Beatriz Neves Gregório

Chemical communication in the Lusitanian toadfish, *Halobatrachus didactylus*

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

2023

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Chemical communication in the Lusitanian toadfish, *Halobatrachus didactylus*

MSc in Marine Biology

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UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

2023

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

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Acknowledgements

A nível académico, gostaria de agradecer aos meus orientadores pela oportunidade de trabalhar convosco. À Teresa Modesto pelo apoio, orientação, entusiasmo e motivação, tanto a nível profissional como pessoal. Ao Peter Hubbard pelo apoio, orientação, por todas as horas de EOGs ao som da rádio e todo o conhecimento transmitido. À Zélia Velez pelo apoio e a orientação principalmente nas técnicas de química analítica.

Gostaria também de agradecer a toda a equipa do CCMar que se mostraram sempre disponíveis para ajudar. À Rita Costa por todo o apoio e conselhos na realização das técnicas de histologia. À Nathalie Marquet por todo o apoio e conselhos na execução da imunohistoquímica. À Vera com a orientação e dicas em relação ao HPLC. Ao José Paulo pela disponibilidade e orientação em relação ao LC-MS.

Quero agradecer ao Paulo Daniel e ao Carlos Estevão e pais pela disponibilidade e fornecimento de "xarroquinhos".

A nível pessoal, quero agradecer à minha mãe, que se sou quem sou e se estou onde estou é tudo graças a ela. À minha irmã, que agora apesar de longe apoia-me sempre. Ao meu namorado, pelas horas infinitas a aturar-me e por todo o apoio constante. À minha família, que sempre me acompanhou e apoiou em todas as fases da minha vida. Aos meus amigos que, de uma maneira ou de outra, longe ou perto, ajudaram-me e motivaram-me ao longo do percurso de mestrado, em especial à Samantha Marum.

Resumo

A comunicação química no ambiente aquático é extremamente importante em vários aspetos da fisiologia e comportamento dos peixes, como atrair parceiros, marcar território, coordenar processos reprodutivos, estabelecer hierarquias sociais, nas respostas de alarme e na definição de rotas de migração. Porém, a comunicação química, mediada pelo sistema olfativo, é ainda uma área pouco estudada da biologia dos peixes. A identidade dos compostos envolvidos e consequente efeitos fisiológicos e comportamentais apenas foram estabelecidos em algumas espécies, particularmente em peixes de água doce. Neste contexto, o objetivo geral deste trabalho é avaliar se a comunicação química está presente numa espécie de teleósteo marinho, o xarroco, *Halobatrachus didactylus*. Esta espécie apresenta características peculiares no comportamento reprodutivo que a tornam um excelente modelo para avaliar a importância da comunicação química, nomeadamente na reprodução. Durante a estação reprodutiva (maio-julho), os machos territoriais que guardam os ninhos (machos Tipo I) emitem vocalizações de corte para atrair as fêmeas e uma variedade de sons pulsados durante as interações agonísticas de defesa territorial com outros machos. Os machos menores (machos Tipo II) imitam as fêmeas morfologicamente e adotam um comportamento oportunista. As glândulas acessórias testiculares dos machos tipo I são maiores em comparação com as dos machos tipo II, sofrem um aumento significativo de tamanho durante a época reprodutiva e produzem diversos esteroides. Durante a época reprodutiva, as secreções produzidas por estas glândulas fluem abundantemente através do orifício urogenital dos machos tipo I. Assim, a hipótese subjacente a este trabalho é que, embora esta espécie utilize comunicação sonora em contexto reprodutivo, as substâncias libertadas pelas glândulas testiculares, ou outros fluidos orgânicos, possam também funcionar como sinais de status social/reprodutivo (ou seja, feromonas) e ter um papel importante na reprodução desta espécie. Neste contexto, os objetivos específicos deste trabalho são: (1) Caracterização morfológica do sistema olfativo; (2) Caracterização histológica do epitélio olfativo; (3) avaliação da sensibilidade olfativa a diferentes fluidos corporais provenientes de machos tipo I em diferentes estados reprodutivos, utilizando técnicas de eletrofisiologia; (4) Identificação química dos principais odorantes presentes nos fluidos olfativos mais potentes, através de técnicas de química analítica. O órgão olfativo foi caracterizado através do uso de técnicas morfológicas, histológicas e imunohistoquímicas. É constituído por duas narinas, uma única câmara olfativa tubular

sem lamelas olfativas, com um padrão de distribuição contínuo do epitélio sensorial, e um único saco acessório. A narina anterior é tubular e alongada com um grupo de pequenas projeções digitiformes e a narina posterior é arredondada sem projeções. Os neurónios sensoriais são encontrados no fundo da câmara olfativa, como acontece em outras espécies de teleósteos. A sensibilidade olfativa a diversos fluidos corporais foi analisada por eletro-olfatograma (EOG). Os resultados mostram que fluidos corporais de conspecíficos, especificamente fluidos intestinais, biliares, das glândulas testiculares anteriores e posteriores são fontes de potentes odorantes para o xarroco. Os fluidos intestinais e da glândula testicular anterior de machos reprodutivos induzem uma resposta olfativa significativamente mais potente do que os dos machos não reprodutivos. Em contraste, a glândula testicular posterior evocou altas respostas olfativas, mas não mostrou diferenças significativas entre as épocas reprodutivas. Além dos fluidos glandulares, os fluidos intestinais e biliares foram os estímulos que evocaram as maiores amplitudes de resposta do EOG. Além disso, a diferença na potência do fluido intestinal de machos de diferentes épocas reprodutivas sugere que estes fluidos poderão transmitir informações sobre o estado reprodutivo do emissor. A extração em fase sólida de fluidos corporais através de colunas C18 mostrou que a maior parte da sensibilidade olfativa estava contida na fração de eluato (fase hidrofóbica), exceto para o fluido biliar. Posteriormente, o fracionamento do eluato dos fluidos intestinais por cromatografia líquida de alta eficiência (HPLC) permitiu separar frações, cuja resposta olfativa foi testada por EOG. A cromatografia líquida com espectrometria de massas (LC-MS) às frações de HPLC que evocaram uma maior amplitude de resposta permitiu a identificação de alguns ácidos biliares, como o ácido cólico, ácido taurolitocólico e ácido tauroquenodesoxicólico. Esses resultados foram posteriormente confirmados por comparação de cromatogramas de massa e tempo de retenção com amostras padrão e reforçados por testes de adaptação cruzada em EOG que indicam a presença de ácido cólico no fluido intestinal. Os ácidos biliares identificados nos fluidos intestinais de animais de época reprodutiva são bons candidatos para explicar os efeitos da sensibilidade olfativa a esses fluidos. No entanto, as diferenças significativas encontradas nas respostas olfativas ao fluido da glândula testicular acessória anterior entre animais de época reprodutiva e não reprodutiva, podem indicar que estas glândulas também podem liberar odorantes importantes, além de outras funções na reprodução.

Os resultados deste estudo sugerem fortemente que o xarroco poderá utilizar uma combinação de sinais químicos juntamente com sinais auditivos durante o processo reprodutivo, que poderão estar relacionados com as estratégias reprodutivas alternativas dos machos. Mais estudos são necessários para uma completa caracterização dos odorantes presentes nestes fluidos e para definir os seus efeitos fisiológicos e comportamentais em conspecíficos.

Palavras-chave: xarroco; olfato; ácidos biliares; glândulas testiculares acessórias reprodução.

Abstract

Aquatic animals use chemicals for communication for a variety of purposes including identification, sensing competitors and social status, detection of predators, and for reproduction. The highly vocal Lusitanian toadfish (*Halobatrachus didactylus*) is very well studied for acoustic communication. Moreover, an early study showed that the Lusitanian toadfish has olfactory sensitivity to conspecific body fluids. Does this species also use chemical communication through biological fluids that may act as signals of social or reproductive status? Detailed knowledge on chemical communication in marine fish is scarce; nevertheless, much evidence exists for its occurrence. This work aimed to characterize the morphology of the olfactory organ of toadfish, using histological and immunohistochemical approaches. The olfactory sensitivity to different body fluids – anterior and posterior testicular accessory gland, bile, and intestinal fluids – and possible differences in their potency is studied by electrophysiological techniques, and analytical chemistry was used to identify the main odorants in the most potent fluid. Olfactory sensitivity assessed by electro-olfactogram (EOG) indicated that conspecific intestinal and anterior testicular accessory gland fluids contain highly potent odorants, especially during the breeding season, suggesting that chemical communication is, indeed, important in toadfish reproduction. Solid-phase extraction of body fluids using C18 cartridges showed that most of olfactory sensitivity was contained in the eluate fraction, except for bile fluid. Further fractionation of eluate of intestinal fluids by high-performance liquid chromatography (HPLC) permitted isolate the most active compounds. Liquid chromatography with mass spectrometry (LC-MS) allowed the identification of some bile acids. Cross-adaptation experiments reinforced the presence of cholic acid in the intestinal fluid. These results are consistent with a role for chemical communication in the Lusitanian toadfish and suggest that bile acids might be involved.

Keywords: toadfish; olfaction; bile acids; reproduction

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List of Abbreviations

EOG	Electro-olfactogram
EDTA	Ethylenediaminetetracetic acid
HPLC	High-performance liquid chromatography
LCMS	Liquid chromatography-mass spectrometry
OE	Olfactory epithelium
ORN	Olfactory receptor neurons
PBS	Phosphate buffered saline
PFA	Paraformaldehyde solution
SEM	Standard error of the mean
SPE	Solid phase extraction

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1. Introduction

1.1. Communication in Aquatic Environments

Animals use different modalities to communicate (visual, acoustic, chemical, mechanosensory and electric cues); the choice of the respective modality depends strongly on the environmental conditions and phylogeny of the species in question (Frommen, 2020). Aquatic environments differ drastically from terrestrial environments in their suitability to transfer information (Pitcher, 1993). For instance, poor light transmission in water, especially in turbid waters, and high habitat complexity may reduce the usefulness of vision in aquatic organisms (Brönmark & Hansson, 2012). Moreover, in contrast to terrestrial environments, long-lasting territorial scent marks are absent in aquatic habitats. This may be due to the high solubility of chemical cues in water causing any scent marks to be diffused by water movements within short time scales (Frommen, 2020). However, chemical messages can last longer than visual and acoustic messages and can occur along with other signals to reinforce or specify the meaning (Hughes, 1996; Sorensen & Wisenden, 2015). Different cues may elicit or enhance the same response in an opponent (Hebets & Papaj, 2005; Partan & Marler, 1999). By sending the same information, either in the same or different modality, receivers are more likely to perceive the signal (Partan & Marler, 1999) and this may lead to the emergence of a reaction that both components alone would not elicit (Partan & Marler, 2005). However, communication using multimodal signals also comes with costs, including the energetic costs of producing, perceiving and integrating such diverse cues as well as an increased attraction of predators or competitors (Partan & Marler, 2005). Thus, the necessity to evolve multimodal communication signals is, again, strongly dependent on the ecological context (Frommen, 2020).

Chemical cues are probably the first signalling systems to have evolved (Wyatt, 2014). All cellular life forms, from bacteria to mammals, are sensitive to chemical information, whether it comes from potential food, predators, the environment, or other members of the same species (Wyatt, 2014). Besides, chemical communication is a universal feature of life that occurs at all levels of biological organization, including the regulation of cells and organs within the body (Agosta, 1992). Contrasting with wave or wave-like propagation of acoustic, visual, and electromagnetic signals, chemical signals disperse through the environment by molecular diffusion and bulk flow (Antunes & Efferth, 2014).

Many studies have shown that aquatic organisms from many different taxa and functional groups respond to minute concentrations of chemical substances released by other organisms (Brönmark & Hansson, 2012).

1.1.1. Chemical Cues

Three independent chemosensory systems can be distinguished in fishes: taste, olfactory sense, and the general chemical sense (Parker, 1912). The olfactory sense is also known as sense of smell. Odorants are chemical compounds that bind to olfactory receptors and thereby stimulate the olfactory sense (P. W. Sorensen, 2015). A particular odorant may cause neurophysiological activity in designated olfactory receptor neurons, but any behavioural response will depend both on context, and the physiological status and/or motivation of the receiver (Baker, 2008). On the other hand, odours are mixtures of odorants and often produce behavioural responses only when presented in a limited range of mixture ratios (Baker, 2008). Semiochemicals convey information regarding the living environment (Korsching, 2016) and result in an adaptive response in the receiver (Brönmark & Hansson, 2012). Furthermore, semiochemicals can constitute two broad categories: interspecific communication, 'allelochemicals', and intraspecific communication, which can be 'signature mixtures' or 'pheromones' (Wyatt, 2014). Allelochemicals are further divided depending on the costs and benefits to the signaller and receiver (Nordlund, 1981). For example, kairomones mediate interactions wherein the information transfer is beneficial for the receiver but not for the sender, allomones mediate interactions where the signal emitter benefits at the cost of the receiver and synomones benefiting both signaller and receiver in mutualisms (Wyatt, 2003). (Wyatt, 2014)) suggests that signature mixtures are a variable chemical mixture that can be learned and used by an animal for individual recognition. In contrast, pheromones are signals emitted by a signaller and received by a second individual of the same species (receiver) wherein they elicit a specific response, for example a stereotyped behaviour ('releaser' effect) or a physiological or developmental process ('primer' effect; (Wyatt, 2014)). Individuals from other species can perceive signals broadcast to the wider world. For instance, the same chemical may be used as a pheromone within a species but may be exploited by specialist predators as a kairomone to locate their prey (Wyatt, 2003).

1.2. Chemical communication in fish reproduction

Fishes evaluate their environment using conspecific and heterospecific cues (Chung-Davidson et al., 2011) and rely on abiotic and biotic olfactory cues to facilitate behaviours critical for foraging, defence, migration, and reproduction (Hara, 1975; Sorensen & Wisenden, 2015). Chemical communication in the aquatic environment is well documented in crustaceans and freshwater teleost, but how marine teleost communicate chemically is understudied (Sorensen & Wisenden, 2015). It would be highly advantageous for marine fish to communicate chemically because the ocean is often dark and turbid making chemical signals more effective than visual cues (P. Hubbard, 2015). Marine fish need to coordinate spawning as the majority are external fertilizers and many species are not sexually dimorphic, which makes chemical signals important to relaying sex and reproductive status to a conspecific (P. Hubbard, 2015). Moreover, marine fish have highly sensitive olfactory systems lending to the idea that they have evolved to communicate chemically (P. Hubbard, 2015).

The main odour classes in fish are charged molecules (Korsching, 2016). The olfactory epithelium of fishes is acutely and specifically tuned to detect amino acids, bile acids, nucleotides, and, in some species, steroids hormones and prostaglandins (Buchinger et al., 2014; Hara, 1994), each of which has been shown to influence behaviour (Hara, 2006; Li et al., 2002; Stacey et al., 2003). Amino acids and nucleotides are believed to be related mainly with feeding behaviour. The majority of reproductive pheromones in fish studied to date are derived from sex steroids (androgens, estrogens, maturation inducing steroids) and prostaglandins and their sulphated or glucuronated conjugates and metabolites that are tightly linked to various stages in the reproductive cycle (Stacey, 2015). In the goldfish, 2 sex steroids and 1 sex steroid metabolite as well as 2 prostaglandins have been identified and shown to be both detected with great sensitivity and specificity, and to function as different types of sex pheromones (Dulka et al., 1987; P. W. Sorensen et al., 1988). Bile acids are also potent odorants in several species of fish. For instance, Arctic charr are acutely sensitive to bile salts (K. Døving et al., 1980), there is evidence that is used as a long-distance migratory pheromone (Jones & Hara, 1985; Nordeng, 1977). However, only in the sea lamprey *Petromyzon marinus* clearly defined roles for bile acids as pheromones been established (Li et al., 2002; P. W. Sorensen et al., 2005). Another option to make a signal species-specific is to add a non-hormonal component to the signal or another sensory cue (P. Sorensen & Baker, 2015).

1.3. The Olfactory System in Fish

The olfactory organ of fishes is of primary importance. It plays an essential role in the lives of fishes in such activities as food search, predator avoidance, parental care, migration, and reproduction (Nikonov et al., 2017). Many researchers investigated diverse views of the olfactory organ of teleost (Ghosh, 2021; Hansen & Zielinski, 2005; Waryani et al., 2013). Variation in the morphology of the olfactory organ correlates with the enormous diversity of life-styles among fishes, their long, divergent evolutionary history, and their ecological adaptations (Zeiske et al., 2009).

Teleost have two nasal cavities, one on each side of the head at the extremity of the snout (Døving et al., 1977; Hansen & Zielinski, 2005). In contrast with higher vertebrates, there is no connection between the olfactory and the respiratory systems, and respiratory sniffing and its implications for odour detection are absent, although some fishes may use muscular contractions for active olfactory sampling (Døving et al., 1977; Nevitt, 1991). Each nasal cavity is composed of an anterior nostril, through which water enters the nose, and a posterior nostril, through which water exits the nose (Kermen et al., 2013; Olivares & Schmachtenberg, 2019). In this case, cichlids are an exception, they only have a single nostril per side (Escobar-Camacho & Carleton, 2015). Commonly, the anterior nostril may be an open hole, a tube or a funnel and the posterior nostril may be an open hole, a slit or a tube.

The olfactory epithelium lies between these two nostrils (Hara & Zielinski, 2007) and may be located on the floor and the sides of the olfactory chamber. In most species, it is arranged in the form of sheets or lamellae, which radiate from a central ridge or raphe and give rise to an olfactory rosette (Hansen & Zielinski, 2005). The number and size of the lamellae increase throughout development of the teleost but remain relatively constant after the specimen reaches maturity (Olivares & Schmachtenberg, 2019). Sensory and non-sensory cells are irregularly interspersed within the epithelia, as are mucus-producing goblet cells (Bazáes, Olivares, et al., 2013). Additionally, certain fish have accessory olfactory cavities as an extension of the olfactory chamber (figure 1.1). However, there are some variations between different taxonomic group of fish depending on their life mode and behaviour.

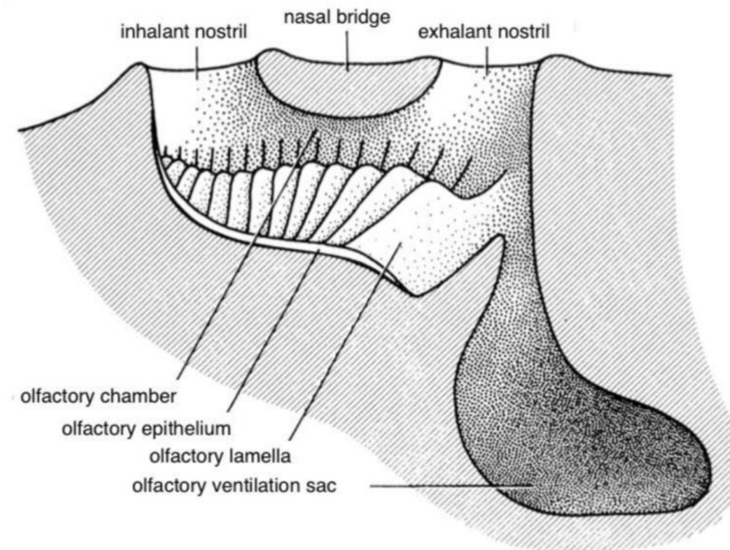

Figure 1.1 Schematic sagittal section of basic structural elements of the olfactory organ in generalized fish (from Zeiske et al., 1992).

1.3.1. Olfactory epithelium

The olfactory epithelium (OE) consists of four main types of cells: olfactory receptor neurons (ORN), supporting, mucous and basal cells (figure 1.2). ORN are the primary sensory cells of the olfactory epithelium. There are five ORNs that can be distinguished by general morphology and relative position: ciliated and microvillous sensory neurons, crypt, pear and *Kappe* cells, the last two only studied to date in zebrafish (G. Ahuja et al., 2015; Hansen & Zielinski, 2005; Olivares & Schmachtenberg, 2019; Wakisaka et al., 2017). Olfactory cells contain the olfactory receptor proteins that are exposed to chemical stimuli in the water (Olivares & Schmachtenberg, 2019). Ciliated cells have long dendrites and few cilia, with the soma situated in the deep layer of the OE (Kermen et al., 2013). Microvillous sensory neurons have shorter dendrites than ciliated neurons, that have the soma located in the intermediate layer of the OE (Kermen et al., 2013). Crypt, pear and kappe have the soma located in the most superficial layer of the OE (G. Ahuja et al., 2015; Kermen et al., 2013; Wakisaka et al., 2017). The distribution of receptor cells within the OE in different fish species may differ. Continuous, large-zone, fine-zone, and irregular types of sensory epithelium distribution are usually distinguished (Yamamoto, 1982; Yamamoto & Ueda, 1979).

ORNs are constantly renewed throughout adult life or following chemical lesion of the epithelium (Bettini et al., 2006; Cancalon, 1982). This regeneration is mediated by the

division of basal cells located in the deepest layer of the olfactory epithelium (Cancalon, 1982).

Supporting cells surrounding the receptor cells are represented in the olfactory epithelium by two different types: the supporting cells with a free surface not bearing any specialized structures, and ciliated cells which bearing numerous kinocilia on the surface facing the cavity of the nasal sac. Kinocilia of the ciliated cells move synchronously, and their activity is responsible for the ventilation of the olfactory cavity in areas located between close lamellae of the olfactory rosette, and in many fish species, ventilation of the whole olfactory organ (K. B. Døving, 1986).

Goblet cells or mucous cells are in upper layer of the OE and may differ in size. It is hypothesized that its mucus creates optimal conditions for the molecular processes involved into the interaction between the signal substance (ligand) and the receptor proteins (P. Sorensen & Caprio, 1998). Furthermore, it is thought that the olfactory mucus has important protective functions, protecting flagella and microvilli of receptor cells from mechanical disturbances (Kasumyan, 2004).

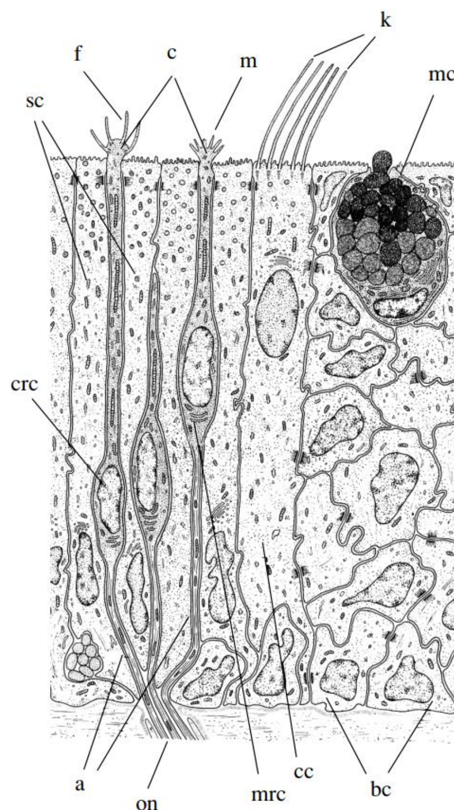

Figure 1.2 Schem of basic cell elements in the olfactory epithelium of fish: (a) axons of receptor cells; (c) club; (bc) basal cells; (f) flagellae; (crc) ciliated receptor cell; (k) kinocilia; (m) microvilli; (cc) ciliate cell; (mrc) microvillous receptor cell; (sc) supporting cells; (on) olfactory nerve; (mc) mucous cell (from Zeiske et al., 1992).

1.3.2. Transduction process: G-protein coupled receptors

The detection of odorants by ORNs is mediated by odorant receptors which, in vertebrates, are constituents of the G-protein-coupled receptor superfamily (Sorensen & Wisenden, 2015). The receptor undergoes through conformational change due to the binding of an odorant that induces the activation of the respective G proteins, which dissociate their alpha subunit. This process changes the functioning of the ion channels in the cell, generation of receptor potential, and its distribution as an electric nervous impulse from the generation location to the primary and secondary olfactory centres (Brand & Bruch, 1992).

Odorant receptors are highly diverse and can be grouped into olfactory receptors (ORs), trace amine-associated receptors (TAARs) and type I and II vomeronasal receptors (V1Rs and V2Rs) (Alioto & Ngai, 2005; Fleischer, 2009; Mombaerts, 2004). It was demonstrated in several teleost species that the different types of olfactory receptor proteins are expressed differentially in the specific ORN types, consequently different transduction processes associated with diverse types of G-proteins (Hansen et al., 2004). Therefore, the response profile of a given neuron is constrained by the receptive field of the receptor it expresses. It has been proposed that ciliated cells express ORs and TAARs, which are associated with $G\alpha_{olf}$ proteins; microvillous express V1Rs, associated with $G\alpha_{i}$, and V2Rs, associated with $G\alpha_{q}$, $G\alpha_{q/11}$ and $G\alpha_{i-3}$; crypt cells express V1Rs, coupled to $G\alpha_{o}$, $G\alpha_{q/11}$ and Gαi1b. (S. Ahuja, 2015; Bazáes, Jesús Olivares, et al., 2013; Hansen et al., 2004; Hansen & Barbara S. Zielinski, 2005). TAARs are expressed in sparse ORNs (Hussain et al., 2009). Some studies have given insights into the ORNs and their interaction with pheromones. In *Oncorhynchus mykiss*, ciliated ORN have a generalist response to a wide range of odorants, among them pheromones and amino acids, whereas microvillous ORNs are specific for amino acid detection (Sato & Noriyo, 2001). In the channel catfish (*Ictalurus punctatus*), amino acids are detected by both ciliated and microvillous ORNs, and bile salts by ciliated ORNs (Hansen et al., 2003). In the crucian carp (*Carassius carassius*), detection of alarm cues occurs in ciliated ORs (K. B. Døving & Stine Lastein, 2009; Hamdani & Kjell B. Døving, 2002) as with some sex pheromones (Lastein et al., 2006).

1.3.3. Odour procession: Olfactory bulb

The processing of odours in vertebrates occurs in the olfactory bulb. In the teleost, in this brain structure four layers can be identified from the periphery towards the centre:

Olfactory nerve layer, glomerular layer, mitral cell layer and granular layer (Satou, 1990). The most external layer is formed by the axonal endings of the ORNs, the dendrites of which extend into the following inner, where glutamatergic synapses are formed with the dendrites of the mitral cells which have their somas located in a deeper layer, the external cell layer (Fujita et al., 1988). The glomerular layer of teleost also contains nerve endings from higher telencephalic centres and projections of granule cells, but it is in the deepest layer of the olfactory bulb that the mitral cells connect to telencephalon and diencephalon brain areas as targets to further process olfactory input (Kermen et al., 2013; Nieuwenhuys et al., 1998). Then, the information is integrated, and it is mediated an appropriate output response.

Knowledge of the cellular organization of the olfactory organ in *H. didactylus* is almost unknown. To date, only (Palazón-Fernández & Sarasquete, 2015) briefly described the olfactory organ in Lusitanian toadfish.

1.4. The species *Halobatrachus didactylus*

The Lusitanian toadfish, *Halobatrachus didactylus* (Bloch and Schneider, 1801) (Pisces: Batrachoididae) is a teleost found in subtropical regions, from the Gulf of Guinea to the Gulf of Biscay, including the western part of the Mediterranean (Bauchot, 1987; Roux, 1986), and in Atlantic islands, Madeira (Roux, 1986; Santos et al., 2000), Canaries and Cape Verde islands (Reiner, 1996). However, there are only significant populations between the Liberian coast and the south of Portugal (Costa, 2004). *H. didactylus* is the only recognized species of the *Halobatrachus* genus (Collette et al., 2006), and it possesses a robust body, similar to other species of the Batrachoididae (figure 1.3). It can reach up to 50 cm in length, although most do not exceed 35 cm (Bauchot, 1987; Roux, 1986). It is a voracious predator and has a wide feeding range (Cárdenas, 1977; Costa et al., 2000; Sobral, 1981), placing it in the top position in estuarine and coastal lagoons trophic webs, where it plays an important role in the structure and balance of the existing biological communities (Costa, 2004).

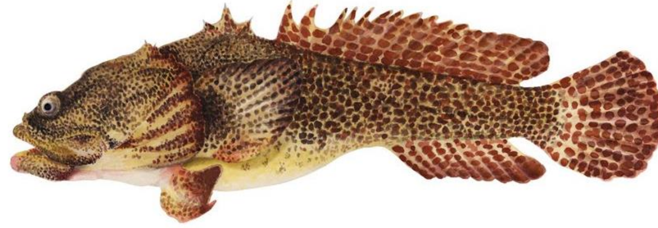

Figure 1.3 Scientific illustration of the Lusitanian toadfish (Martins, 2010).

It is a benthic, solitary and relatively sedentary species (Costa, 2004), mostly found in estuaries and coastal waters living in sand, muddy bottoms, but is also found in hard bottoms, under stones or sheltered in rocky crevices (Bauchot, 1987; Roux, 1986; Santos et al., 2000). It can be found down to 50 m deep, with reports of catches by bottom trawling at 250 m depths (Roux, 1986), where visual communication is often limited, and probably as a consequence relies heavily on acoustic (Vasconcelos, 2011) and possibly chemical signalling (Modesto et al., 2015, 2022) to interact with conspecifics throughout life and to advertise nests and attract mates in the breeding season.

1.4.1. Reproduction

H. didactylus is a gonochoric species with external fertilization (Costa, 2004). The spawning season occurs from March to August (Palazón-Fernández et al., 2001) with a peak from May to June on the south coast of Portugal (Modesto & Canário, 2003b). During the reproductive season, two male morphotypes can be distinguished that differ in morphometric and endocrine characteristics, as well as in vocal behaviour. Type I males are the reproductive nesting males, have smaller testis, larger accessory glands, and sonic muscles, and produce higher levels of 11-ketotestosterone (Modesto & Canário, 2003a). Type II males are generally smaller and sneakers, with higher gonadosomatic index but smaller sonic muscles (Modesto & Canário, 2003b, 2003a) that parasite the nests to attempt opportunistic fertilizations. Sonic muscles of type I males, but not of type II males or females, experience hypertrophy during the breeding season (Modesto & Canário, 2003a), mirroring an increase in vocal activity (Amorim et al., 2006).

The territorial nest-holders (type I) build nests in aggregations in shallow waters under rocks or in crevices. These males attract females to spawn using an extensive vocal repertoire, the ‘boatwhistle’, produced by the swim bladder, which is especially well

developed (Amorim et al., 2006; Santos et al., 2000). Also, type I males also produce other sounds, such as grunt trains, long grunt trains, and double croaks, as well as other less frequent sound emissions such as croaks and mixed croak–grunt, long grunts trains calls (Amorim et al., 2008). The advertisement calls have been also implied in signalling territorial ownership and in spacing out individuals (Remage-Healey & Bass, 2005; Vasconcelos et al., 2012; Winn, 1967).

Females deposit their eggs in the roof of the nest where they attach by an adhesive disc and are guarded by the male until the offspring are free-swimming (Roux, 1986; Vasconcelos, 2011). Females and sneaker males are only known to emit grunt trains. Furthermore, females show lighter and less developed sonic muscles than males, with type II males presenting intermediate sonic muscle mass to females and type I males (Modesto & Canário, 2003a). The sonic muscles of type I males, suffer hypertrophy during the reproductive season; this characteristic does not happen with males type II, nor with females (Modesto & Canário, 2003a), which is related to an increase in the sound activity in males type I in the reproductive period to call the females to the nests (Amorim et al., 2009).

1.4.1.1. Accessory glands

Accessory glands are a collection of reproductive glands and ducts that do not produce gametes and can be found in both males and females across the animal kingdom (Miller et al., 2019). Accessory glands in males serve a variety of reproductive functions such as sperm storage and recycling via lytic activity, and the production of seminal fluid, which is released with the sperm (Chowdhury & Joy, 2007; Hyman, 1992; Scaggiante et al., 1999). For example, sperm duct accessory glands that have high protein content can store sperm in and near the sperm duct in preparation for release, increasing sperm storage capacity (Chowdhury & Joy, 2007; Mochida et al., 1999). Sperm duct accessory glands are also capable of lytic activity, engaging in autophagocytotic and heterophagocytotic processes, the latter allowing the gland to absorb and eliminate aged sperm cells and thus improving ejaculate quality (Chowdhury & Joy, 2007). Glycogen and lipid production occurs in the sperm duct accessory gland of many fishes as well, these products are important for the final differentiation of sperm and as energetic substrates during fertilization (Miller, 2017). Seminal fluid specifically contributes to fertilization by triggering sperm capacitation, enhancing sperm motility, and acting as an osmotic and

ionic shock absorber for sperm entering new environments and maintains environmental pH immediately surrounding sperm (Chowdhury & Joy, 2007; Poiani, 2006; Ramm et al., 2005). Besides, (Gillott, 2003) suggests that male accessory gland secretions act as modulator of female reproductive physiology and behaviour. There are two main types of accessory glands among fishes: testicular accessory glands, which are outgrowths of the testes, and sperm duct accessory glands, which are appendages of the sperm duct (Miller, 2017). The accessory glands type has been indicated as sperm duct accessory gland in *H. didactylus* (Amorim et al., 2006; Miller, 2017; Modesto & Canário, 2003b; Vasconcelos et al., 2012).

H. didactylus has the accessory glands divided into a fan shaped anterior yellow coloured region (AR) and a rounded dark brown to black posterior region (PR). Connecting these two regions was a thin pale yellow middle region (MR). These glands are connected laterally through three regions with the spermatic duct, which open to the exterior through the genital pore situated at the extremity of an elongated papilla (figure 1.4). The accessory glands of type I males, unlike type II males, undergo seasonal changes in weight and fluid production in parallel to the seasonal profile of gonadosomatic index (GSI) and main sex steroids (Modesto & Canário, 2003b). The abundant fluid produced by these glands is composed of mucous substances and proteins, which can be expressed through the genital pore by gentle pressure applied to the surrounding area (Modesto & Canário, 2003b). This fluid has been suggested in other teleost to increase the viscosity of the seminal fluid and help to agglutinate the sperm, increasing the efficacy of fertilisation of the sticky benthic eggs by parental type I males (Cinquetti, 1997; Lahnsteiner et al., 1992). Recent in vitro preliminary studies also shown that gland fluids can prolong sperm motility, possibly enhancing sperm performance during spermiation or fertilization process (Modesto et al., 2022).

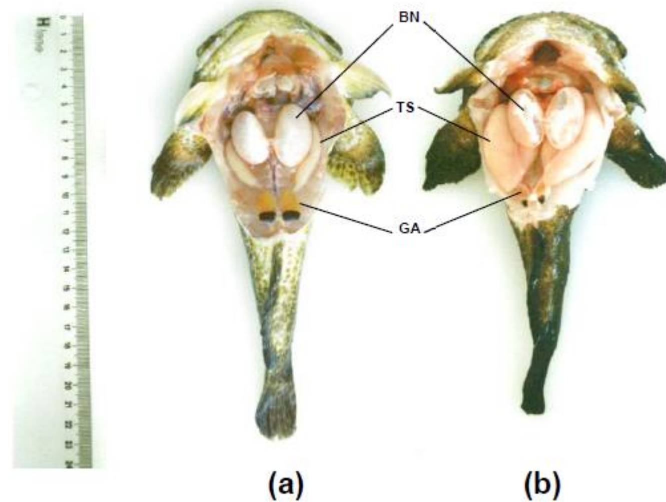

Figure 1.4 Dissected ventral view of the two types of males of *H. didactylus* during the reproductive season: Type I (a) and Type II (b). Testicles (TS), Accessory glands (GA) and swimming bladder (BN). From: Modesto (2003)

In some fishes, the accessory gland secretes chemical compounds either in seminal fluid or in separate secretions that serve as pheromones and similar olfactory signals. For example, the mesorchial gland found in some members of the Gobiidae family synthesizes steroid metabolites that act as olfactory signals likely involved in courtship and competition (Arbuckle et al., 2005; Jasra et al., 2007; Locatello et al., 2002; Serrano et al., 2008). This is also true of the sperm duct accessory gland in the African catfish, *Clarias gariepinus* (Resink, Van Den Hurk, et al., 1987; Resink, van den Hurk, et al., 1987; Schoonen et al., 1987; Schoonen & Lambert, 1987), and evidence supports this function of the accessory gland in the four-eyed sleeper fish, *Bostrichthys sinensis* (Hong et al., 2006), and the dorsal accessory duct gland in two Nemipterid threadfin bream species (Lau & Sadovy, 2001). (Modesto et al., 2015) showed that the accessory glands of the *H. didactylus* have the capacity for steroid biosynthesis and proposed that there are differences in testicular steroidogenic pathways between the two male reproductive phenotypes. Type I males produce at least five steroids (mainly 5β,3α-hydroxyl androgens) that are not found in type II males, which led to the suggestion that different endocrine profiles are involved in the differentiation of different sets of traits (secondary sexual characteristics and reproductive behaviour) that make up alternative phenotypes (Modesto et al., 2015).

(Marcelino, 2020) studied the olfactory sensitivity of females to male body fluids (mucus, urine, intestinal fluid, bile fluid, and fluids from the anterior and posterior accessory glands) and reported that conspecific intestinal and accessory gland fluids contain highly potent odorants, especially during breeding. This suggests that chemical communication, along with sound communication, can be important in *H. didactylus* reproduction.

1.5. Objectives of this work

The main objective of the current work is to establish whether chemical communication in involved in toadfish. For that we aim to characterize the morphology of the olfactory organ and identify the main odorants present in the body fluids as good candidates to be pheromones.

To achieve this main goal, the following specific objectives are outlined:

(1) Morphological characterization the olfactory system (Stero microscopy observation after dissection to characterize the gross anatomy of the olfactory organ and olfactory nerves);

(2) Histological characterization of the olfactory epithelium (basic histological and immunohistochemical techniques to characterize the olfactory epithelium at the cellular level);

(3) Evaluation of the olfactory sensitivity to different body fluids (intestinal, bile and, testicular accessory glands fluids) and investigate possible differences in their potency according to male reproductive status;

(4) Identification of odorants present in the most potent olfactory fluid, by isolation of the odorants by solid-phase extraction (SPE) and High-Performance Liquid Chromatography (HPLC); chemical identification of the odorants by Liquid Chromatography with Mass Spectrometry (LCMS); olfactory responses to fractions and identified odorants by EOG.

2. Materials and Methods

2.1. Experimental Animals

Animal maintenance and experimentation are carried out in certified experimental facilities and following Portuguese national legislation (DL 113/2013) under a “group-1” license by the Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal. Adult Lusitanian toadfish, males and females, were caught in Ria Formosa and maintained in 600 L tanks with continuously running natural seawater, under natural photoperiod and temperature, and fed with mussels and squid in Ramalhete field station (Universidade do Algarve). Tanks were provided with shelters (plastic pipes cut in half lengthways) as environmental enrichment.

2.2. Morphological characterization the olfactory system

To study the anatomy of the olfactory system a specimen (30 cm total length) was sacrificed after being anesthetized with an excessive dosage of 2-phenoxyethanol (5:10000, Sigma-Aldrich). Macroscopic and stero microscopy observation after dissection allowed the characterization of the main structures of the olfactory organ and the location and anatomy of the olfactory nerves. To investigate the internal morphology of the olfactory organ was introduced a moulding paste (Express 2 Light Body Flow, 3M, Douromed, Portugal) into the posterior nostril using a syringe, filling the olfactory chamber and nares. The cast was removed after completely solidified and photographed.

2.3. Histology

2.3.1. Tissue sampling and processing for paraffin embedding

To study the morphology of the olfactory epithelium of the toadfish, two small animals (mean ± s.e standard length = 12.5 ± 0.9cm; mass = 38.1 ± 14.95g) were sacrificed after being anesthetized with an excessive dosage of 2-phenoxyethanol (5:10000, Sigma-Aldrich). The anterior part of the head region, containing the nasal tubules and olfactory chambers, was dissected from the head, and fixed in 4% PFA (appendix I) overnight at 4ºC for histological examination.

Fixed tissues were washed three times for 10 minutes with sterile 1x PBS (appendix I), one time for 10 min with sterile MilliQ water and immediately decalcified with 0.5M

EDTA pH 8.0 (appendix I) for 7 days. The EDTA was renewed every two days with fresh solution in order to increase the strength of the decalcifying process. The decalcified tissues were washed four times for 20 minutes with sterile MilliQ water and put in 70% EtOH (appendix I). The piece of decalcified head tissue was further cut into two pieces to prepare two distinct paraffin blocks: one for transverse sections and one for longitudinal sections. This allowed a better visualization and understanding of the morphology of the olfactory epithelium, maximizing the information got with only one fish. The tissues were put in labelled histological cassettes and prepared for paraffin embedding in an automated tissue processor (Leica TP1020, Leica®) by dehydration in an ascending series of ethanol (70%, 95% and 100%), cleared in xylene, xylene-paraffin (1:1, in volume) and embedded in low melting point paraffin wax Histosec (Merck; for details see appendix II). Paraffin blocks were prepared in a paraffin console (Miles Scientific). Serial 7 µm longitudinal and transverse sections were cut using a microtome (Leica RM2125T, Leica®) with disposable stainless-steel low-profile blades (MX35 Ultra low-profile, Thermo Scientific) and mounted on poly-L-lysine coated glass slide (appendix III).

2.3.2. Haematoxylin-eosin staining

Haematoxylin-eosin staining is a basic histological procedure that allows the morphological identification of cells and tissues. With this staining, negatively charged nuclei stain purple and the basic cytoplasm stains pink.

All staining procedures are performed with dewaxed and dehydrated tissue section. This is done by immersion of the sections for fifteen minutes in two xylene baths and then in a graded series of ethanol baths (100%, 95% and 70%) for five minutes each. The rehydration process is completed by a final immersion in distilled water for five minutes.

After hydration, every 5th slide of sectioned material was immersed in Harris haematoxylin solution (appendix II) for 5 minutes, blued in running tap water, rapidly rinsed in distilled water, immersed in an aqueous solution of eosin Y (appendix II) for 2 minutes and rinsed in distilled water with a few drops of acetic acid. To obtain definitive preparations, tissue sections were dehydrated through an ascending series of ethanol, cleared with xylene and mounted in DPX (Fluka, Sigma), as described in appendix III. Stained sections were analysed using a microscope (Leica DM2000) coupled to a digital camera (Leica DFC480) and linked to a computer for digital image analysis.

2.3.3. Masson's trichrome staining

This method relies upon to similar acid dyes to provide a differential visualization of tissue elements. The following trichrome staining was done according to the protocol described by Witten & Hall (2003). Deparaffinized sections were stained for 10 minutes with Mayer`s acid haematoxylin (Sigma-Aldrich), exposed to running tap water for 10 minutes and rinsed in distilled water. Sections were then stained with freshly prepared Xylidine Ponceau for 2 minutes (appendix I), rinsed in distilled water, treated for 4 minutes with 1% phosphomolybdic acid (appendix I), rinsed again and stained with light green for 90 seconds (appendix I). The excess dye was cleaned from the slides, which were then rapidly passed through an ascending ethanol series (50%, 70%, 90% and 100%), cleared and mounted in DPX (Sigma-Aldrich), as described in appendix III). With this staining procedure connective tissue is stained green and colloidal and mineralized structures (if present) are stained red. Stained sections were analysed using a microscope (Leica DM2000) coupled to a digital camera (Leica DFC480) and linked to a computer for digital image analysis.

2.3.4. Alcian Blue - Periodic Acid – Schiff Technique (PAS)

The following trichrome staining was done according to the protocol described by Mowry (1963). Sections were rinsed briefly in 3% aqueous acetic acid (appendix I), stained for 2 h in 1% Alcian Blue 8GX in 3% acetic acid (appendix I) and rinsed again briefly in water and then in 3% acetic acid, running water and distilled water. Sections were oxidised for 10 minutes in 1% aqueous periodic acid (appendix I) at room temperature, washed in running water for 5 minutes and immersed in Schiff`s Reagent (appendix I) for 10 minutes. The excess dye was cleaned from the slides in running water for 2 minutes, then the slides were rinsed in 3 changes of 0,5% sodium methabisulphite (appendix I), 1 minute each, and washed in running water for 5 minutes. Sections were dehydrated, cleared in K-Clear and mounted with DPX (Sigma-Aldrich). Stained sections were analysed using a microscope (Leica DM2000) coupled to a digital camera (Leica DFC480) and linked to a computer for digital image analysis.

This procedure stains periodate-unreactive, alcinophilic mucosubstances blue; periodate-reactive and alcianophilic components are bluish-purple and periodate-reactive, non-

alcianophilic components are red. Acid mucosubstances stained by this procedure include hyaluronic acid and sialomucins, and all but the most strongly acidic sulphated mucosubstances stain blue or bluish-purple.

2.4. Immunohistochemistry

2.4.1. Tissue sampling and processing for cryostat

For immunohistochemical analysis of the olfactory epithelium of the toadfish an animal (26.2 cm; 234 g) was sacrificed after being anesthetized with an excessive dosage of 2 - phenoxyethanol (5:10000, Sigma-Aldrich). The peduncles were dissected from the head and fixed in 4% PFA (appendix I) overnight at 4ºC. Fixed tissues were washed three times for 15 minutes in 0.1M PBS (appendix I) and cryoprotected by being incubated in an ascending series of sucrose solution (10%, 20% and 30%). The excess of sucrose was removed from the tissue by blotting on paper and the tissue was placed in the centre of a well filled mold with Tissue-Tek optimum cutting temperature (OTC; Sakura). Tissue blocks were freeze on dry ice and stored in the freezer -20°C. Serial 20 µm transverse sections were cut using a cryostat (Cryostar NX50, Thermo Scientific) with disposable stainless-steel low-profile blades (MX35 Ultra low-profile, Thermo Scientific) and mounted on poly-L-lysine coated glass slide (appendix III).

2.4.2. Immunohistochemistry techniques

Immunohistochemistry was carried out using a mouse monoclonal anti-G_{olf} antibody (sc-55545, Santa Cruz, Dallas, TX, U.S.A.). Tissues were washed twice with PBS-T 1x (PBS + Triton) for 5 minutes, treated with 3% of sheep serum in TCT (Tris buffer containing 0.7% carrageenan and 0.5% Triton X-100, pH 7.6) for 2 hours at room temperature, to block non-specific binding, and incubated with the primary antibody (1:50 dilution in the blocking solution; $G_{\alpha s/olf}$) at 4°C overnight. After washing twice with PBS-T 1x for 5 minutes, sections were incubated with the secondary antibody (1:400 in PBS 1x; Alexa Fluor 546-conjugated anti-mouse IgG; A-11030, Molecular Probes) for 2 hours. Control staining was performed using PBS in place of primary antibodies. Tissues were washed twice with PBS for 5 minutes and mounted in glycerol gelatine (Sigma-Aldrich, GG1).

2.5. Body fluids sampling

The collection of body fluids - bile, intestinal fluid and testicular accessory glands fluids from type I males was carried out in two periods, January (N= 6; mean ± s.e standard length = 23.1 ± 0.5cm; mass = 226.2 ± 13.1g) and May (N=7; mean ± s.e standard length = 26.7 ± 1.9cm; mass = 348.4 ± 85.8g), corresponding to non-reproductive season and reproductive season, respectively. The male morphotype type I was easily identified based on body size and secretion of their large accessory glands when gently pressed near the vent (Modesto and Canário 2003a).

Fish were sacrificed after being anesthetized with an excessive dosage of 2-phenoxyethanol (5:10000, Sigma-Aldrich). Accessory gland fluids were collected by gently slicing the anterior (AG) and posterior (PR) region of accessory glands, following by centrifuge and collection of supernatants. Bile fluids were obtained by slightly cutting the gallbladder, and intestinal fluid was collected by cutting the posterior extreme of the intestine, near anus, and forcing the content of the last 10 cm of intestine to a tube. Intestinal contents were centrifuged, and supernatant separated. Samples were pooled mixed thoroughly, centrifuged, aliquotted and frozen at -20°C until they were used in electro-olfactogram (EOG) procedures.

2.6. Electrophysiology

Different methods allow the evaluation of the olfactory sensitivity of fish; the technique used in the current work is the electro-olfactogram (EOG). The EOG is a direct current (DC) ‘field’ voltage measured from above the olfactory epithelium. It is assumed to be a summation of the generator potential of olfactory receptor neurons responding to a given stimulus (Scott & Scott-Johnson, 2002). EOG gives a negative wave that corresponds to a sink caused by positive ions, mainly sodium (Na^+) and calcium (Ca^{2+}), entering sensory neurones. The main advantages of the EOG are the large amplitude responses, it is relatively simple and straight-forward to carry out (usually requires little or no surgery to the fish) and the position of the electrode does not usually affect the relative amplitude of responses. The main disadvantages are the fact that the amplitude of response depends on the conductivity of the water. This means that EOG responses of marine fish are much smaller than those of freshwater fish.

Figure 2.1 EOG experiments were conducted in a Faraday cage to block electromagnetic fields.

The electrophysiology system is composed of a Faraday cage (figure 2.1), to isolate the system from external electric interference, an amplifier, a filter, and an analogue-digital converter. The raw signal is amplified, filtered, and converted into a digital signal before being visualised on a computer. During an experiment, a Lusitanian toadfish were anesthetized by immersion in water containing 2-phenoxyethanol (0.2 ml/L, Sigma Aldrich), followed by intraperitoneal injection of an intramuscular injection of the neuro-muscular blocker gallamine triethiodide (Sigma-Aldrich, Portugal; 10 mg/kg in 0.9% NaCl). Then, the anesthetized fish is placed on a cushioned support with a slight backward tilt; for toadfish a padded support, made in-house. A silicon tube is placed in the mouth, connected to a submersible pump in a reservoir of anaesthetic-containing, aerated seawater, and water is pumped over the gills (approximately 100 mL/100g body weight per minute; figure 2.2).

The tube of the stimulus-delivery system is positioned into the nostril, close to the olfactory epithelium. Contrary to many species of teleost, where peripheral olfactory organ is shown as a multi-lamellar structure lined by olfactory epithelium, in Lusitanian toadfish, the olfactory epithelium is constituted by a simple flat epithelium located in the base of the peduncle of the inhaling nostril and extending to the beginning of the olfactory chamber (see results). Thus, peduncle of the inhalant (anterior) nare was removed to gain access to the olfactory epithelium. The recording electrode was placed above the olfactory epithelium at a position that resulted in the largest response to the "standard" stimulus (10^{-3}M L-cysteine) and the reference electrode placed in non-neuronal tissue closed to the recording electrode. The stimulus delivery system allows the rapid switch from clean

background water to stimulus-containing water; when the valve is switched the water flow switches from background water to that containing the odorant, this starts the recording of the data. The signal was digitised (DigiData 1322A, Axon Instruments, Molecular Devices Corporation, Union City, CA, USA) and stored on Axoscope software (version 9.0, Axon Instruments).

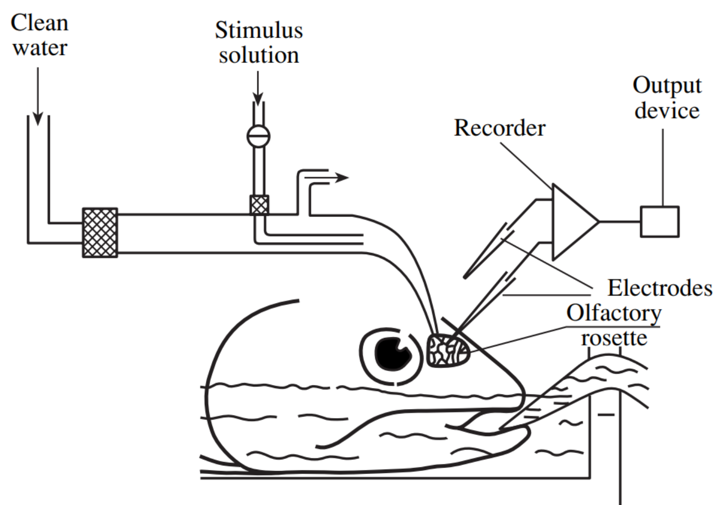

Figure 2.2 Setting for the recording of electro-olfactogram in fish (scheme from Kasumyan, 2004).

Aliquots of pooled samples of each body fluid from reproductive and non-reproductive male, L-cysteine and further solid phase extraction and high-performance liquid chromatography fractions were diluted in charcoal-filtered sea water (35 ppt) to be used in EOG. Besides, all dilutions were prepared on the day of experiment. Concentrations-response curves were obtained with dilutions of body fluids, 1:1,000 to 1:1000,000 and L-cysteine, 1:1,000 to 1:10,000,000. Dilutions of either fractionated pooled fluids were all equivalent to allow direct comparations.

For cross-adaptation, the background water superfusing the olfactory epithelium was replaced by an adapting solution (containing stimulus A) until the response declined and stabilized (2 min), and test stimulus (B) prepared with the adapting solution was tested (Hara, 2005; Laberge and Hara, 2004). The cross-adapted responses were calculated as a percentage of the control (un-adapted) response. The concentrations of the adapting and test stimuli were chosen, based on concentration/response curves, to give similar sized EOG responses (10^{-3} M).

2.7. Analytical Chemistry

2.7.1. Solid phase extraction (SPE)

Analytical chemistry was carried out according to a well-established method. The selected samples body fluids were initially fractionated by solid phase extraction (SPE) using reverse phase C18 cartridges. C18 cartridges feature a hydrophobic reversed phase material which is retentive for most nonpolar compounds and retains most organic analytes from aqueous matrices. Cartridges were first activated with 3mL methanol, followed by equilibration with 3mL water. After this procedure, samples were loaded. Compounds that pass thought the column were immediately collected and constituted the most hydrophilic fraction called filtrate. Compounds that were retained within the C18 cartridges were extracted with 3mL methanol and constitute the more hydrophobic fraction called eluate. Both fractions were stored at -20ºC. The olfactory potency of the eluate and filtrate of each body fluid were be accessed by eletro-olfactogram (EOG), selected active fractions were further fractionated by preparative high performance liquid chromatography.

2.7.2. High performance liquid chromatography (HPLC)

The intestinal fluid eluate was fractionated using a high-performance liquid chromatography (HPLC) system (Shimadzu Nexera series, Shimadzu Corporation, USA) consisting of a quaternary pump, a degassing device, an auto-sampling injector, an automatic sample collector, a column oven with a cooling device, to keep the column at 28 °C and a diode array detector scanning from 200 nm to 300 nm. The column used was an C18 column (Luna Omega, 3 µm, Polar C18). Prior to injection, samples, and all solvents, were vacuum filtered using 1.5 µm glass microfibres membrane filters (VWR, 696) to remove impurities. HPLC was carried according to a standardized methodology; samples were injected and loaded initially with 95% water/ 5% methanol for 10 minutes, followed by a linear gradient of water methanol, going from 5% methanol/ 95% water at time 10 minutes to 100% methanol/ 0% water at time 30 minutes. Samples were injected 1 time (200ul) each and fractions were collected every minute. The olfactory potency of the resulting fractions (figure 2.3) was tested by EOG and the most olfactory active were analysed by liquid chromatography with mass spectrometry (LC-MS).

2.7.3. Liquid chromatography with mass spectrometry (LC-MS)

LC-MS give information about the molecular weight of compounds present in the olfactory active fraction and its mass spectrum, this information and LC-MS available libraries aimed to identify at some of the isolated compounds. Samples were analysed by liquid chromatography coupled to high resolution mass spectrometry. The chromatographic separation was performed on a Thermo Scientific ultimate 3000 UHPLC. The column was a 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 10 % of B for 2 minutes. Then B increased linearly to 30 % in 2 minutes, then to 100% of B in 6 minutes and maintained at 100 % for additional 5 minutes. The mobile phase then returned to 10% of B in 1 min and then was maintained at 10 % of B for 4 minutes. The flow rate was 0.3 mL/min. The injection volume was 5 µl.

Mass analysis was performed on an Orbitrap Elite (Thermo Scientific) mass spectrometer with a Heated EletroSpray Ionization source (HESI-II). 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, in negative and positive polarities. LC-MS profiles were analysed using Compound Discoverer 3.3. The identified commercially available compounds were tested by EOG to access their olfactory activity.

2.8. Data and Statistical Analysis

The amplitude of the peak of the EOG was measured in millivolts. This was blank subtracted (amplitude of EOG in response to water treated in the same way as stimulus solutions but without the addition of odorant). The amplitudes of all stimuli were normalised to the amplitude of response to a 'standard' stimulus, 10^{-3} M L-cysteine, recorded from the same epithelium and similarly blank-subtracted. Standard and blank responses were recorded at regular intervals throughout the recording period. For each odorant only concentrations that elicited responses significantly greater than zero were

chosen. The data was log-transformed as previously described (Hubbard et al., 2003; Hubbard et al., 2011).

Statistical analysis was performed using SigmaPlot Version 14 (Systat Software, Inc.) statistical software. Concentration-response curves of the body fluids collected from reproductive and non-reproductive males were compared using two-way repeated-measures ANOVA with reproductive status and dilution as the two factors using a Fisher's LSD Post-Hoc test. Concentration-response curves of the commercially and stock available bile acids were compared using two-way repeated-measures ANOVA with bile acids and dilution as the two factors. Comparison between reproductive and non-reproductive responses from SPE and HPLC fractions were done through Student’s t tests for paired samples. At all stages of data analysis $P<0.05$ was considered statistically significant.

3. Results

3.1. Olfactory system characterization

The olfactory organ of *H. didactylus* consists of two nasal cavities symmetrically located in the dorsal region of the head rostral to the eye. Each nasal cavity consists of an anterior nostril (figure 3.1A, indicated by a white arrow), through which water enters the nose, and a posterior nostril (figure 3.1A, indicated by a yellow arrow), through which water exits the nose. The anterior nostrils consisted of a peduncle: tubular with a group of small finger-like projections at the distal end (figure 3.1A). Specifically, water enters the anterior nostril through an external lateral opening of the peduncle (figure 3.1A, indicated by a white arrow). The posterior nostrils are simple and rounded. The olfactory organs are isolated from the mouth.

Within the nasal cavity, the olfactory chamber, a tube-like elongated canal, can be discerned. The canal starts in the peduncle or anterior nostril. The sensory olfactory epithelium is in a thickened, plate-like area of the inner wall of the nasal tube-like cavity on the ventral side of the animal, starting at the base of the peduncle and prolonged along the canal (figure 3.1, indicated by Z3). Folds of the sensory epithelium that increase the surface area of the sensory tissue, known as lamellae, were not observed. There is a single olfactory accessory sac as an extension of the olfactory chamber (figure 3.1, indicated by Z6) and a valve situated in the posterior nostril in the form of two thin lips (Figure 3.1, indicated by VAL).

In the beginning of the peduncle (figure 3.1, indicated by Z1 and Z2) is lined by a stratified epithelium rich is mucous cells. The olfactory chamber is supported by a layer of connective tissue, the lamina propria (figures 3.2 and 3.3), with abundant blood vessels and olfactory nerve fibres that represent the axons from the sensory cells.

The sensory epithelium is easily identified as a columnar pseudostratified epithelium (figures 3.3 and 3.4), consists of olfactory sensory neurons, supporting cells and basal cells, with a continuous distributional pattern. The olfactory receptor neurons (figures 3.3 and 3.4) are bipolar cells with nervous terminations both in the apical (the dendrites), and basal (the axons) sides, have an elliptical nucleus and are present in high numbers between the supporting cells. They have nuclei stained strongly with haematoxylin, and their axon and dendric processes are stained faintly with eosin. In the sensory epithelium, the free border, dendrites, somata, and axons of receptors neurons showed immunoreactivity for

G_{olf} (figures 3.5 and 3.6). The supporting cells are cylindrical shaped-cells (figure 3.3) with the nucleus in apical position, and have a rounder nuclei than the olfactory sensory neurons. These cells provide mechanical and physiological support to the olfactory sensory cells and have a more faintly stained nuclei with haematoxylin than the sensory neurons. The basal cells are oriented horizontally or vertically in the basal part of the layer and have a round nucleus.

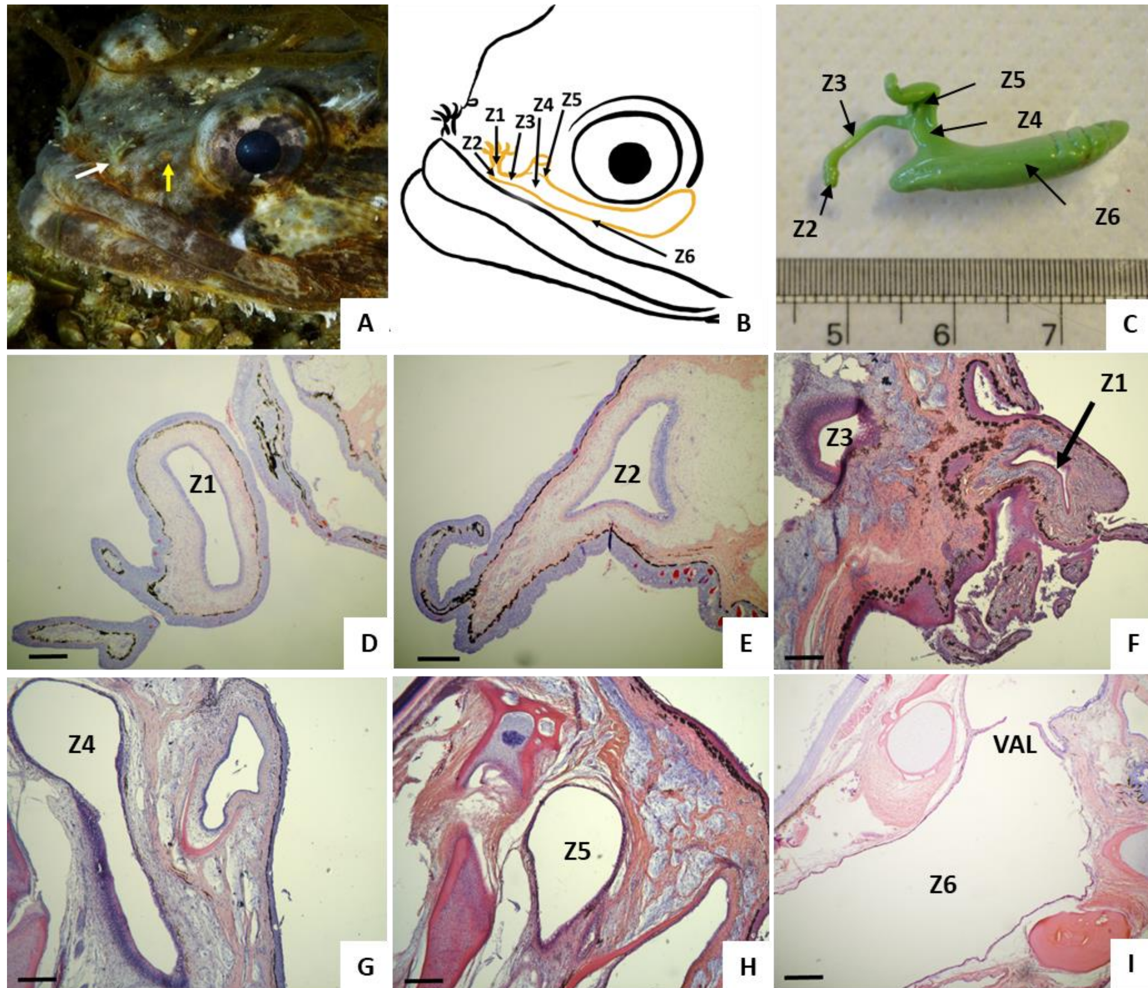

Figure 3.1 Structure of the olfactory organ: (A) *H. didactylus* photograph, white arrow anterior nostril / peduncle, yellow arrow posterior nostril (author João Pedro Silva); (B) Schem showing the location of the olfactory organ is represented in yellow; (C) Cast of the olfactory system; (D to I) haematoxylin–eosin stained histological section. Z1: anterior nostril, in the beginning of the peduncle; Z2: anterior nostril, in the base of the peduncle; Z3: olfactory chamber; Z4: transition between nostrils and accessory sac; Z5: posterior nostril; Z6: accessory sac; VAL: valves. Scales bars (D to I) indicate 300 µm.

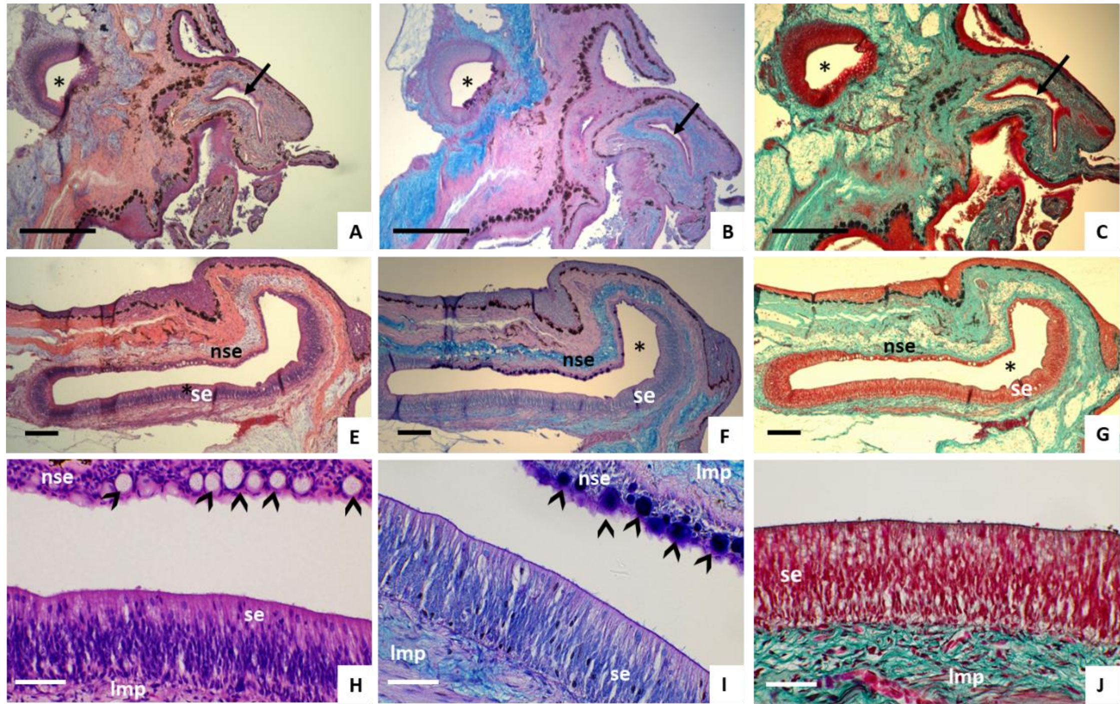

Figure 3.2 (A to C) Transversal sections of the pedunculated anterior nostril with the beginning of the canal indicated by an arrow (→) and olfactory chamber indicated by an asterisk (*) (E to G); Longitudinal sections of the olfactory chamber of *H. didactylus* showing non-sensory epithelium (nse) and sensory epithelium (se); stained with haematoxylin-eosin (A, E and H), Alcian Blue/PAS (B, F and I) and Masson's trichrome (C, G and J). Arrow heads indicate mucous cells. Scales bars indicate 500 µm (A to C) and 50 µm (E to J).

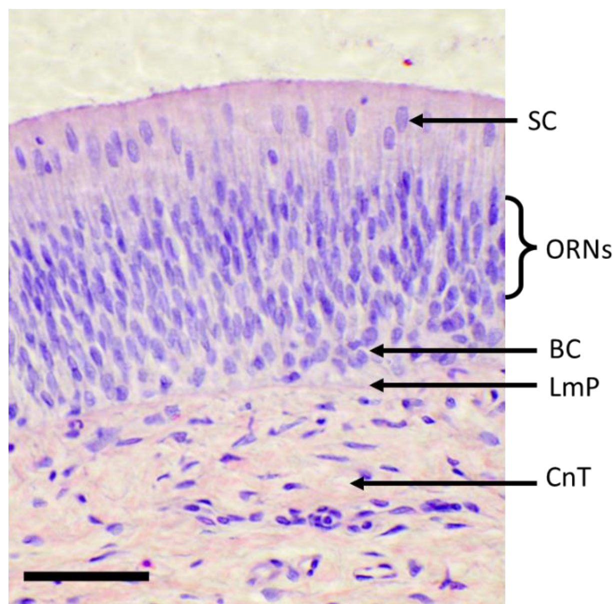

Figure 3.3 Hematoxylin-Eosin staining showing the different layers of the pseudostratified sensory epithelium, with supporting cells (SC), olfactory receptor neurons (ORNs), basal cells (BC), lamina propria (LmP) and connective tissue (CnT).

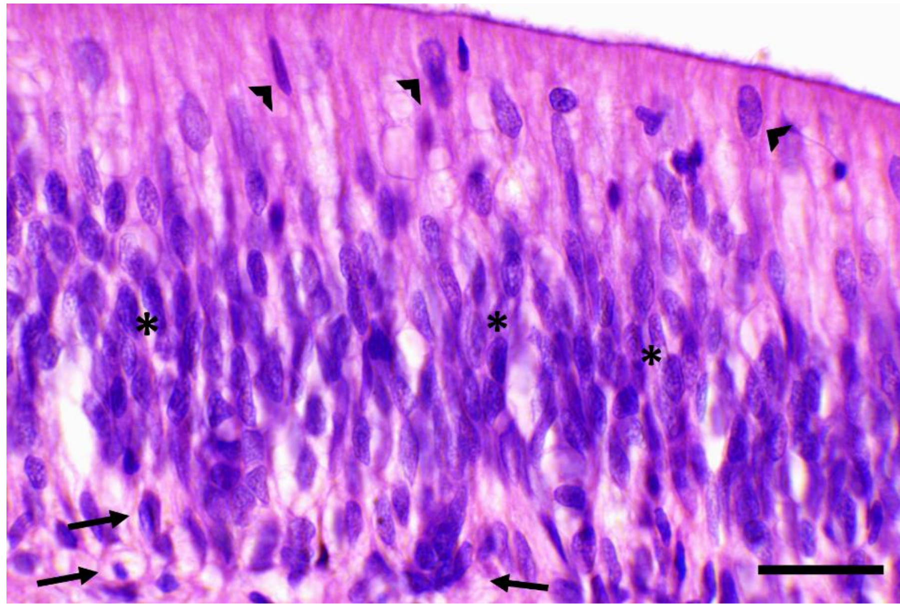

Figure 3.4 Histological characteristics of the sensory epithelium of *H. didactylus* stained with haematoxylin–eosin. The columnar pseudostratified epithelium has olfactory receptor neurons indicated by an asterisk (*), supporting cells indicated by arrow heads (>) and basal cells indicated by arrows (→). Scale bar indicates 20 µm.

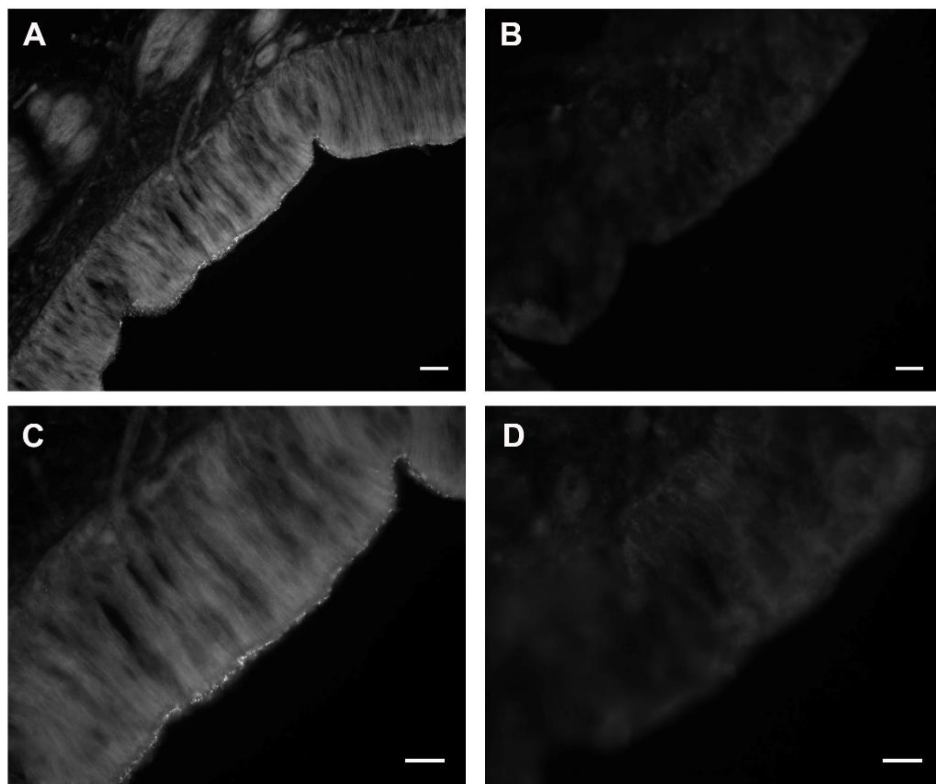

Figure 3.5 Presence and distribution of immunoreactivity for G_{olf} in sensory epithelium. (A and C) Immunostained epithelium, and (B and D) are the respective control. Scale bars indicate 20µm.

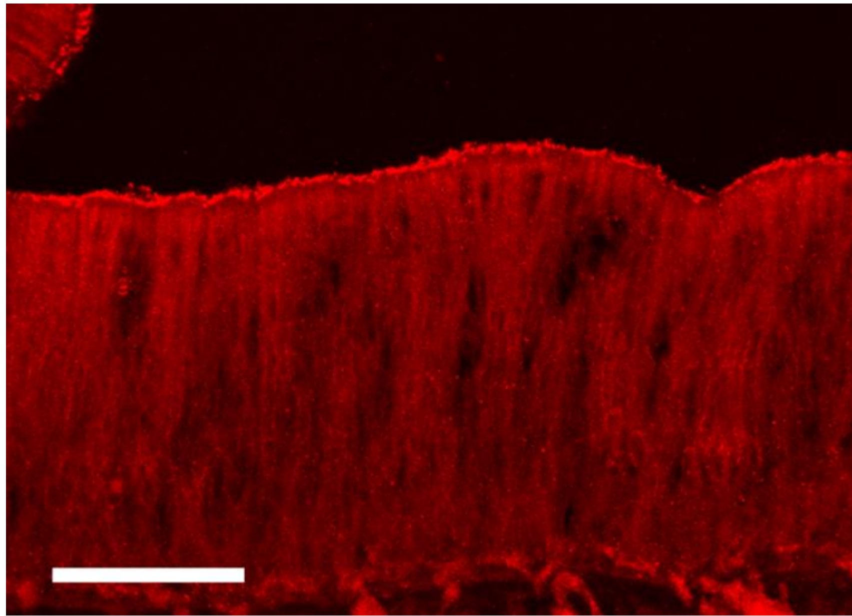

Figure 3.6 Immunoreactivity for G_{olf} in sensory epithelium. The free border, dendrites, somata, and axons of receptors neurons are immunopositive obtained with high sensitivity confocal imaging (Airyscan). Scale bars indicate 50 µm.

The non-sensory epithelium is a stratified squamous epithelium very rich in mucous cells. The roundish mucous cells are found in the outermost layer of the epithelium. These cells are positive to Alcian-blue/PAS and Masson's trichrome staining, but negative to haematoxylin-eosin (figure 3.2). In the olfactory accessory sac, the stratified squamous epithelium on its floor comprises only two types of cells: stratified epithelial cells and mucous cells; and consists of the 2 or 3 cells layer.

3.2. Olfactory sensitivity

The olfactory system of Lusitanian toadfish is highly sensitive to L-cysteine, giving large amplitude EOG responses typical of fishes; a rapid negative deflection upon the arrival of the stimulus at the olfactory epithelium followed by a period of adaptation and a return to baseline after the stimulus was removed (figure 3.7).

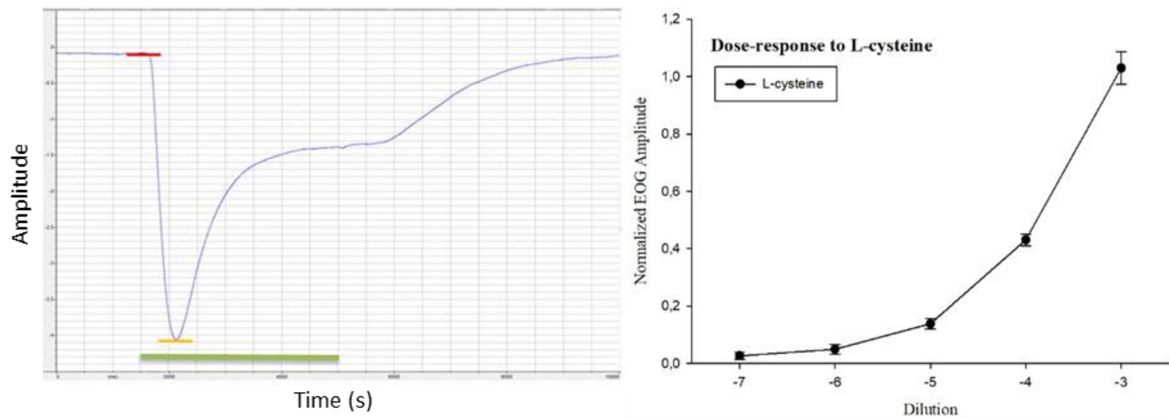

Figure 3.7 A. Typical EOG response to 10^{-3}M L-cysteine ('standard'). The baseline is red, and the peak line is yellow, the EOG amplitude being measured between them. The green line represents the period in which the stimulus is present in the nostril. B. Semi-logarithmic plot of normalised EOG amplitudes responses to L-cysteine. Data are shown as mean ±S.E.M. (n=9). Amplitude = V (amplified).

3.2.1. Olfactory responses to body fluids

The Lusitanian toadfish has olfactory sensitivity to all fluids tested. Semi-logarithmic plots of normalized EOG responses to different concentrations of the stimuli show that the amplitude of response was strongly concentration dependent in all cases, there is a progressive increase response to all stimuli along with increase of stimulus concentration, with no sign of saturation (figure 3.8). In the case of posterior testicular gland fluid and bile fluid, the concentration-response curves, there were not statistically differences between the state of reproduction; reproductive and non-reproductive posterior testicular gland fluid (figure 3.8, B) and bile fluid (figure 3.8, D) evoked statistically indistinguishable curves. However, there were significant differences in the potency of anterior testicular glands fluid (figure 3.8, A) and intestinal fluids (figure 3.8, C) from reproductive compared to non-reproductive fish.

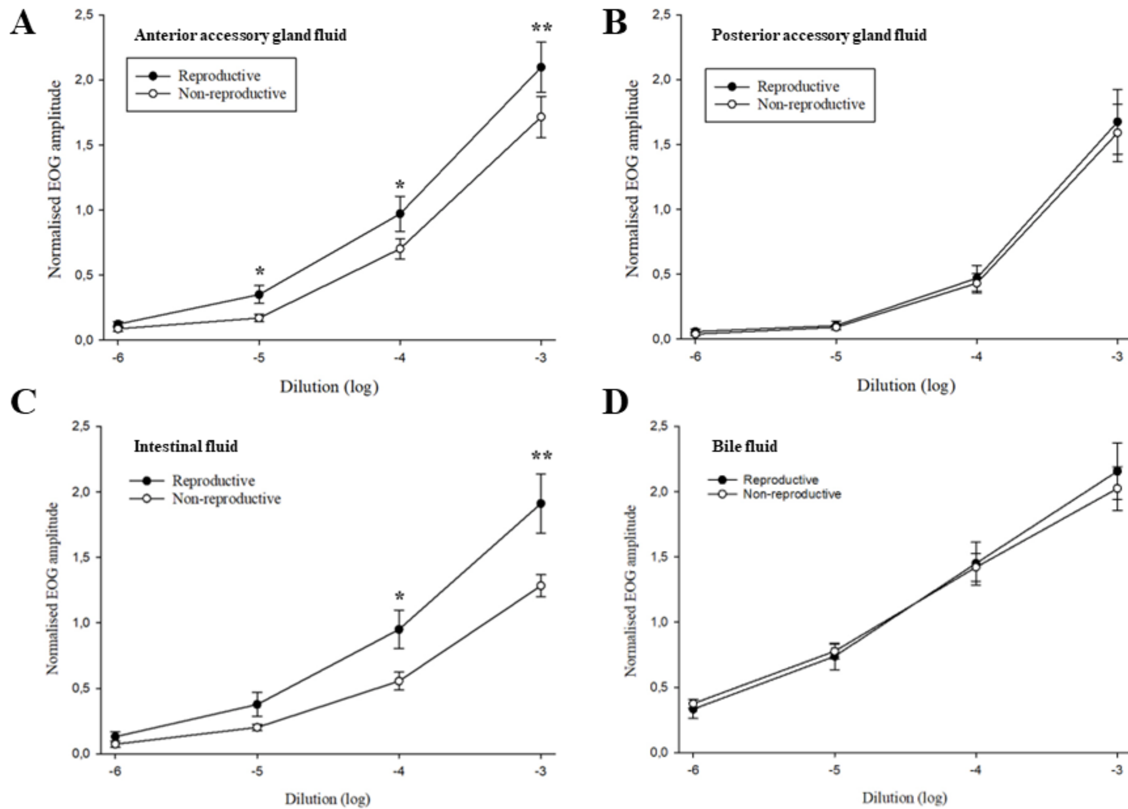

Figure 3.8 Semi-logarithmic plots of normalised amplitude of electro-olfactogram (EOG) responses to dilutions of pools of reproductive and non-reproductive male anterior (A) and posterior (B) testicular accessory glands, intestinal fluid (C) and bile fluid (D) recorded on Lusitanian toadfish. Data are shown as mean ±S.E.M. (n=6) normalised to the amplitude of response to 10^{-3}M L-cysteine. * $P<0.05$, ** $P<0.01$, Two-way RM ANOVA (Fisher LSD).

3.2.2. Olfactory responses to solid-phase fractions of intestinal fluids

After solid-phase extraction with C-18 cartridges, most of the olfactory activity from both reproductive and non-reproductive male intestinal and anterior testicular accessory gland fluids were contained in the eluate (figure 3.9, B and figure 3.10, respectively). Although there were no statistical differences between fractions, reproductive fraction contained a higher proportion of activity than that of non-reproductive male intestinal fluid. The C-18 filtrate of these samples contained correspondingly lower activity. No clear differences were seen between filtrate fractions of reproductive and non-reproductive of male intestinal fluid. In contrast, equivalent fractionation of the bile fluid showed that filtrate from bile fluid that contained slightly more activity than eluate for both reproductive and non-reproductive, but with no statistical difference. There was more olfactory activity in fluids from reproductive donors, similarly to intestinal fluid responses. In the case of the

anterior and posterior testicular glands, fluids from reproductive males were compared (figure 3.10). Most olfactory activity was significantly contained in the anterior testicular gland in all fractions. Besides, there was significantly more activity on eluate fraction than filtrate fraction of the anterior testicular gland (Student's t test for paired samples: p-value = 0,006).

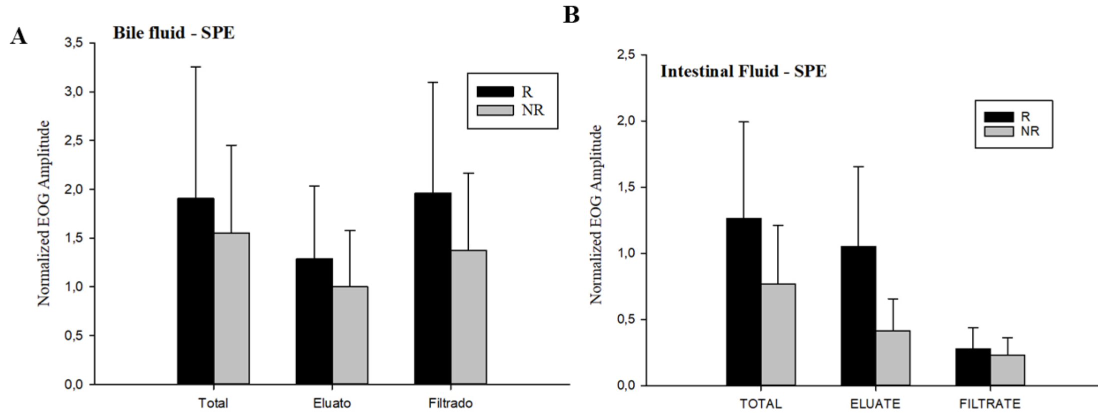

Figure 3.9 Mean + S.E.M. electro-olfactogram amplitudes recorded in *H. didactylus* (n=3) in response to fractions of solid-phase extraction of bile fluid (A) and intestinal fluid (B) from male conspecifics in reproduction (R) or non-reproduction (NR) periods. Amplitude of response normalized to 10^{-3}M L-cysteine.

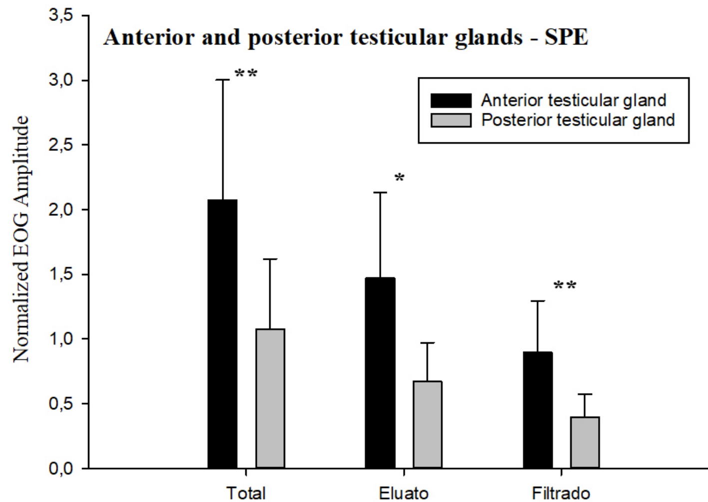

Figure 3.10 Mean + S.E.M. electro-olfactogram amplitudes recorded in *H. didactylus* in response to fractions of solid-phase extraction of anterior and posterior testicular glands. Data are shown as mean ±S.E.M. (n=5) normalised to the amplitude of response to 10^{-3}M L-cysteine. * P<0.05, ** P<0.01, Student's t tests for paired samples.

3.2.3. Olfactory responses to high performance liquid chromatography fractions of SPE eluate fraction of male intestinal fluids

EOG responses to high performance liquid chromatography fractions of SPE eluate (figure 3.11) showed that most of the olfactory activity of reproductive male intestinal fluids was contained between fraction 19 and 30, whilst most of the olfactory activity of non-reproductive males' intestinal fluids was contained between fraction 25 and 28, suggesting that these fractions contained most of the olfactory potency found in the total SPE eluent (figure 3.12). All fractions from reproductive males evoked higher EOG amplitudes than the respective fractions from non-reproductive males, particularly fractions 6, 12, 16, 18 to 24, 29 and 30 were significantly different. Specifically, no significant difference was found between the olfactory potency of the original SPE eluate and HPLC fraction 22 of reproductive males (Student's t tests for paired samples: p-value = 0,056).

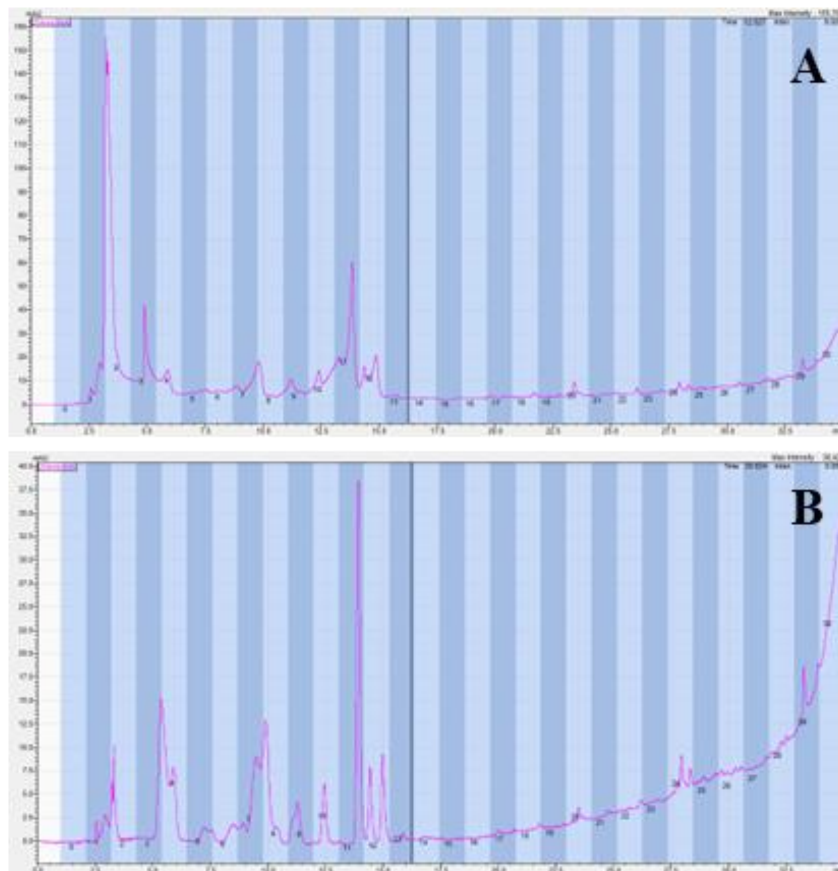

Figure 3.11 High-performance liquid chromatography (HPLC) chromatogram obtained of intestinal fluid eluate from reproductive (A) reproductive and (B) non-reproductive; blue columns represent collected fractions.

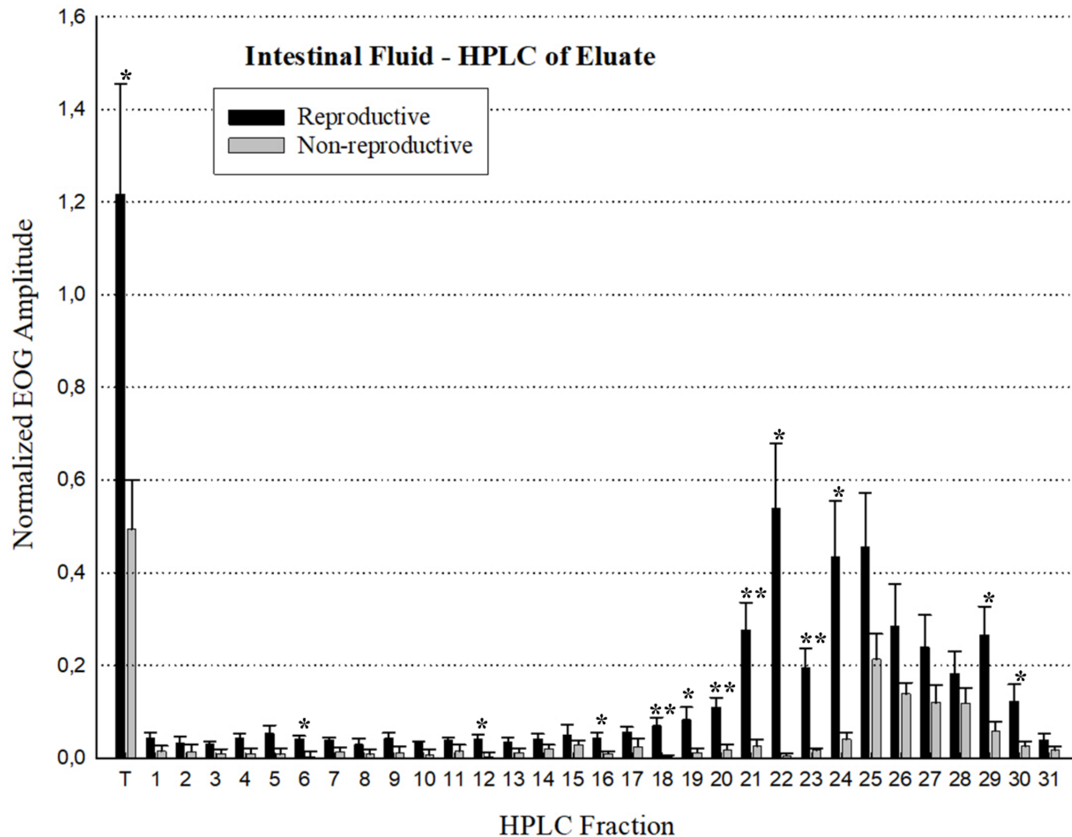

Figure 3.12 Olfactory responses of Lusitanian toadfish to reproductive and non-reproductive male intestinal fluid. Data are shown as mean ±S.E.M., n=6 for total eluate (T) and n=4 for HPLC fractions; normalised to the amplitude of response to 10^{-3}M L-cysteine. * P<0.05, ** P<0.01, Student's t tests for paired samples.

3.2.4. Identification of the main compounds in the intestinal fluid

HPLC fractions of C18 intestinal fluid eluate with most olfactory activity were analysed by liquid chromatography with mass spectrometry (LC-MS). LC-MS available libraries suggested the presence of some bile acids, taurolithocholic acid, cholic acid, taurochenodeoxycholic acid, in some fractions. A mixture of standards of these bile acids was prepared and analysed in LC-MS and chromatograms and mass spectrum compared to samples from HPLC fractioning. For reasons related to the availability of these bile acids in our laboratory and limitations of time for running this technique, our analysis focused on cholic acid. For this bile acid, results show the same retention time peak, 9.47 minutes (figure 3.13), and mass spectrum peak (m/z) 407.28 (figure 3.14), in both standard cholic solution and HPLC fraction 29.

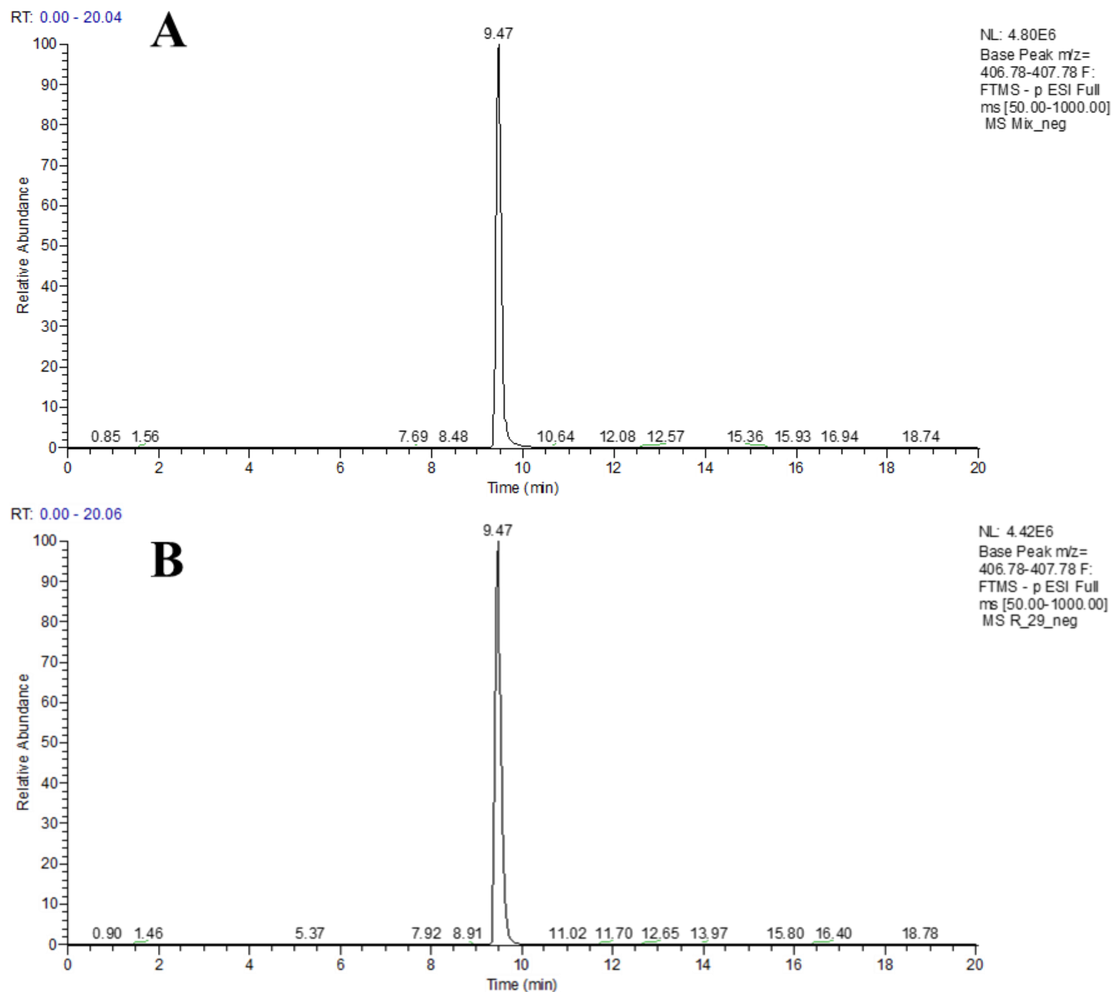

Figure 3.13 LC-MS chromatograms of (A) 'standard' cholic acid, and (B) HPLC fraction 29 of the C18 eluate of intestinal fluid, both with the same retention time (9.47 minutes)

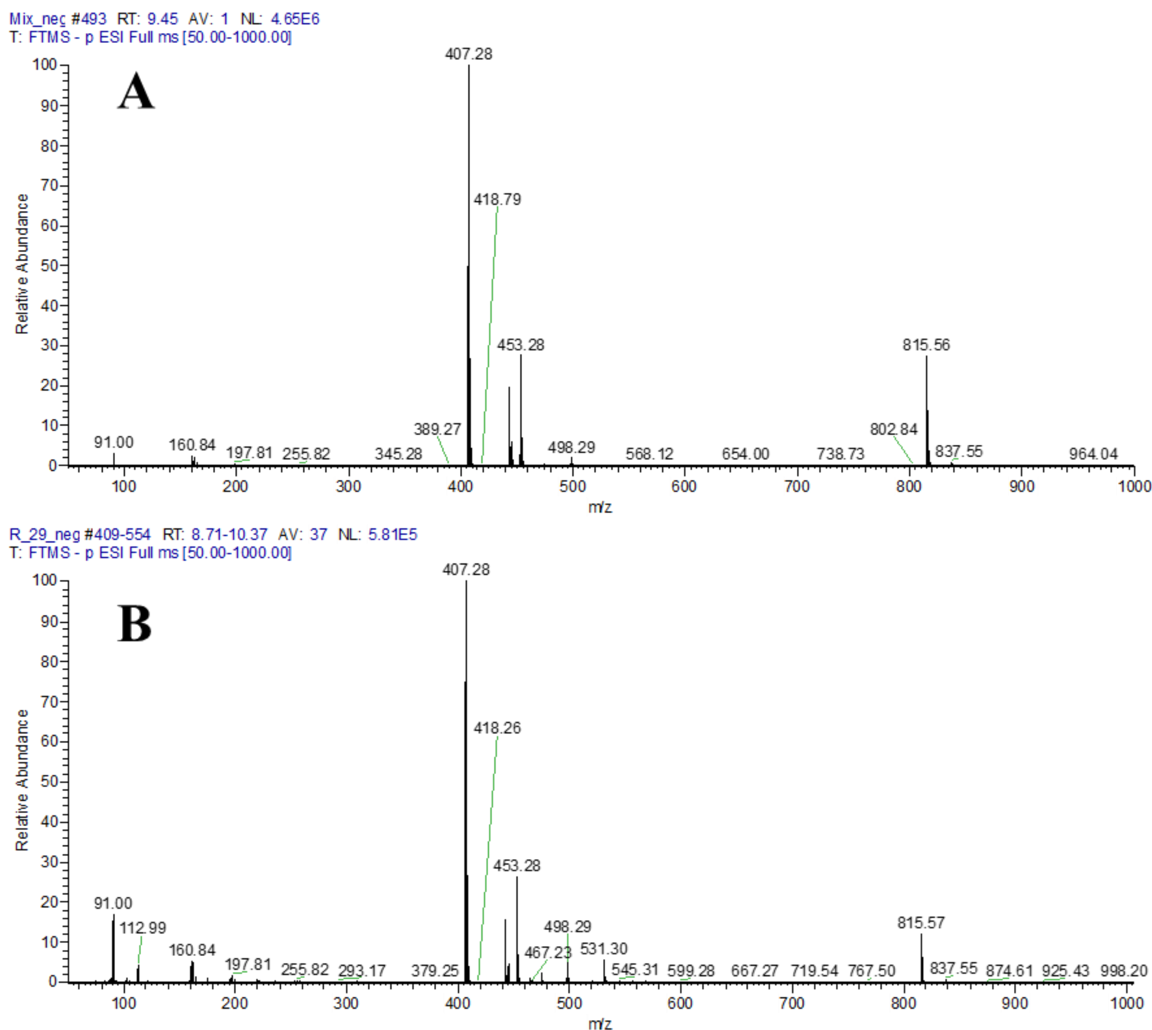

Figure 3.14 LC-MS mass spectrum of (A) 'standard' cholic acid, and (B) HPLC fraction 29 of the C18 eluate of intestinal fluid, both with a molecular peak of (m/z) 407.28.

3.2.5. Olfactory sensitivity to bile acids

The Lusitanian toadfish has olfactory sensitivity to all four bile acids tested (figure 3.15). However, semi-logarithmic plots of normalized EOG responses to different concentrations of the stimuli versus log (C) were not identical in shape for different bile acids. Taurochenodeoxycholic acid was the most potent stimuli at the highest concentration tested (10^{-5} M), although not statistically different, whilst taurolithocholic acid was statistically more potent than the other stimuli at lower concentration (10^{-9} to 10^{-7} M).

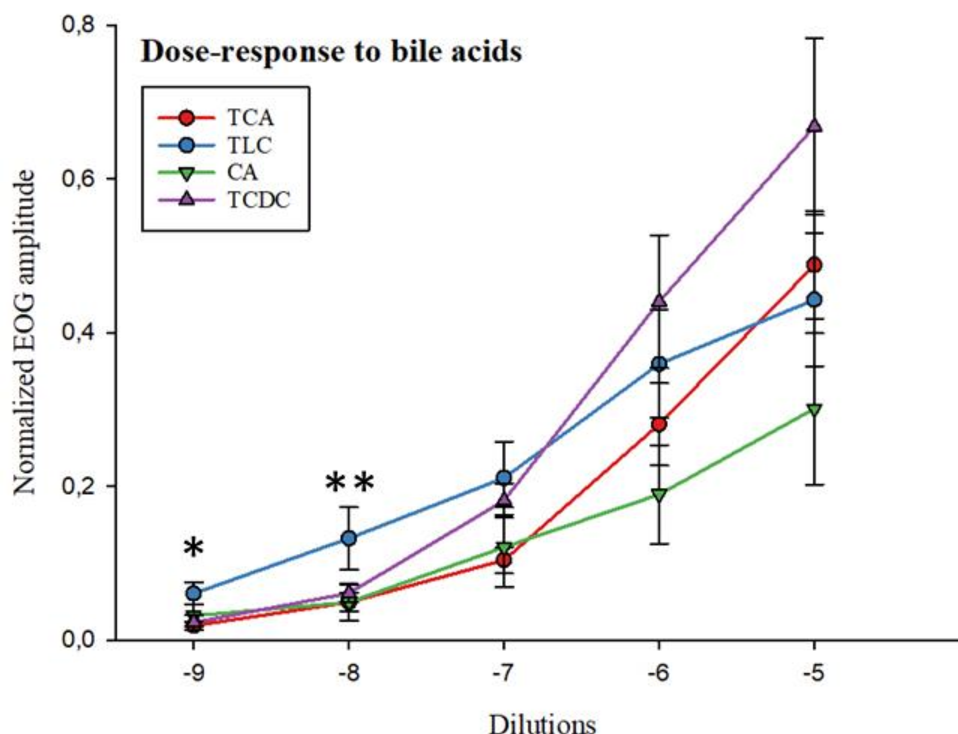

Figure 3.15 Olfactory responses of Lusitanian toadfish to different bile acids. Data are shown as mean ±S.E.M., n=6 for taurocholic acid (TCA) and n=4 for taurolithocholic acid (TLC), cholic acid (CA) and taurochenodeoxycholic acid (TCDC); normalised to the amplitude of response to 10^{-3}M L-cysteine. * $P<0.05$, ** $P<0.01$, Two-way RM ANOVA (Fisher LSD).

3.2.6. Cross-adaptation

Responses to HPLC fractions 21 to 25 from SPE eluate fraction of reproductive intestinal fluid in cholic acid (10^{-5} M)-adapted olfactory epithelium was 56% to 71% of the original response of these fractions (figure 3.16, A). In contrast, fractions 19, 20 and 26 to 30 from the same fluid against the original response of these fractions reduced the responses to 15% to 30%. A higher concentration of the adapting solution (10^{-4} M) was tested and evoked a total inhibition of fraction 19 and 20 and reduced the responses of fraction 26 to 30 to 1% to 8%. Responses to HPLC fractions 25 to 28 from SPE eluate fraction of non-reproductive intestinal fluid in cholic acid (10^{-5} M)-adapted olfactory epithelium was 23% to 41% (figure 3.16, B). A higher concentration of the adapting solution (10^{-4} M) was also tested and reduced the responses of the same fractions to 4% to 13%.

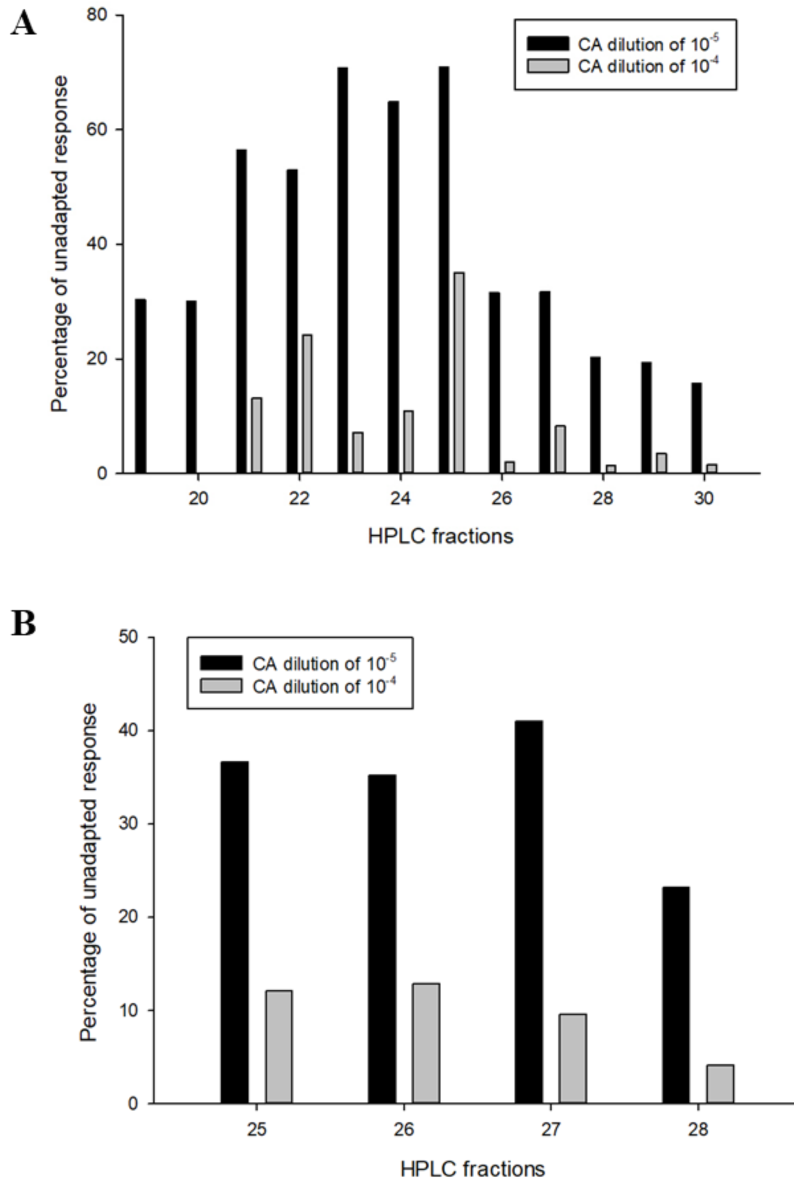

Figure 3.16 Relative EOG response to selected HPLC fractions, 19 to 30 of reproductive intestinal fluid (A) and 25 to 28 of non-reproductive intestinal fluid (B), as percentage of the initial response to these compounds during 10^{-5} M and 10^{-4} M adaptation to cholic acid.

4. Discussion

4.1. Morphological and histological characterization of the olfactory system

In both stereo and light microscopic levels, the structural and histological results on the olfactory organ of *H. didactylus*, are as follows: two nostrils, a single tube-like olfactory chamber without lamellae with a continuous distributional pattern of the sensory epithelium, several mucous cells, and a single accessory sac. In comparison with other teleost fishes, the gross olfactory structure of *H. didactylus* corresponds to the general pattern such as the dorsal position of two nostrils in the head, an olfactory chamber, and an accessory nasal sac (Kasumyan, 2004). However, based on the ecological habits by given species, the detailed structure on the olfactory organ differs significantly across specie. In the Lusitanian toadfish, the olfactory organ has an elongated tubular anterior nostril with a group of small finger-like projections, and a roundish posterior nostril that does not project. It is possible that the projected tubular anterior nostril facilitates suction of water into the olfactory canal in the sandy or muddy bottoms, since this specie is a sedentary species that is often partly buried in shallow water. However, a possible function of the finger-like projections remains unknown, further studies can be done to evaluate the possibility of some role on chemical detection.

In general, the olfactory chamber is somewhat oval or circular and contains a rosette structure that is frequently folded, forming olfactory lamellae (Hara, 1975). *H. didactylus* has an elongated olfactory canal tube, there are neither rosette structures nor lamella. The lack of the structure has been generally addressed to mudskippers of the subfamily Oxudercinae (Kuciel et al., 2011, 2013). For instance, *Periphthalmus barbarous* is characterized by the absence of an olfactory rosette, and the olfactory sensory epithelium is in a chamber-like sac of the nasal cavity (Kuciel et al., 2011). It appears to be closely related to the fish's ecology that has evolved to adapt to intertidal mudflats. Besides, the feeding habits of fishes are reflected on the structure and cellular organization of the olfactory organ (Hara, 1994).

The distribution of sensory and non-sensory areas in the olfactory epithelium is variable among teleost (Yamamoto, 1982). In this specie, the olfactory epithelium is largely divided into the sensory and non-sensory epithelium. The sensory neurons are found on the bottom of the olfactory chamber, as typically in other species of teleost (Hara, 1975), and can be characterized as a continuous type among the categories classified by Yamamoto & Ueda (1979). As observed in teleost fishes, the sensory epithelium is a

pseudostratified epithelium that contains the olfactory receptor neurons, supporting cells, and the basal cells, while the non-sensory epithelium with a stratified layer consists of epithelial cell and mucous cell (Hara, 1975; Kasumyan, 2004). The supporting cells provide a reservoir for the formation of supporting and olfactory receptor cells as they migrate to the upper part of the olfactory epithelium. In general, the types of olfactory receptor neurons are recognized as three distinct cells: ciliated, microvillous, and crypt neurons (Hamdani & Døving, 2007). Further studies should be done on identification of the type of olfactory neurons in this specie. Mucous cells are present only in the non-sensory epithelium and can be find in a great amount along the olfactory chamber and specially in the posterior nostril. The mucous film produced by these cells plays roles on the reduction of a friction force for water ventilation, cell protection against bacterial and physical particles, osmoregulation, and ion exchange (Shephard, 1994). Mucous cells probably help the smooth flow of water through the olfactory chamber by binding microscopic debris which is ejected through the posterior nostril. The mucus layer may help in ion trap, which obstructs the penetration of salts and heavy metals to underlying organs (Banerjee T.K., 1993). Furthermore, the mucin secreted over the lamellae probably forms a suitable medium for diffusion of odorants (Ghosh S.K & Chakrabarti P., 2014). In general, the mucous cell on the olfactory epithelium has been known not be tested positive to haematoxylin-eosin staining (Ghosh S.K & Chakrabarti P., 2014). In this study, mucous cells tested negative to haematoxylin-eosin staining and positive to Alcian-blue/PAS and Masson's trichrome staining, which strongly suggest the mucous cells are rich in glycoproteins.

In *H. didactylus*, one accessory nasal sac was identified at the end of the olfactory canal. Accessory sacs may be direct expansions of the main olfactory chamber (e.g. in the striped eel catfish, *Plotosus lineatus*; Theisen, 1991) or may be separate chambers connected to the olfactory chamber by short ducts (e.g. in the striped panchax; Zeiske, 1974). In the case of the Lusitanian toadfish, the accessory sac is a direct expansion of the tube-like olfactory chamber. The pumping of a nasal sac has been generally established to assist fish's water ventilation via the olfactory organ (Døving et al., 1977). In most teleostean fish that have a single accessory nasal sac, water may be actively drawn into the olfactory chamber, circulated inside, and expelled from the olfactory chamber by pumping action caused by expansion and compression of the accessory sac, mostly during ventilation and foraging (Døving et al., 1977; Nevitt, 1991). The occurrence of accessory

nasal sacs may be an anatomical evidence of sedentary bottom-dwelling nature (Sarkar et al., 2013). One of the factors causing the compression of the chamber-like sacs may also be the pressure induced by mouth opening, but confirmation of this assumption requires further studies. So, it might let the olfactory organ detect new odours from the outside and discharge remaining odours and residues in the olfactory chamber. Further, there was also noticed the presence of a valve in the form of two thin lips in the posterior nostril. This valve can ensure that water flows through the chamber in an unidirectional fashion (Cox, 2008).

4.2. Olfactory sensitivity

The current study shows that conspecific body fluids, specifically intestinal, bile, anterior and posterior testicular glands fluids are sources of potent odorants for the Lusitanian toadfish. There is evidence that the anterior testicular gland and intestinal fluids are related to reproduction status. Furthermore, bile acids were identified as a constituent of intestinal fluid, especially that of reproductive males.

The anterior testicular gland fluid of type I males may play a role in chemical communication during reproduction, as olfactory potency is significantly higher during the reproductive season compared to the non-reproductive season. Furthermore, these glands increase both in size and the amount of fluid produced during the reproductive period (Modesto & Canário, 2003b). Regardless of the breeding season, the anterior testicular glands fluid evoked a high olfactory response. In some fishes, it is known that accessory glands play a role in mate attraction by secreting steroid pheromones (Arbuckle et al., 2005; Hong et al., 2006; Jasra et al., 2007; Locatello et al., 2002; Resink, van den Hurk, et al., 1987; Serrano et al., 2008). In fact, Modesto et al. (2015) have already shown that testicular glands in *H. didactylus*, particularly in type I males, have the capacity to produce steroids (*in vitro*) that may eventually have functions in physiological and behavioural aspects (hypertrophy of sonic muscles, agonistic, territorial behaviours) or act as pheromones. In the black goby, guarder or type I males attract spawning females by releasing a steroid conjugate pheromone produced in one of their accessory glands, a mesorchial gland, which is highly developed in type I males (Colombo et al. 1980). In contrast, in black goby sneaker or type II males, mesorchial glands are reduced, producing low amounts of pheromones, and their ejaculates are pheromonally inconspicuous, thereby avoiding detection by type I males (Locatello et al., 2002). Thus, it is possible

that steroids produced by the anterior gland of *H. didactylus* function as pheromones. In other fishes, the accessory glands can also play a role in parental care by producing antimicrobial compounds that reduce infection-caused offspring mortality (Giacomello et al., 2006). In contrast, the posterior testicular gland evoked high olfactory responses but did not show any significant differences between reproduction seasons. In this context, the role of the posterior testicular gland is unclear. In future studies, behavioural experimentation and identification of the compounds involved in such high olfactory sensitivity are now required to further identify the Lusitanian toadfish accessory gland function.

Intestinal and bile fluids were the stimuli that evoked the highest EOG response amplitudes. Furthermore, the difference in potency of the intestinal fluid from males of different reproduction status suggests that they may convey information of the reproduction status of the donor. This is consistent with other studies, for instance, in female Mozambican tilapia, the olfactory potency of intestinal fluids also depends on the reproductive state (Miranda et al., 2005). It is known that fishes in general have high olfactory sensitivity to bile acids (e.g., P. C. Hubbard et al., 2017) and bile fluid is a concentrated source of bile acids (25–50 mM) where the concentration may reach 300–400 mM after starvation (Grosell & Jensen, 2000). Therefore, the higher potency of bile fluid suggests that bile acids (or other odorants contained in the bile fluid) may contribute to the higher potency of intestinal fluid. Furthermore, the use of bile acids to indicate reproduction status has already been suggested by Huertas et al., (2010) in the case of eels. However, in this study, EOG responses to bile fluid did not show significant differences between reproduction and non-reproduction seasons. One hypothesis is bile fluid would have been diluted during its transit down the intestine, bile salts modified by bacterial action and most bile acids resorbed (Hofmann, 1999), so it is likely that other odorants are also involved. In the case of *Solea senegalensis*, only part of the potency of the intestinal fluids is due to the presence of bile acids (Velez et al., 2009). This may also explain why most of the olfactory activity in the intestinal fluid was retained by the C-18 cartridges, but not in bile fluid. Differences between the two seasons may be also related to changes in diet throughout the year. Moreover, for the population of the Mira estuary of the Lusitanian toadfish, the diet is mainly based on shrimp and mysids in the reproductive season and on fish in the non-breeding season (Costa, 2004). Feeding can

thus intensify chemical signals from both intestinal fluid and bile in the reproductive season.

Due to time limitations and the complexity of the procedure, it was only possible to isolate and identify some of the active components of one body fluid. Two factors were considered to choose the body fluid. Firstly, unlike bile fluid, intestinal fluid is released directly into the environment. Marine teleost, in general, drink as part of their osmoregulatory adaptation to a hyperosmotic environment (Marshall & Grosell, 2005). Therefore, there is a constant excretion of watery fluid from the rectum of marine fish (Wilson et al., 2002), even if they do not feed. Thus, intestinal fluid can be an important route of release for pheromones in marine fishes in general. Secondly, intestinal fluid had greater significant differences in responses between reproductive and non-reproductive, in compassion with other body fluids in this study. LC-MS available libraries suggested that the potent odorants contained in the intestinal fluid are cholic acid, taurolithocholic acid and taurochenodeoxycholic acid. Those results were further confirmed by comparison of mass chromatograms and retention time of standard samples. Indeed, in teleost, the principal identified bile acids are sulphated bile alcohol, mainly 5-cyprinol and 5-chimaerol; C24 bile acids, mainly cholic acid, chenodeoxycholic acid, deoxycholic acid, and haemulcholic acid (Goto et al., 1996; Haslewood, 1967). Olfactory sensitivity to bile acids have been reported in several other species (e.g., Velez et al., 2009a) Cross adaptation reinforced those results, however, evoked nearly total response inhibition, which may be provoked by other molecules in the intestinal fluid with the same receptor as those identified.

Further studies should also focus on the quantity released by these odorants to identify if those bile acids are released in sufficient quantities to be detected by conspecifics and/or potential predators and prey (Velez et al., 2009). Besides, hormonally derived pheromones and bile acids are limited in physiological functions and are conserved throughout vertebrates, so if toadfish are producing these types of pheromones, it may be a specific mix to make their signal only detectable by conspecifics (Stacey, 2015). It would be also important study behavioural effects of these odorants to better understand their role. The results of this study strongly suggest that the Lusitanian toadfish may be using a combination of chemical along with auditory signals during the reproductive process, and possibly related to the male's alternative reproductive strategies. Such multimodal signalling would not be unusual (Starnberger et al., 2014; Still et al., 2019;

Woodley, 2014) and would provide potentially important additional information about the animal's social/reprodutive in an environment where vision cannot be employed (Frommen, 2020).

5. Final Considerations

This study constitutes the first scientific contribution to the complete characterization of the olfactory organ of *H. didactylus* and to support the hypothesis that this species communicates by chemical signals, in addition to sound.

The olfactory organ of the Lusitanian toadfish is constituted by two nostrils, a single tube-like olfactory chamber and an accessory sac that may be used to pumping the water into the nasal cavity. The sensory epithelium can be found layering the olfactory chamber, and have a continuous distributional pattern, not associated to olfactory lamellae. The presence of a layer of olfactory epithelium coupled with an accessory sac is found in other benthic species and is probable an adaption to the environment.

This work indicates that Lusitanian toadfish has high olfactory sensitivity to accessory testicular glands, intestinal and bile fluids. However, the anterior accessory gland and intestinal fluid evoked significant higher responses to fluids from reproductive donors compared to non-reproductive. This points to a possible important role of these fluids in chemical communication during the reproductive period.

The bile acids identified in the intestinal fluids from reproductive donors are good candidates to explain the effects of olfactory sensibility to these fluids. However, considering the significant differences found in olfactory responses to anterior accessory gland fluid between reproductive and non-reproductive donors, these glands may also release important odorants, in addition to their other multiple functions in reproduction.

Further studies are needed for a complete characterization of the odorants present in these fluids and their physiological and behavioural (pheromonal) effects on conspecifics.

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Annexes

Appendix I – solutions used in the protocols

Ethylenediaminetetraacetic acid (EDTA) 0.5M and 0.2M pH 8

Preparation of 100 ml solution:
7.6 g (0.2M) $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$
19 g (0.5M) $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$

Dissolve the desired amount of EDTA in 90 ml of distilled water and adjust the pH to 8 with NaOH. Check the pH until complete dissolution and adjust the final volume to 100 ml. Autoclave for 20 minutes at 121ºC. Store at room temperature.

1M Phosphate Buffer Saline (PBS) pH 7.4, for the preparation of PFA solutions

Preparation of 100 ml solution:
4.68 g Sodium hydrogen phosphate, dehydrated ($NaH_2PO_4.2H_2O$)
12.46 g Disodium hydrogen phosphate, dehydrated ($Na_2HPO_4.2H_2O$)

Dissolve in 90 ml of distilled water. Check the pH to 7.4 and make the volume up to 100 ml. Autoclave for 20 minutes at 121ºC. Store at room temperature.

Paraformaldehyde (PFA) 4% pH 7.4, for tissue fixation

Preparation of 1L solution: 40 g PFA (Sigma-Aldrich) Sodium Hydroxide (NaOH) 1M PBS pH 7.4

Dissolve the PFA in 900 ml MilliQ water; add 100 µL of NaOH and heat at 65ºC until complete dissolution. Let it cool to room temperature and add 100 ml of sterile 1M PBS pH 7.4. Keep at 4ºC up to two weeks.

Eosin Y 1% aqueous solution

Preparation of 100 ml solution:
Dissolve 1g Eosin Y (Sigma-Aldrich) in 100 ml of double-distilled water and store until use.

Harris haematoxylin solution

Preparation of 100 ml solution:
1 g Haematoxylin
10 g Aluminium potassium sulphate
0,25 g Mercury oxyde
4 ml Glacial acetic acid
5 ml Absolute ethanol

Dissolve the hematoxylin in absolute ethanol. Dissolve the aluminium sulphate in 100 ml of warm distilled water. Combine the solutions and boil for 4 minutes, remove from the heat and add the mercury oxide, mix well and boil until the dye becomes a dark purple color. Cool the solution rapidly under running water, add the glacial acetic acid and filter the solution. Immediately before using dilute 50:50 in absolute ethanol and filter the resulting solution. Store the stock solution in the dark at room temperature.

Light green dye solution

Preparation of 100 ml solution:
0.2 g Citric acid
10 ml distilled water
0.2 g Light green

Prepare a solution of 2% citric acid by dissolving it in 10 ml distilled water. Add the light green and mix until complete dissolution. Store the stock solution in the dark at room temperature. To obtain the working solution of 2% light green, dilute 1:10 in distilled water immediately before use.

Xylidine Ponceau solution

Preparation of 100 ml solution:
0.25 g Xylidine ponceau 2R (Sigma-Aldrich)
0.25 g Acid Fucsin (Sigma-Aldrich)
100 ml 1% acetic acid (1ml Glacial acetic acid + 99 ml distilled water)

Dissolve the xylidine ponceau 2R in 50 ml of 1% acetic acid. Dissolve the acid fucsin in 50 ml of 1% acetic acid. Store the stock solution in the dark at room temperature. Just before use, mix the two solutions 1:1 to obtain the working solution for the stain.

1% Phosphomolibdic acid

Preparation of 100 ml solution:
Dissolve 1 g of phosphomolybdic acid in 100 ml of distilled water. Store at room temperature.

1% Alcian Blue 8GX in 3% acetic acid

Preparation of 50 ml solution:
Dissolve 0,5 g Alcian Blue 8GX in 50 ml of 3% acetic acid. Agitate until complete dissolution. Store in the dark at room temperature.

1% Aqueous Periodic Acid

Preparation of 50 ml solution:
Dissolve 0,5 g Periodic Acid in 50 ml of Elix water. Stir until complete dissolution. Store in the dark at room temperature.

Schiff`s Reagent (from Tomasi, 1936)

Preparation of 50 ml solution:
Dissolve 1 g Basic Fuchsin in 200 ml of boiling distilled water. Agitate for 5 min, let it cool to exactly 50 ºC, filter and add to the filtered 20 ml N-HCl. Let it cool to 25 ºC and add 1 g Sodium Methabisulphite ($Na_2S_2O_5$). Keep in the dark for 14h to 24 h. Add 2 g charcoal and agitate for 1 minute and filter. Store the reagent in the dark, between 0 ºC and 4ºC. Let Shiff`s Reagent reach room temperature before use.

0,5% Sodium Methabisulphite

Preparation of 150 ml solution:
Dissolve 0,025 g Sodium Methabissulphite in 150 ml Elix water. Agitate until complete dissolution.

Appendix II – Paraffin embedding and slides coating

Paraffin embedding

Tissue samples were placed in appropriate histological cassettes and immersed in 70% ethanol in an automatic tissue processor (Leica TP 1020; table 1).

Table 1. Program for automated dehydration, clearing and paraffin (Low melting point 56- 58ºC, Histosec, Merck) embedding was set up following the instrument instructions and consisted of:

Solution	Treatment	Times (minutes)
70% Ethanol	Dehydration	10
95% Ethanol	Dehydration	30
95% Ethanol	Dehydration	30
100% Ethanol	Dehydration	60
100% Ethanol	Dehydration	60
100% Ethanol: Xylene (1:1)	Clear	60
Xylene	Clear	60
Xylene	Clear	90
Xylene: Paraffin (1:1)	Infiltration	120
Paraffin	Infiltration	120

Once the program finished paraffin blocks were prepared with the processed material using moulds of appropriate size.

Coating of slides with Poly-L-lysine (Sigma-Aldrich) for mounting sections

Histological glass slides (Normax) were coated with poly-L-Lysine (P8920, Sigma-Aldrich), which is nonspecific attachment factor for cells that promotes cell adhesion to solid substrates by enhancing electrostatic interaction between negatively charged ions of the cell membrane and the solid surface (Sigma-Aldrich).

Glass slides were washed for 5 minutes by immersion in a 1% acid/alcohol solution (1% Hydrochloric acid, 70% ethanol, 29% distilled water, v/v) and then rinsed in running tap water for 1 – 2 minutes before being briefly immersed in distilled water for 1 minute. Glass slides were finally immersed in a 0.01% aqueous solution of poly-L-Lysine for 5 minutes and then allowed to dry at 37ºC overnight.

Appendix III – Tissue sections dehydration and clearing to obtain definitive preparations

Tissue sections dehydration and clearing to obtain definitive preparations

The procedure for the preparation of definitive preparations is the same for histological stains, histochemistry, and immunohistochemistry. On completion of staining of sectioned material, definitive preparations were prepared by dehydration, clearing (table 2), mounting with DPX (Sigma-Aldrich) and covering with a glass coverslip.

Table 2. Dehydration, clearing series to obtain definitive preparations:

Solution	Treatment	Time (minutes)
70% Ethanol	Dehydration	5
95% Ethanol	Dehydration	5
100% Ethanol	Dehydration	5
Xylene I	Clearing	10
Xylene II	Clearing	10