight increase in steroids and milt volume (Agulleiro et al., 2007, 2006; Cabrita et al., 2011; Guzmán et al., 2011b, 2011a). When these treatments were complemented with a 11-ketoandrostenedione implant (a 11-KT precursor) or pimozide (an antidopaminergic drug), a bigger increase of steroids and milt volume was obtained (Agulleiro et al., 2007; Guzmán et al., 2011a). However, the most successful treatment was the application of human chorionic gonadotropin (hCG) using a multiple injection protocol (Guzmán et al., 2011b). Promising results

in enhancing sperm production were obtained using homologous recombinant gonadotropins, although sustained administration of high amounts may become harmful to Leydig cells (Chauvigné et al., 2017). Besides these optimistic results described for some of the therapies tested, applications in aquaculture are still limited, since the total sperm volume obtained is still reduced in this species, in addition to their relative complexity and need of trained personnel (Cabrita et al., 2006; Martín et al., 2014; Rasines et al., 2013, 2012).

1.7. Techniques

1.7.1. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a frequently used method for detection of various compounds, including hormones. Since it is a quick and simple method, is usually used for research and diagnostic purposes. There are four types of ELISA, each with different advantages. The simplest one is the direct ELISA, where only primary antibodies are used to link directly to the target antigen. The primary antibody's enzyme then reacts producing a measurable signal. The second type is the indirect ELISA, which differs from the first one in the use of a secondary antibody, linked to the enzyme, producing a measurable signal. The third method is the sandwich ELISA, where the antigen is sandwiched between a captured antibody, immobilized to the solid surface, and a primary and a secondary detection antibody. The fourth and final method, the competitive ELISA, is considered the most complex of all. The basic principal is that the target antigen competes with an inhibitor antigen for primary antibodies to bind to. In contrast to the other methods, the signal is inverted, meaning that the less signal produced, the more target antigen is present. Each ELISA method has their advantages being the first method the simplest, fastest, and cheapest in comparison with the fourth which is more sensitive and flexible. For example, Guzmán developed a protocol for a competitive ELISA used to quantify E_2 and T levels in plasma samples in Senegalese sole (Guzmán et al., 2008).

1.7.2. Histology

Histology is defined as the study of tissues under light microscope, allowing the observation of the cells and structures present in the tissues evaluated. This technique is extremely useful in gonadal tissues, to validate the sex and maturation stage, and assessment of reproductive point or atresia quantification (Blazer, 2002). For this purpose, the target tissue needs to be fixed, embedded in

paraffin wax or resin for further cut into thin sections, between 2 and 8 µm depending on the staining method. The stains will also depend on the desired structure evaluation, where the most common is the use of Haematoxylin/Eosin to stain the cells nuclei and unspecific proteins, which will create a contrast in the tissue perceptible in the microscope.

1.7.3. Liquid Chromatography – Mass spectrometry (LC-MS)

Liquid chromatography-mass spectrometry is a widely used technique that separates compounds within a sample, like urine, and identifies them. The equipment for this technique includes several components: an atmospheric pressure ionization source, an ion-inlet and focusing component, two mass filtering devices, a collision chamber, and an ion-impactor detector (Grebe and Singh, 2011). Combining liquid chromatography with the detection specificity of mass spectrometry, it can separate compounds according to their physical and chemical properties. This technique can process large, polar, ionic thermally unstable and volatile compounds.

1.7.4. Electro-olfactogram

Chemical communication is the most primordial and a common system to exchange information between organisms. The message is transferred *via* chemicals liberated to the environment by an emitting organism to be received by the receiver organism. These chemical emissions have become specialised as hormones, neurotransmitters, or other products with highly specific activities. To communicate chemically, animals rely on their olfactory epithelium, where in case of the fish, the olfactory system is one of the most important to survive and perceive the environment. When the epithelium receives a stimulus, such as the urine of a rival or mate, it produces an electrical potential change in the olfactory neurons, present in the olfactory epithelium. By measuring this potential change, the potency of a stimulus may be quantified (Knecht and Hummel, 2004). Electro-olfactogram (EOG) is commonly used in electrophysiology to understand and obtain more information about chemical communication (Hubbard et al., 2014, 2002). Some studies used this technique to test whether humic acid blocks chemical communication in goldfish, finding that in the presence of humic acid, EOG amplitude evoked by a steroid was significantly reduced, suggesting that it may "trap" steroid pheromones (Hubbard et al., 2002). It was also used to compare the Nile and Mozambique tilapia species, finding that the urine from one is equally potent to the other (Hubbard et al., 2002). Finally, Fatsini et al. 2017 and Velez et al. 2013 also used this

technique, in studies that helped gaining crucial information regarding Senegalese sole chemical communication. The first suggested that urine and faeces released reproduction related odorants, and the second study found evidences for distinct functions of both sole olfactory epitheliums, upper and lower (Fatsini et al., 2017; Velez et al., 2013).

1.8. Objectives

1.8.1. Determine the Effect of kisspeptin treatment in advancing puberty in Senegalese sole juveniles

The first specific objective of this thesis was to investigate if kisspeptin treatment allows to control the onset of puberty in juvenile Senegalese sole. This effect was tested by measuring sex steroids' levels (T and 11kt for males and T and E_2 in females) in blood plasma by ELISA. In addition, gonadal maturation stages were evaluated through histology, in both control and treated groups. The comparison among groups allowed to detect any influence in sole puberty caused by kisspeptin treatment.

1.8.2. Test the effect of kisspeptin treatment in reproduction metabolites in urine of Senegalese sole breeders

The second experiment aimed to test if the exogenous administration of kisspeptin on F1 sole breeders had an impact on their chemical communication. To achieve this goal, urine from control and treated breeders was analysed for reproductive metabolites through LC-MS/MS, to identify any significant changes in urine composition, and providing a baseline for potential reproductive metabolites in this species. In addition, the same urines were used as stimulus for an EOG analysis, to find specific evidences that kisspeptin treatment has effects on the chemical communication of this species.

2. Material and methods

2.1. Fish and rearing conditions

2.1.1. Experiment 1: Effect of kisspeptin treatment in advancing puberty in Senegalese sole juveniles

Senegalese sole juveniles used for the first experiment (205 ± 25 g and 24.75 ± 2.25 cm), were obtained from IPMA research station, in Olhão, Portugal. A total of 72 fish were selected from a group originated from a single batch, with 1.5 years old, age at which this species is thought to enter puberty. Homogenizing the developmental stage among individuals, to guarantee similar maturation stages at the beginning of the experiment, the selection of fish was performed by choosing animals weighting between 180 and 230 g. This selection was done assuming a sex ratio 1:1, since identifying males from females at this stage is unfeasible due to the absence of sexual dimorphism. During fish selection, soles were anaesthetized with 2-phenoxyethanol at a dose of 250 ppm, to safely measure, weight and tag them (ID100 Implantable Transponder, *Trovan*, The Netherlands). These pit-tags allowed individual tracking of fish at consecutive samplings along the experiment. Afterwards, fish were transported to Ramalhete station, were they were distributed randomly between 6 tanks of 200 L, under similar rearing conditions. Each tank had 12 soles with a mean density of 4.92 kg/m^2. Tanks were covered with shad nets to avoid possible stress effects derived from people performing operational tasks and high light intensity (Figueiredo et al., 2020; Morais et al., 2016), as well as to prevent fish jumping out of the tanks. Water quality parameters were monitored daily (temperature, oxygen saturation in %, dissolved oxygen concentration in mg/L and salinity; Table 1) and tanks were cleaned and purged. Sole juveniles were fed seven days a week, at approximately 2 - 2.5 % biomass with a commercial feed from SPAROS Lda. (MARINE Sole, mixed pellets of 3 and 4 mm).

Table 1. Water quality parameters as an average among all tanks (oxygen saturation (%), dissolved oxygen (mg/L), temperature (°C) and salinity concentration (‰)) taken daily for three consecutive weeks. Mean ± SD are shown.

Average values			
Oxygen Saturation (%)	Dissolved Oxygen (mg/L)	Temperature (°C)	Salinity (‰)
93.57 ± 3.15	7.34 ± 0.49	16.86 ± 2.16	35.86 ± 0.28

2.1.2. Experiment 2: Effect of kisspeptin treatment in reproduction metabolites in urine of Senegalese sole breeders

This experiment took place in Ramalhete experimental station, using the established Senegalese sole F1 broodstock, maintained in 4 indoor tanks, 12 fish per tank at a sex ratio of 1:1, under natural conditions of photoperiod and temperature (17.67 ± 2.29 ºC). Fish were fed *ad libitum* once a day, five days a week with a commercial diet from SPAROS Lda. (BROODFEED LEAN 7). Similarly to the juvenile experiment, all fish were identified with a Pit-tag system.

2.2. Experimental design

2.2.1. Experiment 1: Effect of kisspeptin treatment in advancing puberty in Senegalese sole juveniles

The sole juveniles experiment took place in November 2019, after a 2-week acclimation period. For both juveniles and adult trials, 1ml syringes and 1.5 ml microtubes for blood sample collection were previously heparinized (heparin solution at 4000 u/ml, Aldrich chemistry, Germany) to avoid blood coagulation. Blood was collected from the caudal vein and centrifuged at 3000 RPM for 15 min at 4°C to separate the plasma from the haematocrits. Plasma was transferred to a new 1.5 ml microtube and stored at -80°C for further processes. A first sampling (T0) was performed before the treatment to obtain the basal values of sex steroids in blood plasma. At this point all fish were sampled. Three days after, Kisspeptin and PBS were administrated to the fish in three of the six tanks (treatment day). PBS was chosen as a control, since it has no direct physiological effect, functioning as a placebo. The concentration used for the kisspeptin decapeptide Sskiss2_v2 (GeneBank HM116743) administration was 250 µg/kg of fish total weight, synthetized by CPC

scientific based on the previously described sequence for the species (Mechaly et al., 2009a). Afterwards, new sampling trials were performed at 4 hours (T1.4), 2 (T3) and 4 days (T5) post injection. These samplings, however, were done in a rotation system, to avoid the stress of the fish as much as possible due to repetitive handling; half of the fish of each treatment were sampled at T1.4 and T5, while the other half was sampled at T3.

At the end of the experiment, fish from one tank of each treatment (control and kiss) were euthanized by anaesthetic overdose with 2-phenoxyethanol, in order to dissect the gonads for histological procedures. These fish were first weighted to calculate the gonadosomatic index (GSI) and then fixed using paraformaldehyde solution at 4 %.

2.2.2. Experiment 2: Effect of kisspeptin treatment in reproduction metabolites in urine of Senegalese sole breeders

The breeder's kisspeptin treatment was performed during the reproductive season for this species (Spring), in April and May 2018. Similarly to the juveniles experiment, Senegalese sole from 2 out of the 4 tanks were treated with kisspeptin decapeptide, with a single intramuscular injection using the same dose of 250 μg/kg total body weight, while the other 2 tanks were treated with PBS used as a control. Blood plasma and urine samples were collected before the treatment (T0) and 2 days post-injection (T3). Blood collection was performed as explained in experiment 1 and urine was collected from the urogenital pore using a 1 ml syringe, after gently pressing the abdominal area. Blood plasma samples were previously analysed to determine hormonal levels of gonadotrophins (Fsh and Lh) and sex steroids (T and 11KT in males and T and E_2 in females) (Oliveira et al., 2019). The results obtained with the sex steroids and gonadotrophins in Senegalese sole breeders treated with kisspeptin suggested the possibility of changes in urine samples which were analysed during the present work.

2.3. Analytical procedures

2.3.1. Experiment 1: Effect of kisspeptin treatment in advancing puberty in Senegalese sole juveniles

2.3.1.1. Hormonal levels determination

In experiment 1, juvenile's steroid levels were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits from Cayman Chemical (testosterone, 11-ketotestosterone and

Estradiol, with the last two being exclusively used for males and females respectively, item kit No's - 582701, 582751 and 582251), previously used for the same species (Chauvigné et al., 2012; Fernández et al., 2019; Oliveira et al., 2019). Samples were first extracted, following a protocol adapted from (Rodríguez et al., 2000), where 5 µl of thawed plasma were diluted (or 10 µl depending on the final dilution pretended) in 100 µl of ELISA buffer from the kit, followed by the addition of 600 ml of methanol and centrifugation at 4 ºC and 9000 rpm for 10 min. After transferring the resulting supernatant to a second microtube, the same process was repeated 2 times with 300 ml of methanol. The resulting supernatant was submitted to a dry bath at 37 ºC for approximately 2 days until methanol was totally evaporated.

Afterwards, the ELISA assays were conducted according to manufacturer's instructions. Firstly, samples were resuspended in 250 µl ELISA buffer and the standard curve was prepared. All samples and standard curves points were added to a previously coated 96-well plate, provided with the kit. The AChE tracer and the antiserum were then added to each well according to protocol specifications. This step was followed by a 1 / 2h incubation (depending on the assay), at room temperature and in an orbital shaker. When completed, the plate was rinsed five times with washing buffer, after which 200 µl of Ellman's Reagent were added into each well. A final developmental period took place in the orbital shaker for 1:30 h to 2 h in complete darkness. At the end, the optical density was measured using a microplate reader (Synergy 4, Biotek Instruments, Inc., Vermont, USA) at a wavelength of 405 nm. The absorbance values obtained were inserted into an excel spreadsheet given by the manufacturer, where the results were calculated.

The 11-KT kit used had a range of 0.78 to 100 pg/ml, a sensitivity of approximately 1.3 pg/ml, and an intra and inter-assay coefficient of variation, at 50 % of binding, of 13.5 and 6.9 respectively; the T kit had a range of 3.9 to 500 pg/ml, a sensitivity of approximately 6 pg/ml, and an intra and inter-assay coefficient of variation, at 50 % of binding, of 4.6 and 2.8 respectively; finally, the E_2 kit had a range of 6.4 to 4000 pg/ml, a sensitivity of approximately 15 pg/ml, and an intra coefficient of variation, at 50 % of binding, of 13.6.

2.3.1.2. Histology procedures and analysis

Samples from the excised testis were first fixed in a paraformaldehyde solution at 4 % for 48 h, after which, they were transferred to methanol and stored at -20 ºC. For the process, sampled were embedded in paraffin passing first through a bath of xylol/methanol (50/50 %) followed by pure xylol 1 h each. This allowed the complete removal of the methanol from the tissues, since if

present, ethanol impedes paraffin wax infiltration. Samples were then inserted into paraffin blocks using a paraffin dispenser (KD-BM Tissue Embedding System, Kedee KD-BM). Serial 5 µm sections were cut using a microtome (Rotary Microtome Microm HM 340E, Thermo Scientific) and stained with haematoxylin and eosin (H&E).

The processed slides were observed under a light microscope (Eclipse E200, Nikon) where photos of the cortical and medullar region were taken using a DXOMARK Camera 25 megapixels, an aperture of f/1.7 and 26 mm wide lens connected to the microscope using an adapter (Slokey Discover The World, Spain) directly to the microscope.

2.3.2. Experiment 2: Effect of kisspeptin treatment in reproduction metabolites in urine of Senegalese sole breeders

2.3.2.1. Electro-olfactogram (EOG)

The fish used in this analysis were some of the remaining juveniles of Senegalese sole from experiment 1, eight females and five males (n = 13), maintained under the same conditions as previously described, at Ramalhete experimental station, in the University of Algarve (533 ± 81.46 g weight).

Urine belonging to the adult breeders used in experiment was used as the stimuli. The urine samples were divided into sexes for treated and untreated (control), with a total of 16 urine stimulus, 10 females (5 treated and 5 control) and 6 males (3 treated and 3 control).

Prior to use, urine samples were aliquoted at 10µl and stored at -20 ºC until use. Each sole was anaesthetized by immersion with tamponed ethyl 3-aminobanzoate methanesulfate, MS-222, (Aldrich chemistry, Germany) and was quickly transported from Ramalhete station to the testing site. The fish was then placed in the EOG platform at a slight backwards tilt with aerated water containing the anaesthetic being pumped into the gills, to remain deeply sedated during the EOG procedure (approximately 100 ml 100 g^{-1} body weight min^{-1}). Additionally, an intramuscular injection of gallamine triethiodide (Aldrich chemistry, Germany) was given to immobilise the fish (0.6 mg per 100 g) (Velez et al., 2013).The whole fish was covered with damp paper towels and the olfactory rosette was exposed by surgically cutting the skin and muscle (Fig. 1). The test was performed as previously described by Hubbard et al., 2002 and Velez et al., 2005. Briefly, the recording electrode was placed according to the position of the largest response to the standard stimulus, M-cysteine. The reference electrode was placed in the skin near the nostrils. The signal

was digitalized using a Digidata (DigiData 1322A, Axon Instruments, now Molecular Devices, Sunnyvale, CA, USA) and stored on a PC using AXOSCOPE (Axon Instruments, Inc.). The urine stimuli were diluted 1:1,000 by mixing 10 µl of urine with 10 ml of sea water from the system followed by delivery via a glass tube placed near the rosette. This was done with a finely pointed tube, to reduce the accumulation of mucus on the epithelium, which could damage the recordings. At least 1 min was allowed between successive stimuli, to allow the membrane to recover from the last stimuli. At the end of the experiment, the fish were euthanized with a lethal dose of anaesthetic MS-222.

Normalization was calculated using the amplitude of M-cysteine standard stimulus after blank subtraction (amplitude of EOG using sea water tested in the same way as the stimulus) to compare the effectiveness of the treatment. Both blank and standard were recorded at regular intervals between stimuli.

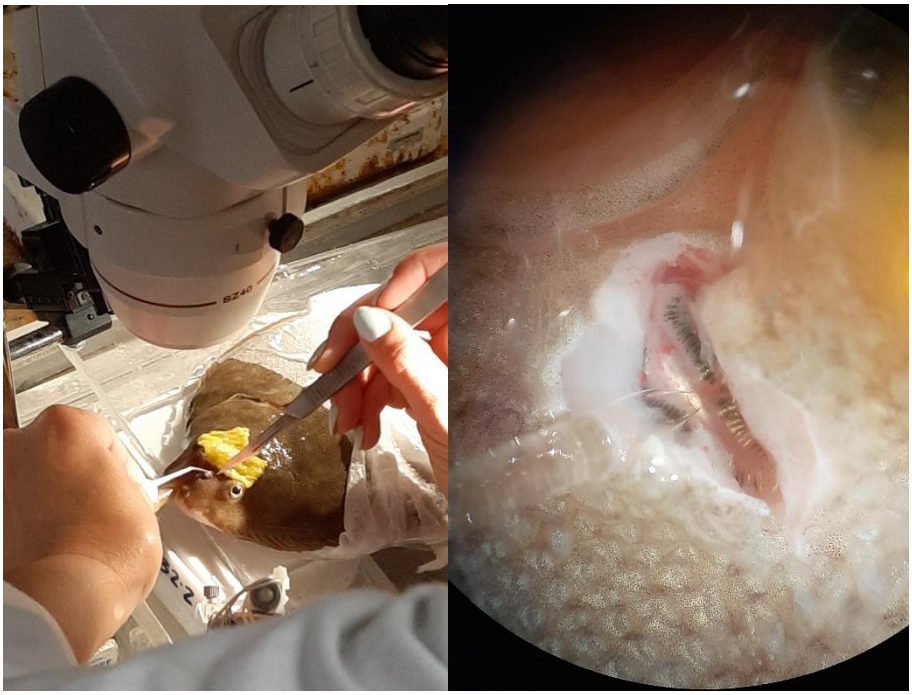


Figure 1. Photos of the Electro-Olfactogram analysis on Senegalese sole. On the left, the surgical procedure with fish laid at a backwards tilt with a damp paper on top, being surgically operated. On the right, we can see the exposed olfactory rosette with the glass pipette pointing at the olfactory epithelium.

2.3.2.3. Liquid chromatography-Mass spectrometry analysis (LC-MS)

Urine samples from sole breeders were thawed and filtered using a 20 µm filter, to eliminate any solid particles, and injected in the LC-MS analysis (UHPLC-HR-MSn). Chromatographic separation was performed on a Thermo Scientific ultimate 3000 UHPLC using the column Thermo Scientific Accucore RP-18 (2.1 × 100 mm, 2.6 µm). The mobile phase composition was prepared with water (A) and acetonitrile (B), both containing 0.1 % of formic acid. The gradient (in v/v %) started with 100 % of A for 2 min, increased linearly to 30 % of B in 13 min, to 100% of B in 16 min; maintained at 100 of % B for 4 min, returned to 100% of A in 1 min and then was maintained at 100 % of A for 4 min. The flow rate was 0.3 ml/min. The injection volume was 5 µl.

Mass analyses were performed on an Orbitrap Elite (Thermo Scientific™) mass spectrometer with a Heated EletroSpray Ionization source (HESI-II). Acquisition was performed under positive and negative polarities. HR-MSn data were acquired using the following ionization parameters: spray voltages, 3.7 kV (positive polarity) and 4.0 kV (negative polarity); sheath gas, 40 arbitrary units; auxiliary gas, 10 arbitrary units; heater temperature, 300 °C; capillary temperature, 350 °C; S-Lenses RF level, 64.9 %. Scan range was 100-1000 m/z. Fragmentation spectra were obtained by running the system in data dependent mode using dynamic exclusion. LC-MS profiles were analysed using Compound Discoverer 3.1 and Xcalibur (Xcalibur™ Software Version 4.1, Thermo Scientific™).

2.4.Statistical analysis

The softwares used for statistical analyses where GraphPad Prism (version 8.0.0 for Windows, GraphPad Software, San Diego, California USA) and SPSS (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Steroid levels' data are presented as a comparison between the treated and the control groups for each sampling point. The data was first treated by removing any outliers using $mean \pm (2 \times SD)$ strategy, after which the normality was checked with a Kolmogorov-Smirnov test and their homogeny of variance with a Leven's test. An unpaired Student's *t*-test was then performed between treated and non-treated groups. Additionally, a repeated measures ANOVA was conducted to compare the different sampling points for statistical differences, followed by a Tukey *post hoc* test.

GSI was calculated as: $\left(\frac{gonad\ weight}{total\ weight} \times 100\right)$, with statistical differences being checked with an unpaired Student's t-test for both sexes. Regarding male gonad histology, each cell type for both

regions was compared between treated and non-treated with a student's t-test. Lastly, for the EOG analysis, a one-way ANOVA followed by a Tukey or Dunnett's *post hoc* test was performed. For all the tests the level of significance was considered p-value < 0.05.

3. Results

3.1.Experiment 1: Effect of kisspeptin treatment in advancing puberty in Senegalese sole juveniles

3.1.1. Plasma sex steroids' levels

Regarding male's plasma levels of 11-KT (Fig. 2A), a significant decrease in values was observed at point T3 for both treatments, which was maintained during T5 for the control group, while in treated fish, values increased again, although not significantly. When analysing T levels

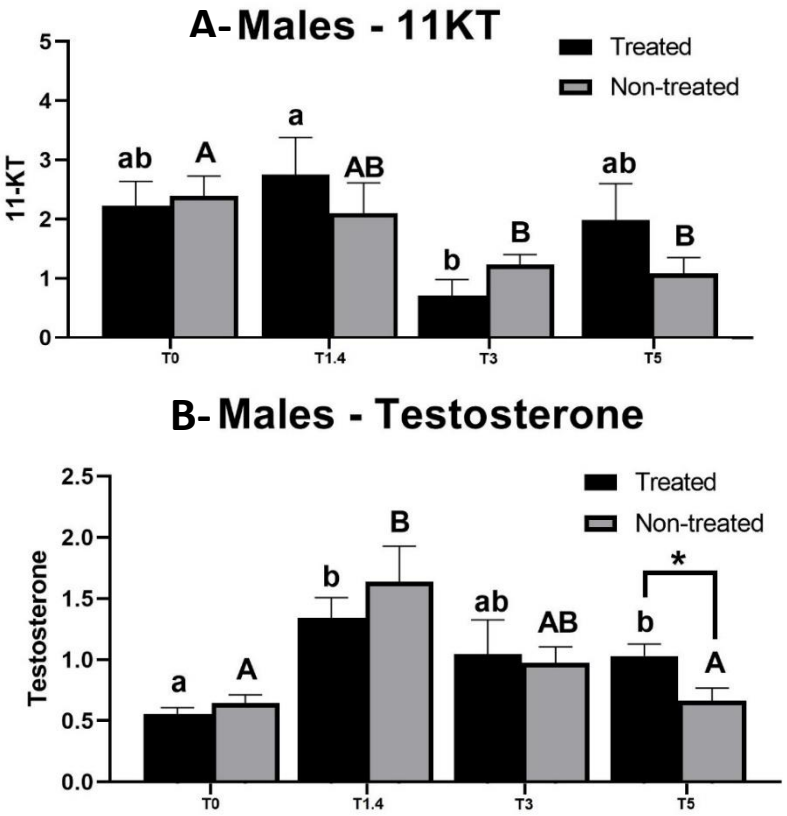


Figure 2. Male plasma steroid levels of A) 11-KT and B) T of Senegalese sole pubertal juveniles treated and non-treated with kisspeptin, during consecutive samplings (before treatment, T0, 4 hours, T1.4, 3 and 5 days, T3 and T5, after treatment). Data expressed as mean ± SEM. Different lowercase letters, denote significant differences along the sampling points for treated males, with uppercase following the same pattern for the non-treated males (Repeated Measures ANOVA, $p < 0.05$). Additionally, an asterisk (*) indicates a significant difference between treated and non-treated groups at each sampling point (Student's *t*-test, $p < 0.05$).

(Fig. 2B), a significant increase was observed for both groups at T1.4, with the following points maintaining higher values in the group of treated fish, and a significant decrease in concentrations observed in the control group at point T5 (Repeated measures ANOVA, $p < 0.05$). Regarding the comparison between groups, the only statistical difference found was for testosterone levels at T5 (Student's *t*-test, $p < 0.05$).

Regarding female's plasma levels of E_2 and T (Figs. 3A and 3B), an initial statistical difference between groups was found at T0 in both steroids (Student's *t*-test, $p < 0.05$). In the following sampling points, despite an overall increase in E_2 levels in fish from the treated group, no statistical difference were found in any of the experimental groups (Repeated Measures ANOVA, $p < 0.05$). Regarding testosterone levels in the treated group, in addition to the initial difference in comparison to the control, the steady increase observed in the following points, resulted in concentrations at point T5 significantly higher in relation to T0 (Repeated Measures ANOVA, $p < 0.05$) and to the non-treated fish at T5 (Student's *t*-test, $p < 0.05$).

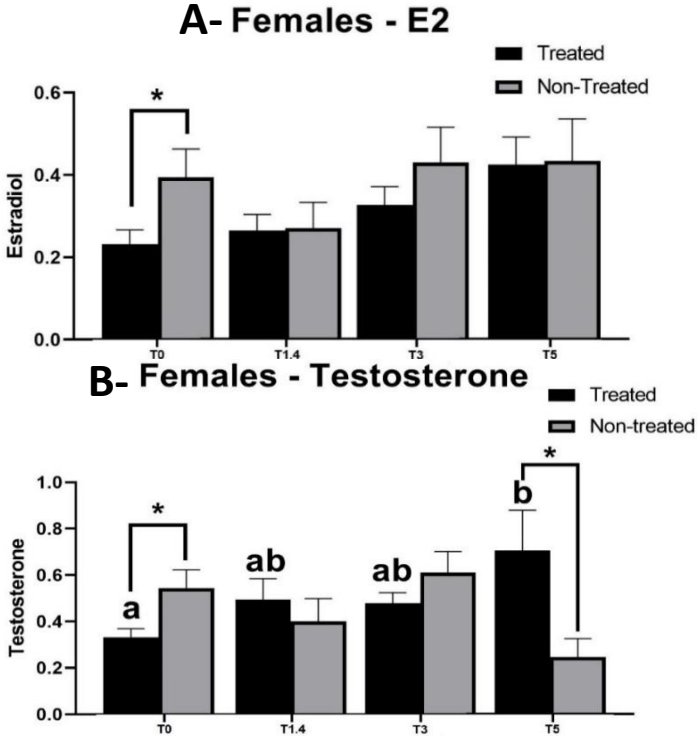


Figure 3. Female plasma steroid levels of A) E_2 and B) T of Senegalese sole pubertal juveniles treated and non-treated with kisspeptin, during consecutive samplings (before treatment, T0, 4 hours, T1.4, 3 and 5 days, T3 and T5, after treatment). Data expressed as mean ± SEM. Different lowercase letters, denote significant differences along the sampling points for treated females, with uppercase following the same pattern for the non-treated females (Repeated Measures ANOVA, $p < 0.05$). Additionally, an asterisk (*) indicates a significant difference between treated and non-treated groups (Student's *t*-test, $p < 0.05$).

3.1.2. Gonadal development

The GSI index of the gonads excised at the end of the experiment, showed a slight increase with the kiss treatment, despite not being significant (Fig. 4, Student's *t*-test, $p < 0.05$). The treated group had a sex ratio of 7:4, with the males having a GSI of 0.062 ± 0.01 % and females 2.160 ± 0.212 %, while the non-treated group had a sex ratio of 1:3, with males having a GSI of 0.056 ± 0.001 % and females 1.943 ± 0.184 %.

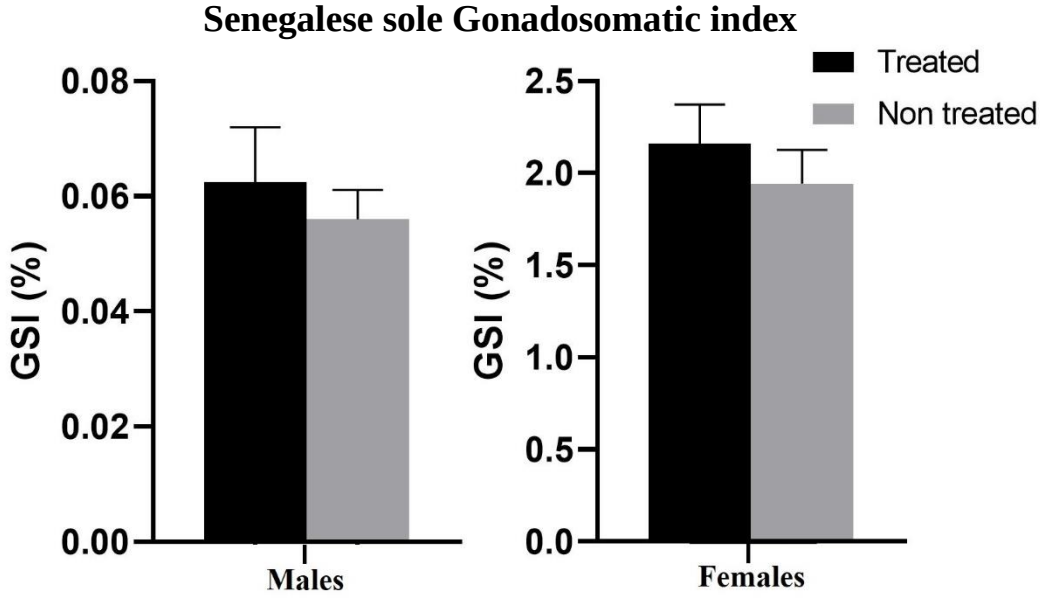


Figure 4. Gonadosomatic index of males and females for each treatment (mean ± SEM), control and kisspeptin. Statistical significance was checked with an unpaired Student's *t*-test ($p < 0.05$).

The histology analysis of the testis of both treated and non-treated fish revealed similar developmental stage, Mid spermatogenesis, which could be defined when testis contained all stages of development, including spermatozoa (SPZ), although the latter type was by no means abundant. This stage so is characterized by a cortex region with a reduced number of spermatogonia (SPG) and an increased number of spermatocytes (SPC) and a medullar region with more spermatids (SPD) and SPZ.

The relative abundance of each germ cell type can be observed in Fig. 5, as percentages of the total amount of cells counted. The cortical region had different abundance distribution of germ cells between groups. The non-treated fish had 41 % SPD, 38 % SPC and 21 % SPG, and the treated fish had a statistically significant higher SPD amount of 60 % ($p = 0.042$) and lower SPC of 17 % ($p = 0.022$) with a similar composition of SPG to the non-treated fish of 23 % (Fig. 5A).

In the medullar region, percentages of germ cells were observed to be similar between treated and untreated males: the untreated group had around 87 % SPD and 9.5 % SPZ, while the treated fish had 95 % of SPD and 4.6% SPZ (Fig. 5B).

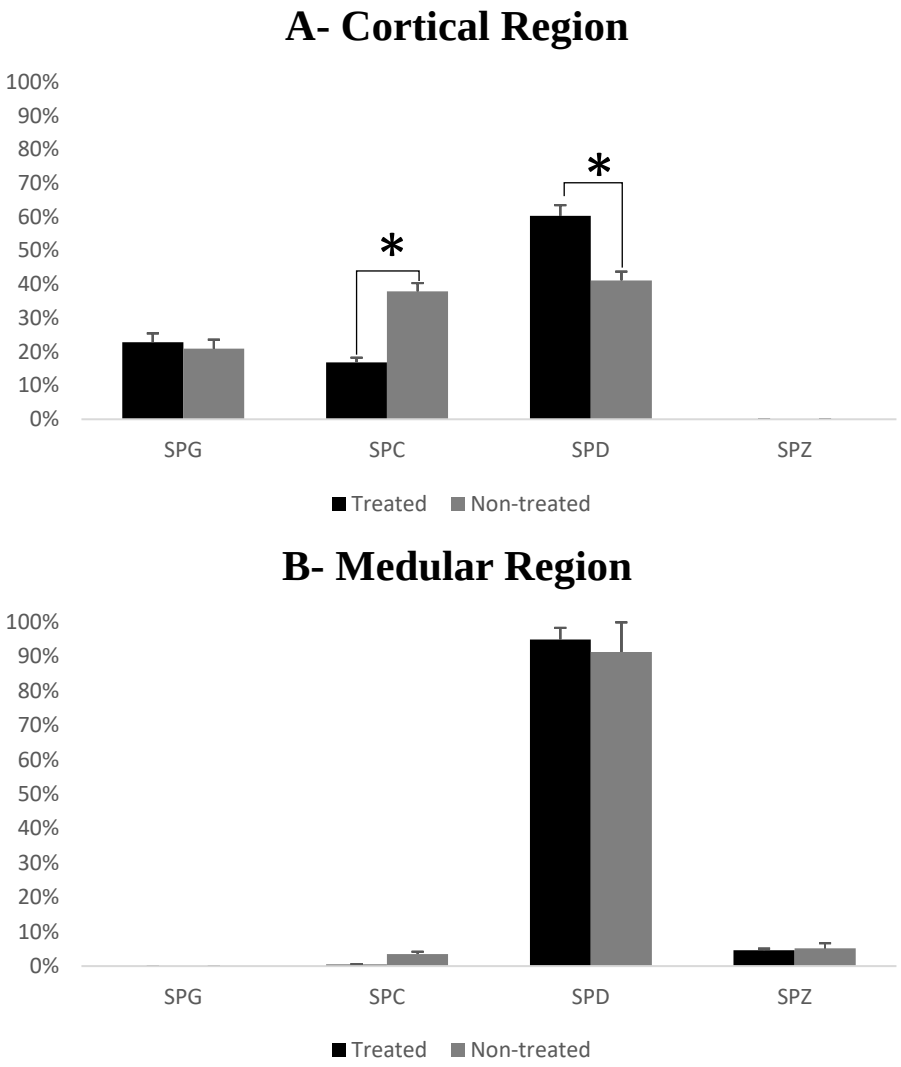


Figure 5. Percentage of the different germ cell types of the testis present A) in the cortical region and B) in the medullar region of the Senegalese sole pubertal juveniles treated and non-treated with kisspeptin. Data expressed as mean ± standard deviation. An asterisk (*) represents a significant difference between treated and non-treated fish for that germ cell type (Student *t*-test, $p < 0.05$).

In Fig. 6 an example of the resulting treated (B and D) and non-treated (A and C) testis histology images can be observed, in which A and B correspond to the cortical region for non-treated and treated fish, and C and D belong to the medullar region of non-treated and treated fish.

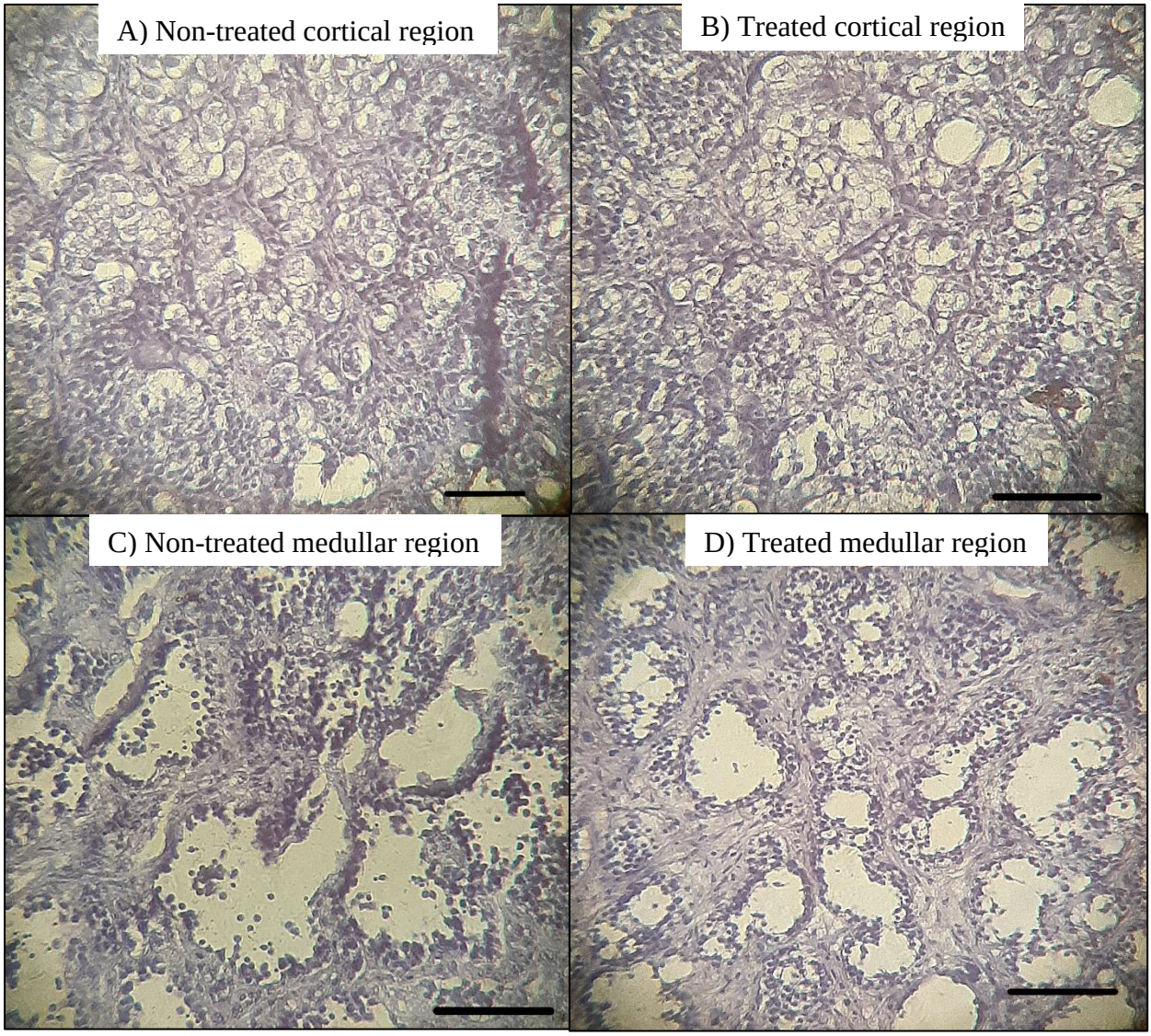


Figure 6. Photomicrograph of sole's testis histological sections for both non-treated (A and C) and treated (B and D) and fish. A and B images belong to cross sections of the cortical region and C and D images to sections of the medullar region. Scale bars: 50 μm.

3.2.Effect of kisspeptin treatment in reproduction metabolites in urine of Senegalese sole breeders

3.2.1. Identification of reproductive metabolites by Liquid chromatography mass spectrometry (LC-MS/MS) analyses

Regarding the urine of adult sole treated with kisspeptin , when analysed both by a photodiode array (PDA, sensing ultraviolet light) and a mass spectrometer detectors (MS, recording the current generated by the passing of ions), two compounds were found to be more represented when compared to the urine from the control fish. One of the compounds had a retention time of 12.98 min, which was absent in the control group (Fig. 7A) and present in the treated group (Fig. 7B), corresponding to an m/z of 151.04, $[M-H]^-$ (Fig. 8). This peak was identified as 3-hydroxyphenilacetic acid and was more abundant in the treated female's urine.

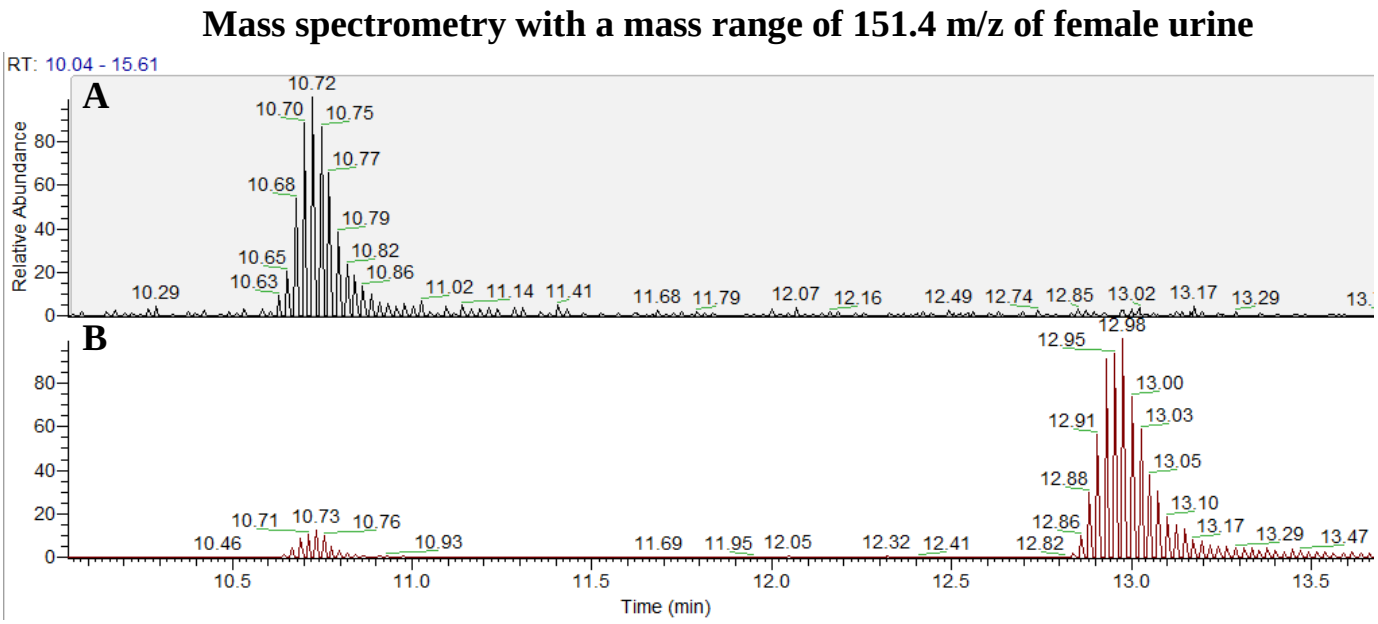


Figure 7. Comparison of mass spectrometry with 151.4 m/z mass range in Senegalese sole female urine from A) non-treated group, and B) treated females.

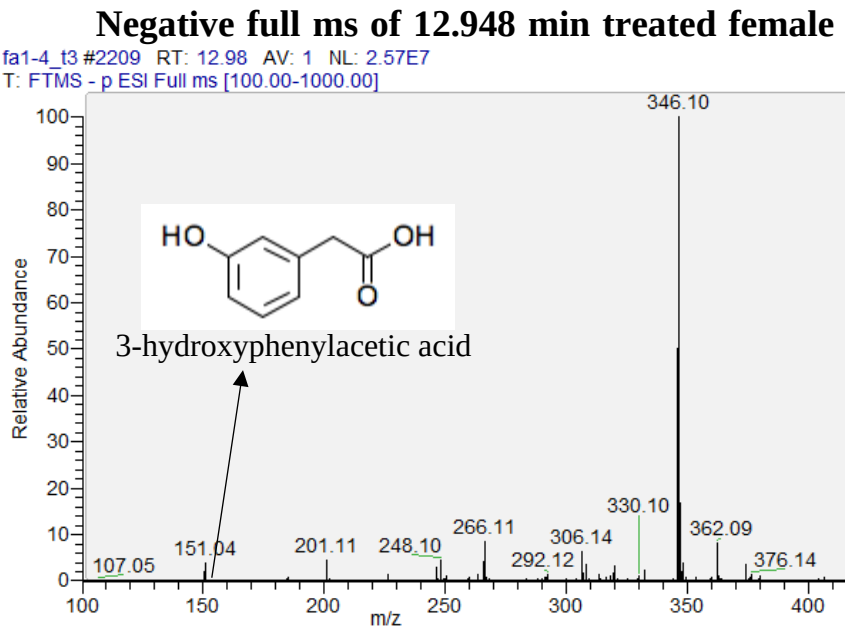


Figure 8. Full mass spectrum of the 12.98 min peak negative run treated female profile urine.

The second compound, which was found to be also more represented in the treated group (Figs. 9 and 10), was present both in males and females, with females having a much higher intensity (Fig. 11).

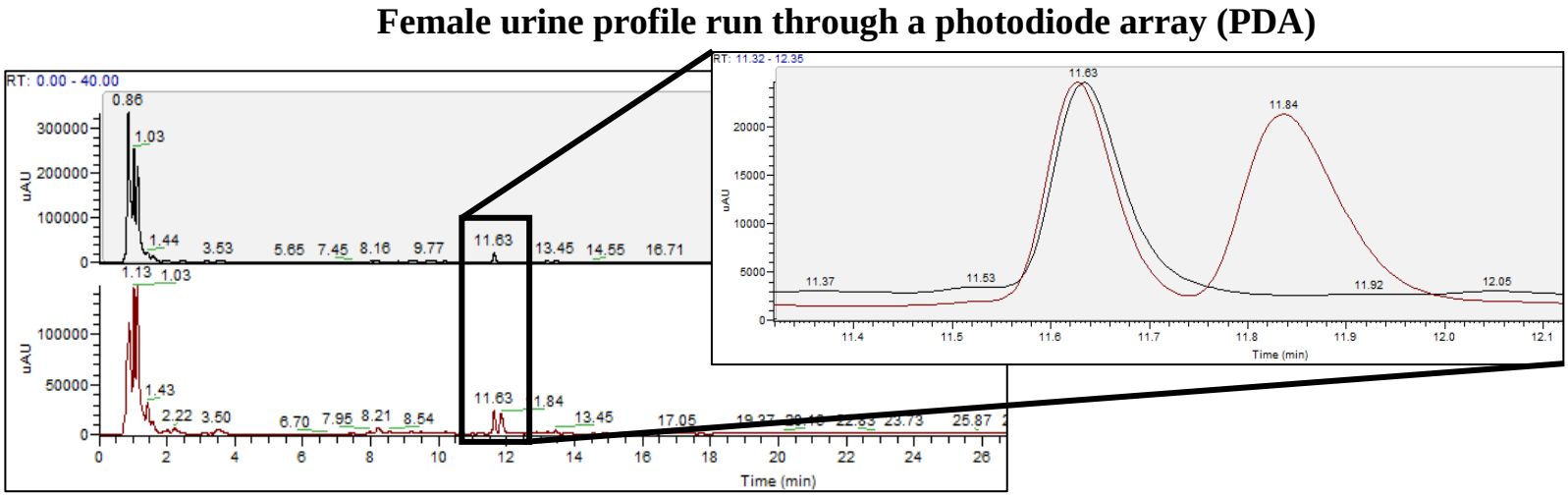


Figure 9. Urine profile from Senegalese sole female using a photodiode array (PDA) detector measuring the UV spectrum. In the upper graph (black line) is depicted an untreated female profile, while bottom one (red line) is related to a female treated with kisspeptin under a negative polarity. The close-up image shows the peak at 11.84 min in detail.

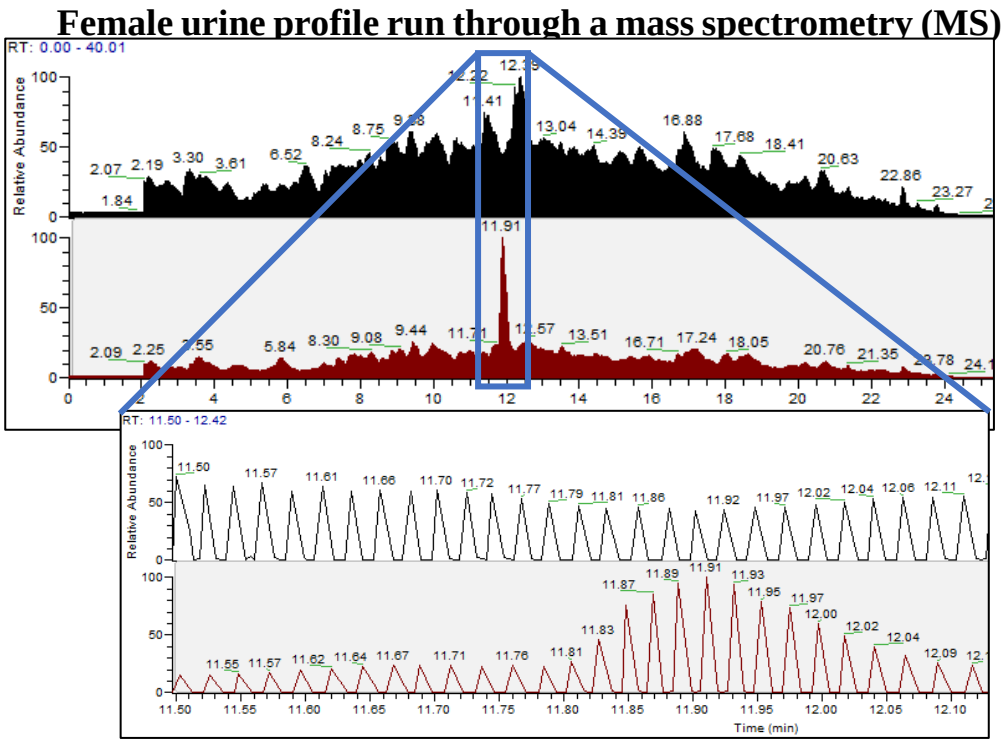


Figure 10. Female urine profile differences using a mass spectrometer (MS). The upper graph (black line) represents the urine profile from an untreated Senegalese sole female, while bottom one (red line) to urine from a female treated with kisspeptin, under a negative polarity. The close-up image shows the difference at 11.84 min.

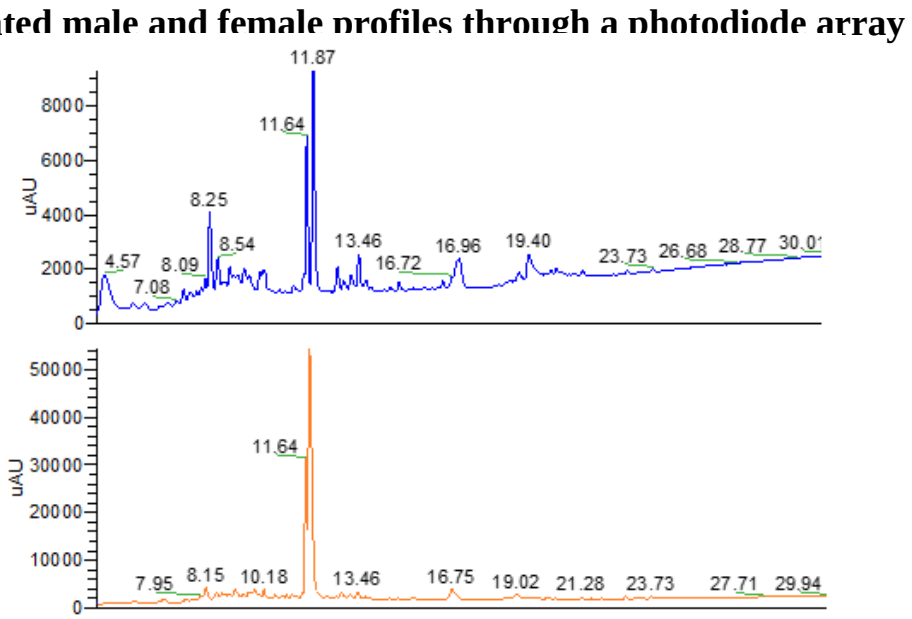


Figure 11. Profiles of Senegalese sole male and female treated with kisspeptin, and respective comparison using a PDA detector. The upper profile (blue) represents male urine and the bottom profile (red) showed female urine, both under a negative polarity.

This compound had a retention time of approximately 11.84 min, corresponding to an m/z 313 under a negative polarity (Fig. 12). The peak observed at the m/z 313, $[M-H]^-$ corresponds to the compound. Additionally, a second smaller peak at m/z 627.18, $[2M-H]^-$ corresponds to its dimer.

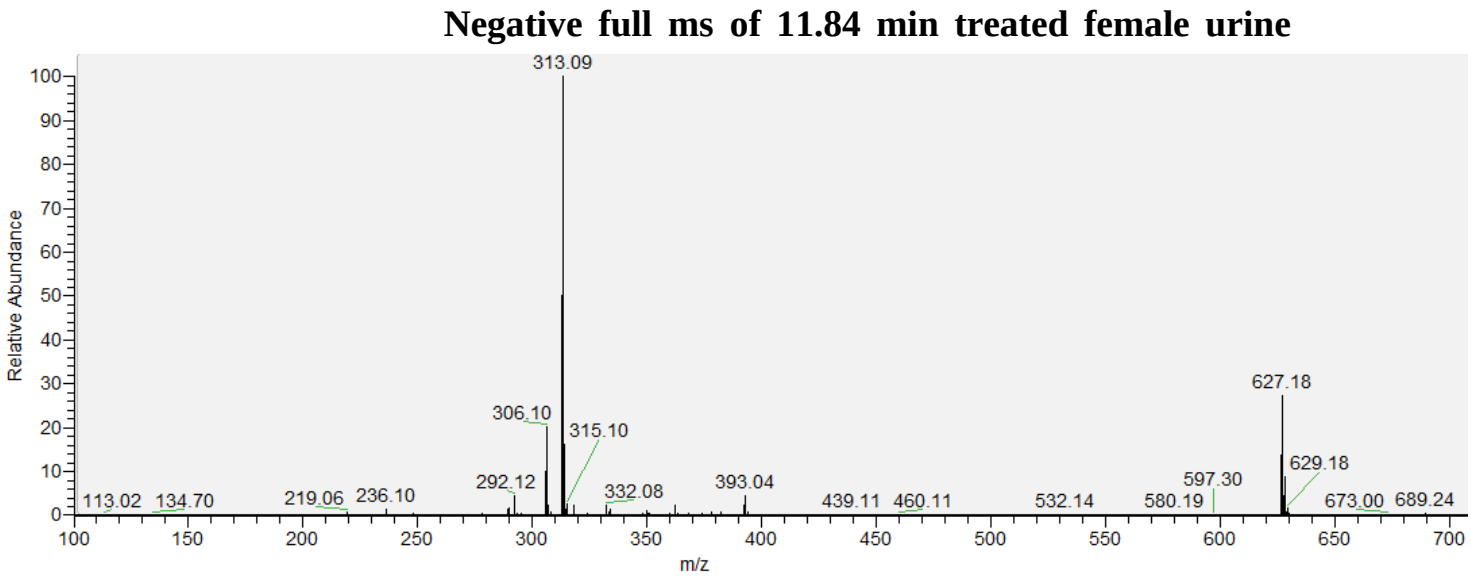


Figure 12. Full ms of treated female urine peak at a retention time of 11.84 min in the negative polarity. It shows the molecule at 313.09 m/z and its dimer at 627.18 m/z.

MS^2 of 313 m/z peak, gave a major peak at m/z 295.08 (Fig. 13). This differs from the original by 18.01 Da, coinciding with a water molecule, suggesting that this unknown compound has at least, an OH group in the structure. Several other peaks can be observed, such as the 253.07 m/z, the difference of which coincides with a loss of 42 Da to the 295.08 m/z peak. This loss could be from an acetyl group (C_2H_2O), or a C_3H_6 group, meaning that they would be part of its structure (Nicolescu, 2017).

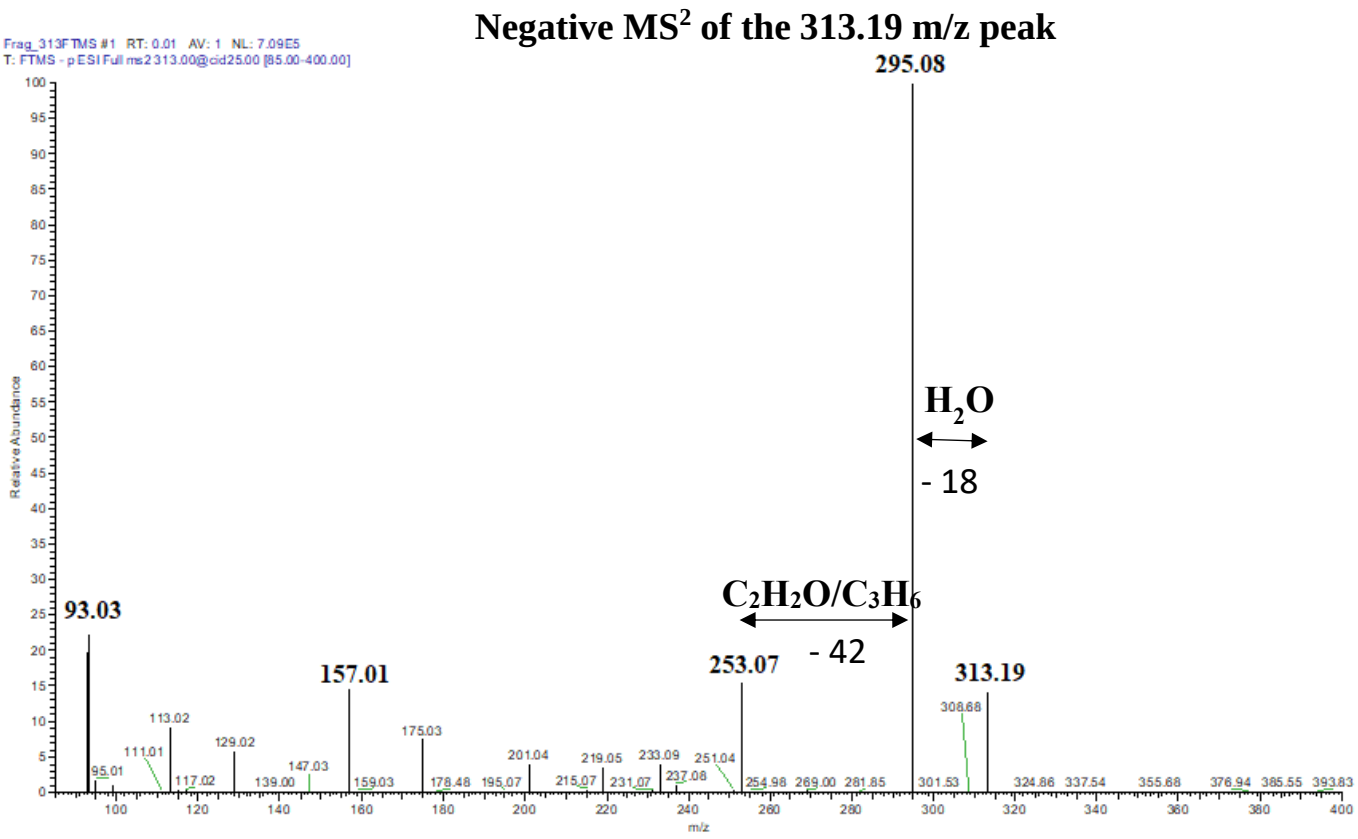


Figure 13. MS^2 of 313.19 m/z present at 11.84 min of the negative run profile of the treated fish urine. It demonstrates the loss of the water molecule, giving the 295.08 peak and the two possibilities for the loss of the 42 Da to the 253.07 m/z peak.

In MS^3 of the 295 m/z peak, $MS^3(313 \rightarrow 295)$, 4 additional peaks of varied weight were obtained (Fig. 14). The m/z 201 differed from the m/z 295.08 by 94 Da, suggesting a possible loss of a phenol molecule (C_6H_6O); the m/z 157 differed from the m/z 201 by 44 Da, possibly a C_2H_6N or a C_2H_4O molecules; the m/z 129 peak differed by 28 Da from the m/z 157, possibly a C_2H_4 or a carbonyl group (CO); The final m/z 93 may correspond to a phenoxy ion $[M-H]^-$, similar to the 94 Da excised by the formation of the m/z 201 (Nicolescu, 2017).

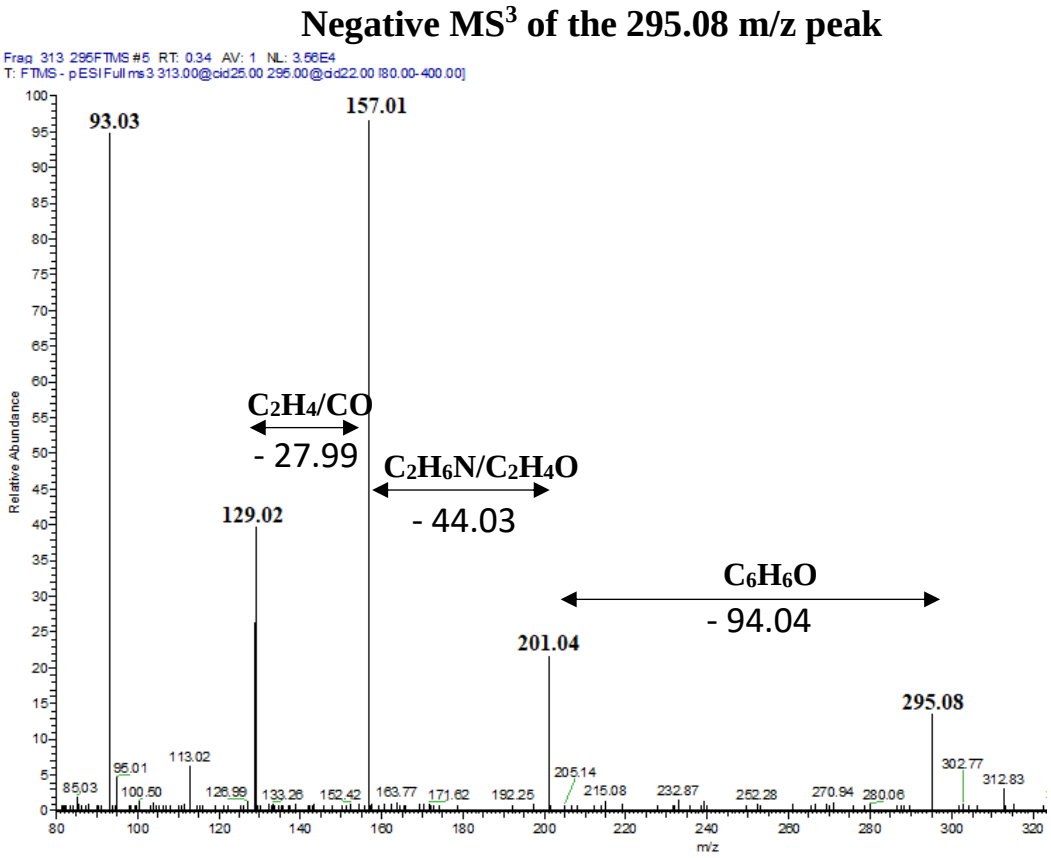


Figure 14. MS^3 (313.19 → 295.08). It demonstrates the several possible losses for each peak from urine of treated fish.

The same compound was observed to be also ionized in the positive polarity (Fig. 15). By analysing the positive polarity, more information may be determined regarding the unknown compound, when analysed in conjunction with the known information from the negative polarity. The original peak had 315.24 m/z, $[M+H]^+$, and by fragmenting it, 2 consecutive losses of 18 Da were obtained. As previously, this also corresponds to water molecules, signifying that the unknown compound probably had 2 hydroxyl groups (Nicolescu, 2017).

Positive MS^2 of the 315.24 m/z peak

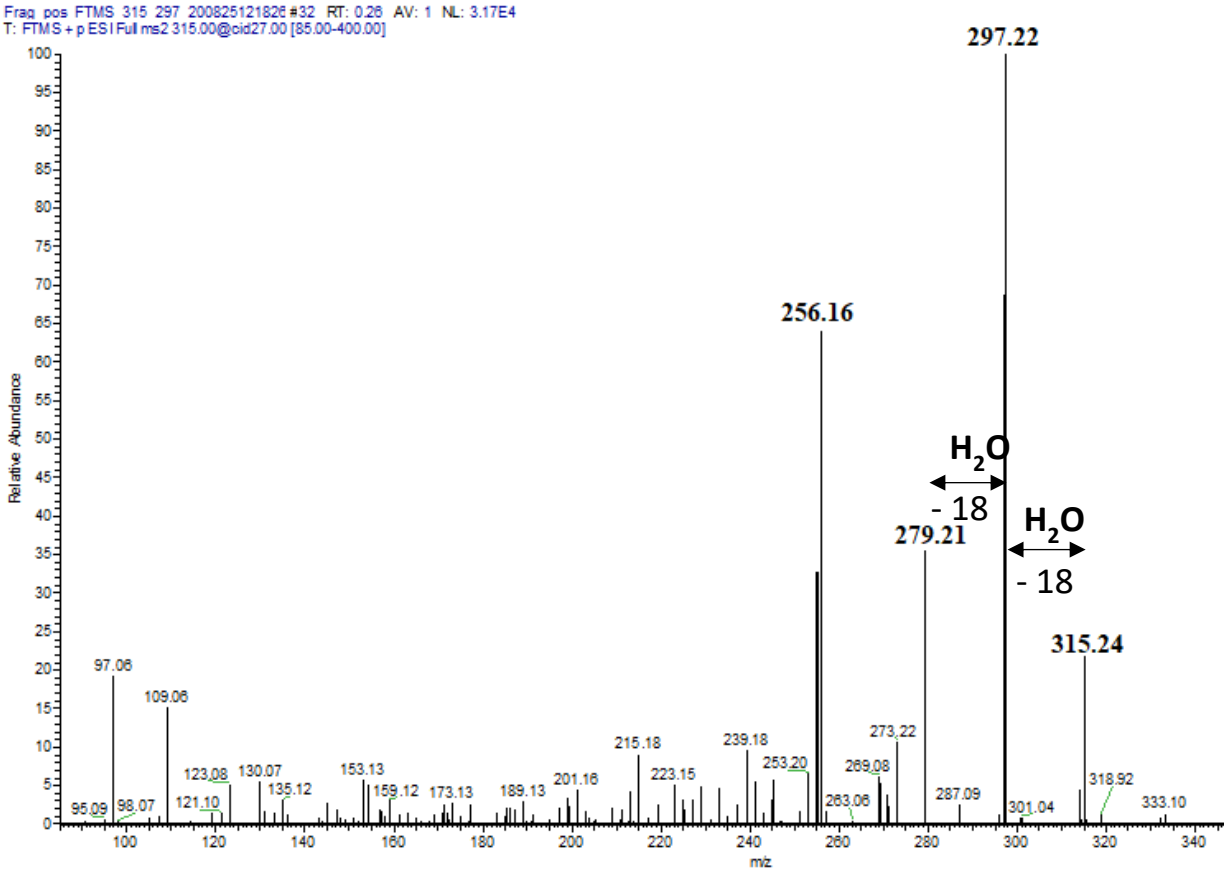


Figure 15. MS^2 (315.24) run through a positive mode. It demonstrates the loss of the two water molecule, giving the 297.22 and 279.21 m/z peaks.

MS^4 (315→297→279) shows a direct loss of 15 Da and 28 Da from the m/z 279, possibly a methyl and carbonyl (CO) groups respectively (Fig. 16). Following this MS^2, starting at m/z 251, a sequential loss of several 14 Da (CH_2) and 12 Da (C), can be observed. This suggests that the main core of the unknown molecule to be aliphatic, possibly a steroid or a fatty acid.

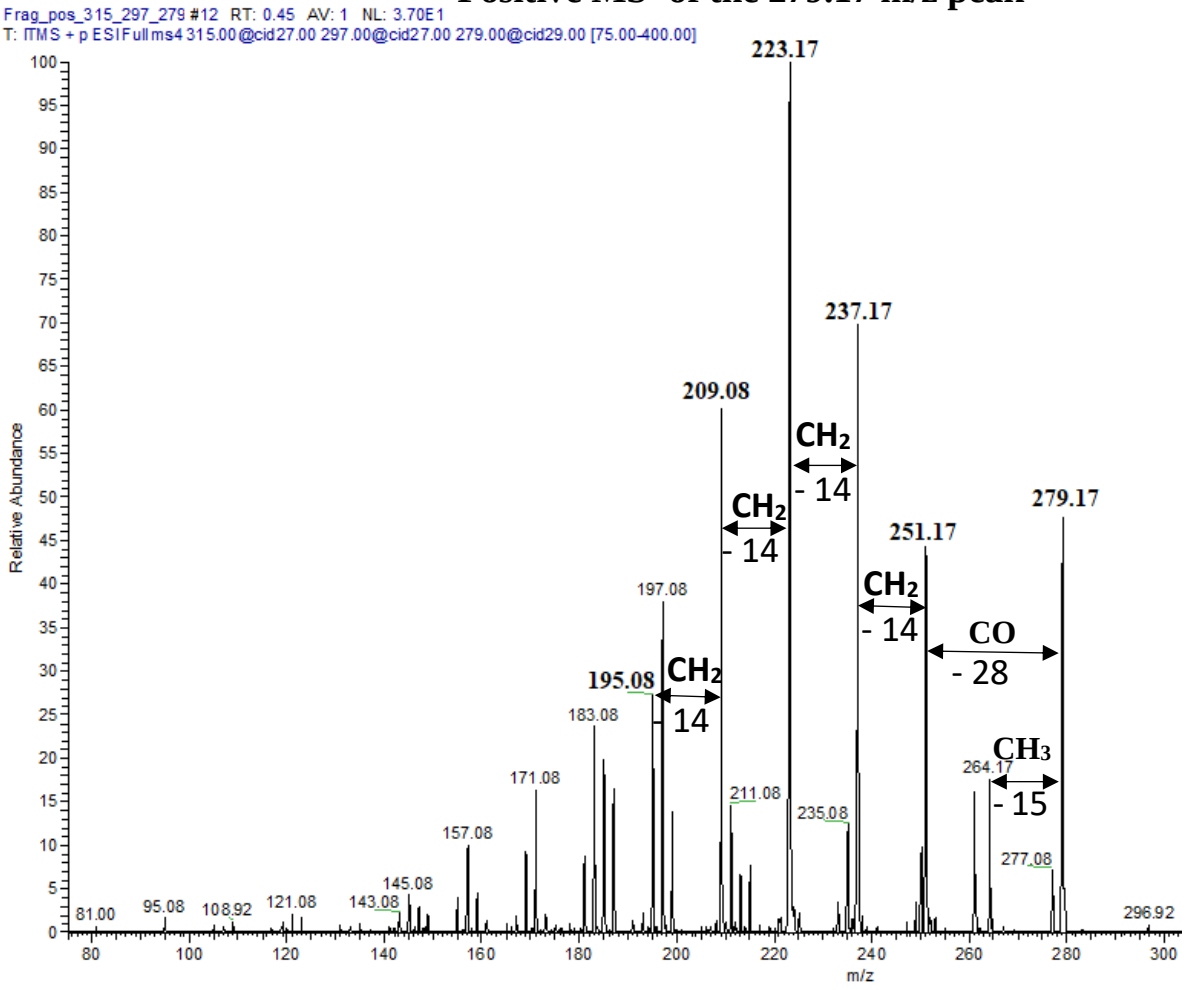


Figure 16. MS^4 (315→297→279). It shows the initial two losses of 15 and 28 Da followed by the sequential 14 Da mass differences.

Our hypothesis is that the unknown compound is a steroid with an aromatic ring, with at least 2 OH groups to coincide with the water losses. The sequential losses of CH_2 molecules by the fragmentation of the 279 m/z peak showed the typical behaviour of steroids (Suppl. Fig. 1 in Keller-Costa et al., 2014). An example of an steroid with similar characteristics than the unknown compound can also be found in Fig. 17 (adapted from Qiao et al., 2012).

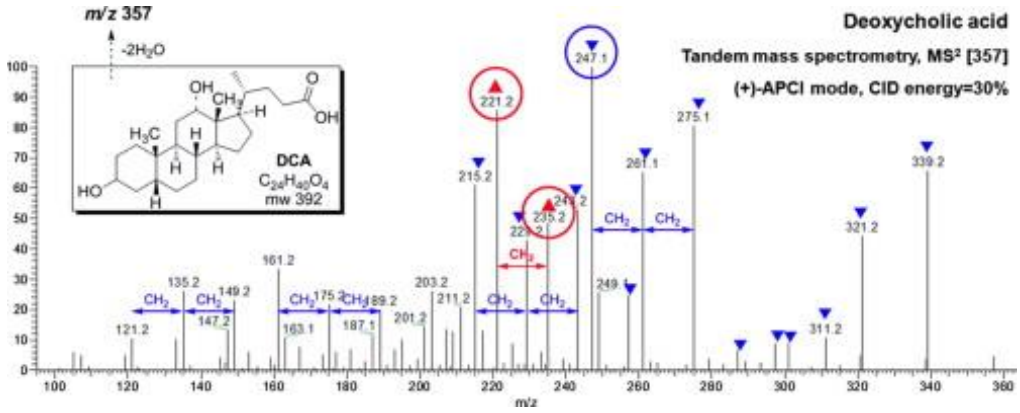


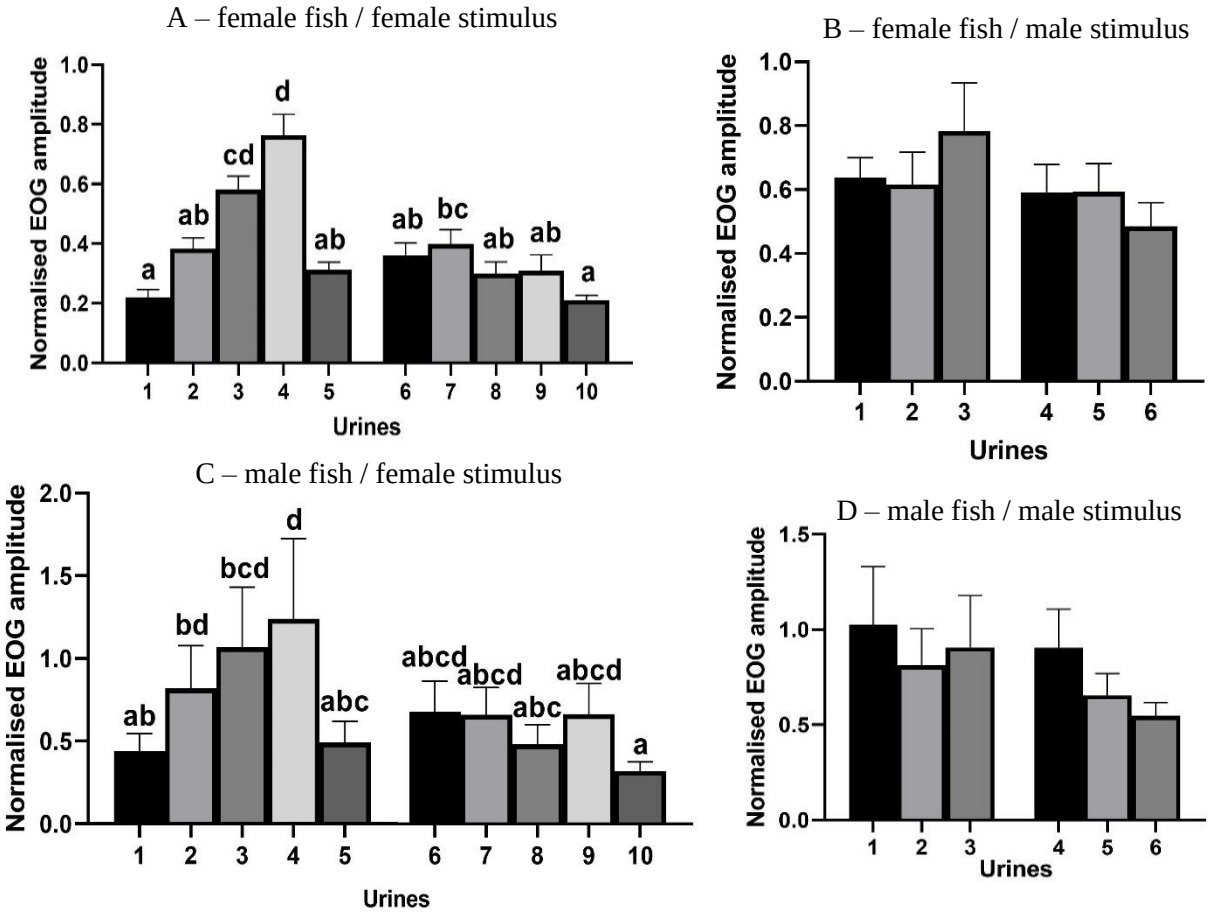
Figure 17. Fragmentation example of Deoxycholic acid, which is a steroid molecule similar to the obtained MS^4 profile in Fig. 16 (adapted from Qiao et al., 2012).

3.2.2. Electro-Olfactogram (EOG)

The olfactory responses towards urine from Senegalese sole treated with kisspeptin, both males and females, tended to produce larger amplitudes in the EOG compared to the control.

Regarding females, significantly differences were observed with the female stimulus. The urine samples 3 and 4, belonging to females treated with kisspeptin, evoked the largest responses, significantly different from all other urines (Fig. 18A). Despite the increased potency of these urines, the remaining treated stimulus (urines 1, 2 and 5) failed to evoke similar responses. Values were similar to the ones produced in response to the urine of the control group (urines 6 to 10). In case of the male urine stimulus, no significant differences were noted in the response from urine treated and untreated fish (Fig. 18B).

Despite no statistical differences when stimulated by the male urine, male receivers showed similar responses as the female receivers. Males stimulated with female stimulus obtained responses similar to the female receivers (Fig. 18C), where the sample urines 3 and 4 evoked also a significantly higher response in comparison to other urines, included those from untreated females. When stimulated with the urine from males, despite the overall higher values, no significance difference was observed (Fig. 18D).



Figures 18. Individual olfactory sensitivity to Senegalese sole urine from untreated and treated fish with kisspeptin hormone. A) females to female urine (1 - 5 kiss treated, 6 – 10 control), B) females to male urine (1 - 3 kiss treated, 4 – 6 control), C) males to female urine (1 - 5 kiss treated, 6 – 10 control) and, D) males to male urine (1 - 3 kiss treated, 4 – 6 control). Data shown as mean ± SEM. Different letters indicate statistically significant differences using One-way ANOVA followed by a Tukey *post hoc* test for the female / female profiles and Kruskal-Wallis test for the remaining profiles ($p < 0.05$).

Observing the compiled data, significant differences were detected between treated and non-treated female stimulus to female receivers. Additionally, higher EOG amplitudes were observed for the male receivers (Figs. 18C and 18D) including higher variability.

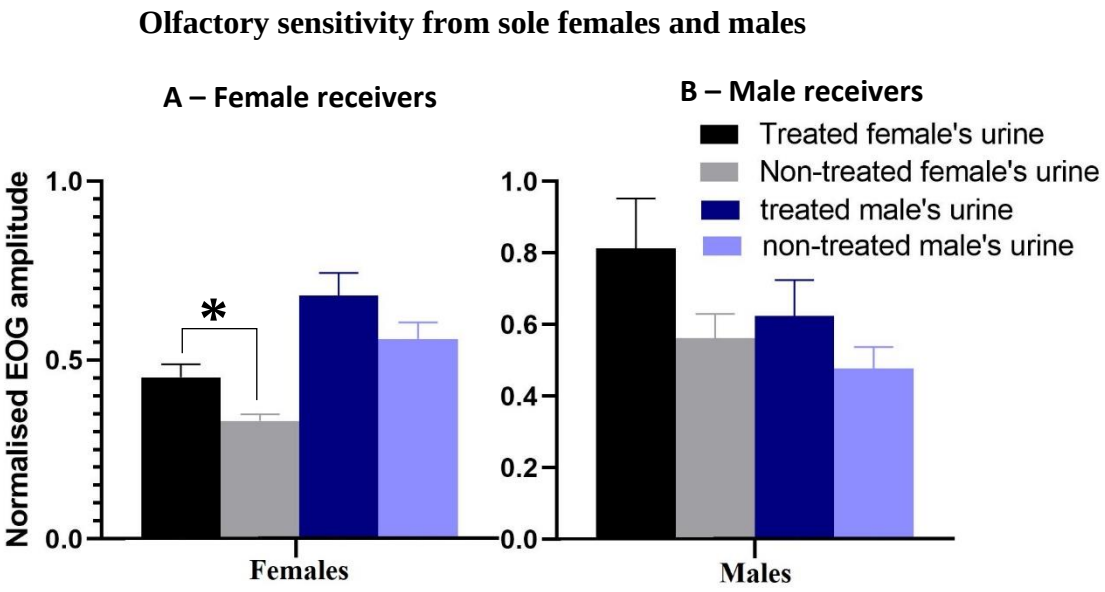


Figure 19. Olfactory sensitivity to Senegalese sole urine from treated and non-treated fish with kisspeptin. A) Female fish stimulated by treated female (black) and non-treated female (grey) fish urine and treated male (dark blue) and non-treated male (light blue) fish urine. B) Male fish stimulated with the same previous colour code. Data as mean ± SEM. Asterisk (*) represents a significant difference between urine stimulus belonging to treated and non-treated groups using a Student's *t*-test ($p < 0.05$).

To summarize, these results showed that kisspeptin administration on pubertal Senegalese sole significantly increased plasma levels of T for both sexes at sampling point T5, with an additional tendency to increase E_2 female plasma levels despite lacking statistical significance. Kisspeptin's effect in the gonadal maturation also resulted in increased amounts of more developed germ cells in the cortical region of male treated gonads, with higher number of SPD and a reduced number of SPC. In addition, the GSI index had a slight tendency to increase despite no statistical difference was found. Regarding kisspeptin's effect on Senegalese sole breeders, two compounds were found in the urine of treated fish, one being identified as 3-hydroxyphenylacetic acid and the other one being separated into several fragments, however, with no identification. This suggests that this compound is an undocumented compound, mainly steroid. Additionally, through an EOG analysis, our results suggested that males had higher sensitivity than females, smelling better urine from females. A possible kisspeptin effect on sole chemical communication was also implied, with overall increased olfactory responses from urine stimulus belonging to treated fish, with female having a statistically significant response to female stimulus.

4. Discussion

4.1.Experiment 1: Effect of kisspeptin treatment in advancing puberty in Senegalese sole juveniles.

Controlling fish maturation is a crucial aspect for the fish farming industry and is primarily performed through the control of environmental parameters and/or exogenous hormone administration. These procedures allow farmers to have more control through restricting/increasing spawning cycles, better management and better resource allocation (Mylonas et al., 2010).

In this first experiment, the positive actions of kisspeptin administration on Senegalese sole puberty were investigated, by evaluating its effects on sex steroid levels and gonadal maturation stage. The significant increase in T levels, observed for both males and females treated with kisspeptin, as well as a tendency of E_2 to increase, demonstrated that administration of this hormone has a potential to control the onset the puberty in this species. Furthermore, the increase in gonadal development in treated males supported this hypothesis.

Regarding male endocrine levels, T significantly increased with kisspeptin treatment at the end of the experiment, T5, being statistically different when compared both to its initial value, T0, and the control group T level at T5. A similar increase in T levels was found in kisspeptin treated adult Senegalese sole, at 4 days post injection (Oliveira et al., 2019), and in lined seahorse (*Hippocampus erectus*), where *kiss2-10* administration resulted in increased T levels measured 6 hours post injection (Zhang et al., 2018). Unfortunately, the effects of kisspeptin on fish testosterone levels have not been so well studied as in mammals, instead focusing more on their effect in gonadotropin levels. According to some researchers, kisspeptin administration in the brain of rats and intravenous treatments in humans significantly increased T levels (George et al., 2011; Patterson et al., 2006), which is in line with the present results. This might suggest a conservative effect of kisspeptin over testosterone, despite being poorly studied.

Concerning the treated fish 11-KT levels, no clear positive trend could be observed. This is possibly due the fact that kisspeptin acts via gonadotropin release at a high level of the BPG axis, and thus a higher induction would be needed to cause a noticeable effect of a sex steroid near the end of the axis. Similar results were observed in adult Senegalese sole, where kisspeptin at the same dose failed to induce 11-KT circulating levels (Oliveira et al., 2019). Previous studies in other species, showed evidence of positive 11-KT induction derived from kisspeptin injection. In both juvenile and sexually immature adult chub mackerel males, an increase in circulating 11-KT levels

was observed (Selvaraj et al., 2013a, 2013b), together with Nile tilapia juveniles (Park et al., 2016), male adult cinnamon clownfish (*Amphiprion melanopus*) (Kim et al., 2014) and, despite a lack of statistical significance, a tendency was also observed in Yellowtail kingfish (*Seriola lalandi*) juveniles (Nocillado et al., 2012). Possibly in the present study the dose or administration method used were not sufficient to elicit such an effect in Senegalese sole juveniles.

Considering T and 11-KT circulating levels, unexpected significant changes in values were observed in both groups. T levels increased at T1.4 sampling point while 11-KT decreased at T3, both significantly. These variations may be explained by some external fluctuations, such as water temperature, that during the course of the experiment varied between 19 °C and 14.7 °C. It has been shown that Senegalese sole plasma hormone levels changed depending on water temperature conditions (García-López et al., 2006a, 2008; Oliveira et al., 2009). The presence of this environmental bias may explain the observed hormone fluctuation levels, independently of the treatment administered. However, no clear justification could be discerned from the available data.

Regarding females, the kisspeptin treatment was effective in inducing a steady statistical increase throughout the sampling points for T plasma levels. E_2 levels had a similar increasing pattern, showing a slight tendency to rise, despite the lack of statistical differences. The stimulation on female's endocrine levels was in concordance with studies performed in other species. In both juvenile and sexually immature adult chub mackerel females, kiss1-15 induced both circulating levels of E_2 and T, with E_2 increasing only for the adults fish (Selvaraj et al., 2015, 2013a); in prepubertal female yellowtail kingfish, adult female cinnamon clownfish and Nile tilapia, kisspeptin also induced E_2 circulating levels (Kim et al., 2014; Nocillado et al., 2012; Park et al., 2016). Several studies managed to induce T and E_2 circulating levels in a variety of species with kisspeptin treatment. Despite the final E_2 levels being approximately twice the initial treated values, the increase was not sufficient to cause any noticeable distinction between the control group. Similarly to male 11-KT levels, in female adult Senegalese sole, treatments with kisspeptin also failed to achieve statistical significance on E_2. The same proposed explanation for juvenile 11-KT levels is also applicable in this case. It exists the possibility that increasing the dose of kisspeptin administration, more noticeable plasma E_2 levels could occur.

The initial difference between groups observed for both female hormones, suggested that treated females were at a less developed stage than non-treated ones at the beginning of the experiment, even after the random distribution of the fish among tanks. This procedure was

expected to homogenize fish developmental stage among experimental groups, mitigating this potential factor. However, this was not the case in females. This resembles the importance of having two sorts of controls in this type of study: before treatment and placebo effect.

Kisspeptin stimulated sexual gonadal steroid release, likely through its action on the BPG axis, stimulating GnRH release into the pituitary, where Fsh and Lh are in turn produced. This was demonstrated in a study in Senegalese sole breeders, where plasma levels of Fsh and Lh were significantly increased after the treatment with kisspeptin, starting to decrease thereafter. T levels however, only increased at the end of the experiment (Oliveira et al., 2019). In several other kisspeptin treatment studies, an increase in gonadotropin release was also observed. In *catla catla* female juveniles, kiss1-10 induced GnRH, Lh and Fsh brain mRNA levels (Rather et al., 2016); In chub mackerel, kiss2-12 and kiss1-15 injections induced Fsh and Lh mRNA together with increased circulating 11-KT and E_2 levels (Ohga et al., 2015; Selvaraj et al., 2013a); in cinnamon clownfish, kiss1 induced GnRH, Lh and Fsh plasma expression levels slightly earlier than any E_2 and 11-KT noticeable effects (Kim et al., 2014); in Nile tilapia juveniles, kiss2 stimulated GnRH, Fsh and Lh mRNA in the brain in addition to plasma 11-KT and E_2 (Park et al., 2016) and in seabass juveniles and yellowtail kingfish, kisspeptin also stimulated gonadotropin release (Felip et al., 2009; Nocillado et al., 2013, 2012). In line with this evidence, and considering the increase of some of the tested steroids in our study, and their lower position in the reproductive axis, it is probable that the gonadotropins that stimulate their release, also increased shortly after treatment, mirroring the aforementioned studies. However, further studies are needed to ascertain this possibility.

In general, for steroid studies our results could imply that further studies are needed to help to determine the specific kisspeptin effect on sole and the minimum dose to produce more pronounced effects at this level.

Kisspeptin action on gonads, caused a slight increase in GSI of the treated fish when compared to the non-treated group, despite not having a statistical difference. Moreover, an exchange of spermatocytes for the more developed stage, spermatids was seen, suggesting an advance in gonadal maturation derived from kisspeptin. The importance of this exchange is derived from the increased presence of certain germ cells in distinct spermatogenesis stages, with spermatocytes being more common in earlier stages than spermatids (García-López et al., 2006b). This effect can also be observed in several species treated with kisspeptin, demonstrating its ability to stimulate

gonadal development. In chub mackerel, kiss1-15 induced spermiation, along with a GSI increase and a higher number of spermatozoa and spermatocytes in the testis (Selvaraj et al., 2013a, 2013b). In white and striped bass, following kisspeptin treatment, an increase in GSI in both species, in conjunction with higher number of male spermatocytes and oocyte diameter in white bass females were observed (Beck et al., 2012). In cinnamon clownfish, kiss1 also caused an increase in GSI (Kim et al., 2014), and in yellowtail kingfish, kisspeptin stimulated a higher number of mature gonads with higher number of developed germ cells (Nocillado et al., 2013, 2012). Altogether these studies suggested that kisspeptin administration has the capability of advancing and stimulating gonadal maturation. In the present study, a higher presence of spermatids in the cortical region of treated juvenile males, demonstrated that, both the dose and application method used, were sufficient to cause a slight increase in gonadal development in Senegalese sole. Regarding the kisspeptin administration on adult Senegalese sole however, the high variability present in both males and females made any comparison difficult (Oliveira et al., 2019). Altogether, our results may indicate, a positive kisspeptin interaction on the modulation and control of male puberty.

One possible explanation for the lack of GSI significant differences in males, would be related to the small size of the testis in this species. Testis weight is almost vestigial in relation to the rest of the body, making it difficult to show GSI fluctuations related with hormonal treatments (Agulleiro et al., 2007; García-López et al., 2005). Another possible explanation, as proposed by Oliveira et al., 2019, was that the application method, a single kisspeptin injection, was not enough to cause a noticeable effect on the maturation of the gonads. The study hypothesized that, either with a multiple injection protocol or a slow releasing medium, the results could have been further increased. The same suggestion could also improve GSI results in juveniles, and even strengthen the already positive gonadal development results obtained. However, the correct dosage and administration method seems to be species dependant. Although optimization is still required for most species, in the yellowtail kingfish a 50 ng/g slow releasing *kiss2* implant was enough to cause effects in the plasma E_2 levels of prepubertal females, while in chub mackerel, three injections at 250 ng/g in a slow releasing medium caused a, still significant, but much less pronounced result (Nocillado et al., 2012; Selvaraj et al., 2015). In line with this idea, a proposed future study would be the optimization of both dose and application method for kisspeptin treatment in Senegalese sole. The current study would provide valuable clues for a multiple injection protocol trough the intervals between the initial injection and final sampling point and the dosage used.

4.2. Experiment 2: Effect of kisspeptin treatment in reproduction metabolites in urine of Senegalese sole breeders

Having in mind the reproductive problems affecting this species under captive conditions, a distinct approach was taken in order to increase the current knowledge regarding Senegalese sole reproduction. Trough exogenous treatments of kisspeptin, and examination of the resulting fish urine via, liquid-chromatography mass-spectrometry (LC-MS) coupled with an electro-olfactogram (EOG) analysis, we potentially uncovered crucial information regarding the chemical communication of this species.

To our knowledge, the use of kisspeptin treatment in reproduction in tandem with LC-MS and EOG analysis has not been performed in any species. The use of LC-MS in kisspeptin studies is often done in pharmacological studies in mammals like rats (Ishikawa et al., 2018). Generally, it is used to study the other functions that kisspeptin has, like its antitumor properties (Ishikawa et al., 2018; Matsui et al., 2014).

In this study, when performing the LC-MS analysis in urine from kisspeptin treated and non-treated fish, two compounds were the most representative inside of the urine from treated fish profile. One of them was identified as 3-hdroxyphenylacetic acid by the compound discoverer software, however, the other could not be identified. The identified compound was found to be very similar in structure to another chemical structure named 4-hydroxyphenylacetic acid, differing only in the position of the hydroxyl group in the aromatic ring (Behrens et al., 2014). This compound has been previously described in a variety of different species, including being involved in the reproductive behaviour in some felines species (Pageat and Gaultier, 2003). More importantly, it was also found in the zebrafish, where it was proposed to function as a putative reproductive pheromone (Behrens et al., 2014). Reproductive pheromones play a crucial role in fish reproduction, facilitating spawning synchronization between males and females and allowing an easier partner selection. Generally, they are secreted outside of the body via urine, faeces or ovarian/seminal fluid (Hubbard, 2018). The exact chemicals used can vary greatly, however some of the more commonly used are steroids and prostaglandins. In addition to their role in the gonad maturation process, when released outside of the body might also function as a potential pheromone (Stacey and Sorensen, 2005). For example, in goldfish, a well-known model species in terms of

reproductive pheromones, the compound 17α,20β-dihydroxy-4-pregnen-3-one, a maturation inducing steroid, and prostaglandin $F_{2\alpha}$ in females and androgens like the 11-KT in males function as pheromones, in addition to their roles in gonadal maturation (Kawai et al., 2015; Kobayashi et al., 2002). However, this is not always the case, since occasionally, a seemingly reproduction unrelated compound, like L-kynurenine, a metabolite of L-tryptophan (an essential amino acid), was found to function as a potent pheromone in the masu salmon (Yambe et al., 2006).

The aforementioned putative reproductive pheromone in zebrafish, 4-hydroxyphenylacetic acid, was tested for its effectiveness, where it was concluded that the carboxyl group in its structure, together with its particular distance from the aromatic ring, were the key factor to represent a potent pheromone. The hydroxyl group present in the aromatic ring, however, merely serves as an enhancement of its potency (Behrens et al., 2014). The similarities between the compound found in kisspeptin treated sole, 3- hydroxyphenylacetic acid, and 4-hydroxyphenylacetic as a possible constituent of both a feline and a zebrafish reproductive pheromone, suggested that this compound also plays a part in chemical communication in Senegalese sole. Furthermore, the presence in the same urine of an unknown chemical compound, with a potential steroid-like structure, may also suggest it as putative reproductive pheromone. This study showed for the first time, the implication of a hormonal treatment in the chemical communication of a potential fish species for aquaculture like Senegalese sole. However, further studies are needed to verify the exact structure, function of this new metabolite and the level of implication in the chemical communication field.

One additional possible effect caused by kisspeptin treatment is related to its antioxidant properties (Abou-Khalil and Mahmoud, 2020; Akkaya et al., 2014). Our putative reproductive pheromone 3-hydroxyphenylacetic acid, has also been suggested to have antioxidant properties (Catapano et al., 2019). In reproduction, antioxidants protect the gonads from oxidative damage, helping to prevent certain diseases and conditions, in addition to increase fertility in some cases (Agarwal et al., 2006). In Senegalese sole, it has been proposed that captive males suffered from oxidative damage, affecting their performance in reproduction (Forné et al., 2009). Their spermatozoa were shown to possess high levels of oxidative stress, regardless of male origin (Valcarce and Robles, 2016). In consequence, an increase in antioxidative capacity can have a positive impact in terms of welfare and reproductive success. In addition to the antioxidant ability of 3-hydroxyphenylacetic acid, one possible connection that can be made is with a molecule named DOPAC (3,4-Dihydroxyphenylacetic acid), a very similar compound to both, 3 and 4-

hydroxyphenylacetic acids, as seen in its formula (Tang et al., 2016; Zabela et al., 2020). These molecules take part in a few important pathways, like the tyrosine and phenylalanine metabolisms (map00350 and map00360), styrene degradation (map00643), metabolic pathways (map01100) and microbial metabolism in diverse environments (map01120) (Kanehisa et al., 2019). DOPAC, in addition to having antioxidant properties, is also a metabolite product of a molecule called dopamine, through its degradation (Kanehisa et al., 2019; Meiser et al., 2013; Nakazato and Akiyama, 2002; Tang et al., 2016). Dopamine is a crucial metabolite in the body, having several functions, including the antioxidant capacity (Byun et al., 2020; Kanazawa and Sakakibara, 2000; Zisapel, 2001). One of those functions, the regulation of the circadian rhythm, shared with melatonin (Korshunov et al., 2017), which is also a potent antioxidant, is known to regulate and modulate kisspeptin gene expression levels (Alvarado et al., 2015; Choi et al., 2015; Maitra and Hasan, 2016; Pazarci et al., 2020; Roseweir and Millar, 2009). In conclusion, it might be hypothesized that kisspeptin injection, trough melatonin regulation and its relationship with additional antioxidant components in the body, may improve reproduction do to a possible increase of antioxidant capacity. However, further research would be needed to concretely ascertain this hypothesis, namely measuring the antioxidant activity in sperm and ovarian fluid after kisspeptin treatment.

The proposed pheromone effect was investigated using EOG analysis. A similar procedure was conducted previously in the Mozambique tilapia (*Oreochromis mossambicus*) (Keller-Costa et al., 2014) where,. these researchers found two steroid pheromones namely, 20b-P-3-G and 20a-P-3-G using an Ultraperformance liquid chromatography coupled high-resolution mass spectrometry, UPLC-HRMS, (Keller-Costa et al., 2014). These compounds were used for EOG analysis and were proven to be responsible for the priming effect of the dominant male's urine, consisting in stimulating female 17,20β-P, that at the same time, stimulated gonad maturation. In Senegalese sole, this technique has been used, for example, to identify differences between both olfactory epitheliums (Velez et al., 2013), upper and lower, having in mind this species is asymmetric. Moreover, the olfactory sensitivity of urine and faeces was determined to be the vehicle for reproduction odorant release, combined with differences between developmental status and sex (Fatsini et al., 2017). When comparing our results with Fatsini et al., 2017 study, we can notice a similar EOG response pattern, with males responding better to conspecific female urine. However, given the methodology differences, no further comparisons could be made. When comparing

between treated and non-treated fish's urine EOG response, it becomes evident that the kisspeptin treatment affected the potency of the urines. The female's stimulus was more affected than the male's, having a statistical increase on the female receivers, with male receivers also having a big increase, despite lacking statistical significance. The urine potency increase derived from the kisspeptin treatment suggested that, the administration affected the chemical communication in this species. This possibility can be linked to the increase of the chemicals, found in the treated sole's urine, with the LC-MS analysis. this linkage would reinforce their putative effect as pheromones; however, further studies are recommended to definitively confirm these results. Considering urines from different individuals, a pattern of maximum response to the female urine 4 appeared in receivers from both sexes. The remaining stimulants all followed a similar response profile when comparing female and male receivers, with males showing slightly higher responses, however, with more variability than the females. This variation might indicate that kisspeptin treatment has a different effect on each individual and in case of some urine samples were more diluted than others, evidenced by a more transparent appearance (Data not shown).

5. Conclusions

- Kisspeptin treatment caused a positive effect in juvenile Senegalese sole sex steroids levels and gonadal maturation, with increased levels of T in both sexes and a tendency to increase E_2 in females and slightly more developed male gonads. These results demonstrated the potential of this treatment in inducing puberty in this species.

- Kisspeptin treatment increased the presence of two metabolites in urine from Senegalese sole breeders, one unidentified with the possibility of being an steroid with an aromatic ring and the other 3- hydroxyphenylacetic acid, both potentially classified as pheromones, likely improving sole chemical communication, via increased olfactory responses to treated sole's urine.

- Kisspeptin showed a great potential as a hormonal treatment in Senegalese sole, with further studies needed to more closely establish its effect on the reproduction of this species, in addition to the need of optimization of both application methods and dosage.

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