Zélia Cristina Pereira Velez

Neural Mechanisms of olfactory discrimination in the Senegalese sole, *Solea senegalensis* Kaup, 1858

Orientadores:
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Doutor Joerg Hardege (Senior Lecturer, University of Hull, U.K.)

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Universidade de Évora 2008

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The work included in this thesis was funded by

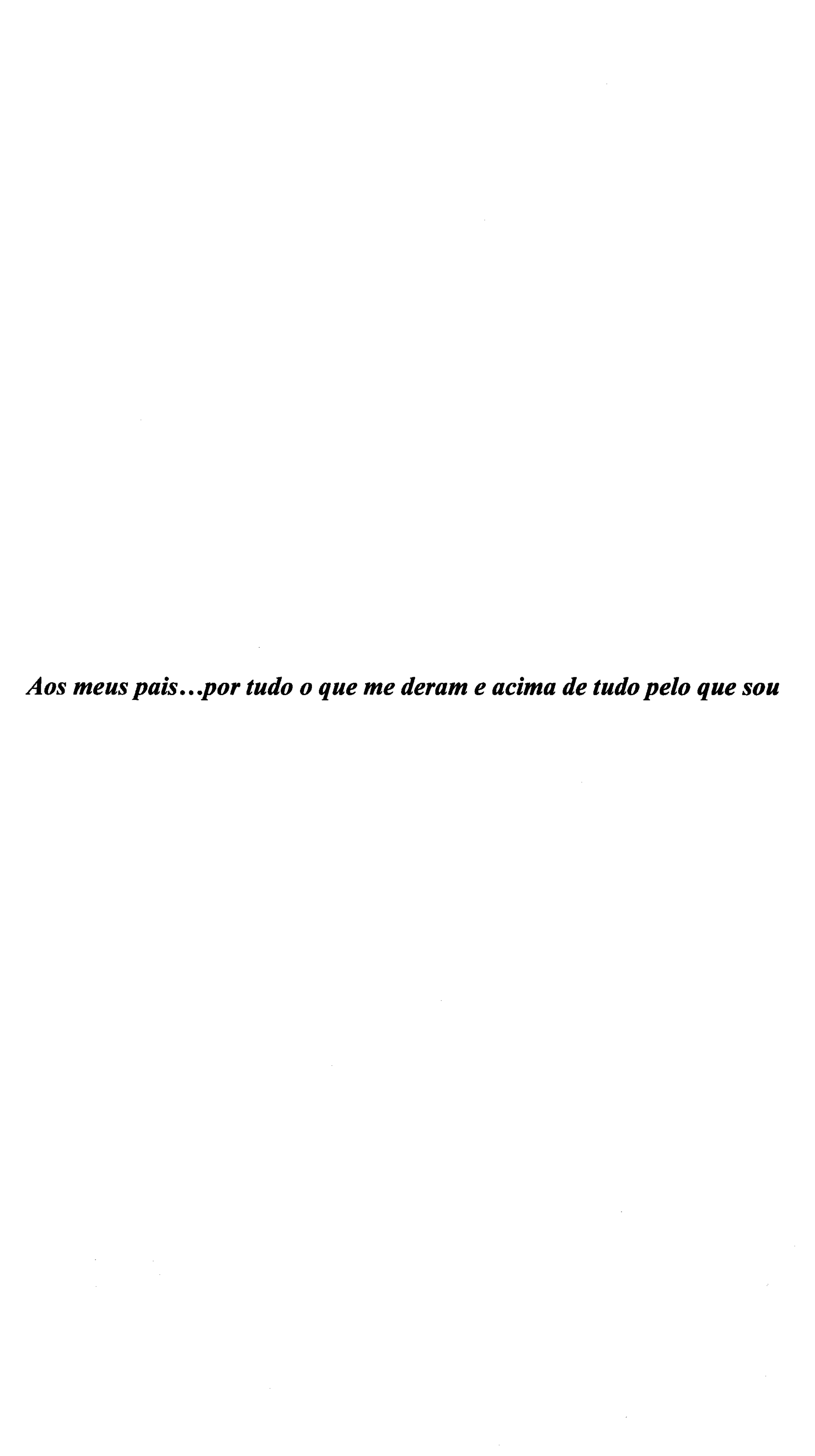
Ph.D fellowship SFRH/BD/16242/2004

The function of olfaction in feeding behaviour of Solea senegalensis,

POCT/CVT/38831/2001

Mechanisms of olfactory sensitivity to inorganic cations in teleosts

POCI/BIA-BMC/55467/2004



Aos meus pais...por tudo o que me deram e acima de tudo pelo que sou

Acknowledgements / Agradecimentos

Depois de tantos meses de escrita vou terminar com aquela que supostamente deveria ser a parte mais fácil, os agradecimentos. A verdade é que estive horas a olhar para esta página em branco na esperança que aflorassem as primeiras palavras. No entanto todas as abordagens me parecem banais e pouco elucidativas da emoção deste momento.

Ver terminada esta obra significa a concretização de um sonho e o edificar de novas metas. Confesso que olho o futuro de forma bem mais receosa do que há quatro anos atrás, quando tudo parecia tão fácil de atingir! Hoje sei, que tudo na vida exige grande esforço e persistência. Estes anos de Doutoramento constituíram uma lição de vida em que cresci como pessoa e como profissional.

Fazer uma retrospectiva de tudo o que vivi significa experimentar um misto de sensações únicas. Os jantares de convívio, as noites de copos, os congressos e os momentos de boa disposição passados no laboratório em plena hora laboral, constituem memórias que nunca esquecerei e espero repetir. No entanto, muitas foram também as horas de dúvidas, desespero e angustia, em que pensei não ter mais força para continuar. Estou certa, que chegar aqui só foi possível graças a uma imensidão de pessoas que com a sua amizade inigualável me motivaram e obrigaram a seguir em frente. A todos eles agradeço do fundo do coração.

Durante estes quatro anos muitos foram aqueles que entraram e saíram da minha vida, alguns deles talvez nunca mais voltarei a encontrar. A todos os amigos que fiz durante as muitas viagens em especial aqueles que me ajudaram a sobreviver durante as minhas estadias em Hull, o meu obrigado.

Isabelita, Pau, Thorid, Ralf, Domino, Steve, Joerg, Helga, Melissa, Íris, Victor, Thomas and his family and all my friends from the University of Hull, tanks for all your friendship and for being my family when I was alone.

Aos meus orientadores Professor Adelino Canário, Doutor Eduardo Barata, Doutor Peter Hubbard e Doutor Jörg Hardege agradeço toda a disponibilidade e ajudas preciosas nos momentos difíceis.

Ao Professor Adelino quero agradecer por, apesar de sempre muito ocupado, ter estado sempre disponível nos momentos em que mais precisei.

Eduardo...apesar de muitos momentos difíceis...muitos foram também aqueles que me deixaram boas recordações. Parece que conseguimos acabar isto juntos (vitória)!

Peter, obrigado pela tua amizade, pelo apoio e acima de tudo por tudo o que me ensinas-te.

Joerg tanks for your constant smile ... one day you will teach me how to play golf! I am a bit tired of chemistry.

A todos os meus colegas que no laboratório, de uma forma ou de outra contribuíram para que este trabalho fosse possível o meu muito obrigado. Trabalhar convosco foi uma experiência única. Durante estes anos muitos foram aqueles que adquiriram o estatuto de amigos, de certo que me vou esquecer de alguns...Rui Serrano, Olinda Almeida, Sofia Engrola, Sandra Soares, Adriana Silva, Mar Huertas, Pedro Frade, António Miranda, Marco, Bruno, Nádia, Laurence, Francois, Patrícia, Rita, Ângela, João Quintela, João Sendão e tantos outros...

A todos os meus amigos de Albufeira, por me aturarem desde pequenina e por acreditarem tanto em mim. Obrigado por me fazerem sempre lembrar que existe uma vida fora do meu laboratório. Obrigado pelas loucuras de Sábado à noite que tanto me ajudaram a manter a alegria nos momentos mais difíceis. Sem querer esquecer ninguém:

Ana (Pita), Vanessa Vieira, Mónica Bizarro, Jammie, Margarida Corado, João Duque, Carla Bento, Margarete Bento, Cláudia Bento, Joana Bishop, Joana Santos, Paulinho Santos, Dinis e Vera Almeida e a tantos outros que têm um lugar bem especial no meu coração.

Não posso deixar de agradecer a todo o staff do Mitto Bar por me proporcionarem os melhores momentos de lazer, que tanto me ajudarem a desanuviar do stress da semana.

A toda a minha família obrigado pelo orgulho e confiança que depositam em mim. Em especial aos meus pais que me proporcionaram todas as oportunidades que tive na vida. É a vocês em especial que devo este Doutoramento e tudo o que sou. Á minha irmã, a coisa mais preciosa do mundo. Obrigada por te orgulhares de mim tanto como me orgulho de ti. A mana conseguiu acabar sem ficar "varada", grande vitória. Á minha tia Sandra (a minha irmã afectiva); sabes que és muito especial. Obrigada pelas nossas pequeninas, Catarina e Iara a quem agradeço a maravilhosa ilustração desta tesa.

E por fim...ao Bruno. Pela paciência, por estares sempre ao meu lado e pelo constante apoio incondicional. Por aturares as minhas neuras nestes últimos momentos de grande pressão, e nos momentos em que mesmo sem pressão acordo com a "Lua". Obrigado por gostares tanto de mim e me permitires gostar tanto de ti.

A todas estas pessoas e a todas aquelas de quem me esqueci um grande Beijo de obrigado, vocês construíram uma parte desta tese.

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I. Abstract

Fish face many problems in finding their way around their environment, which is immense, dense, often dark and often devoid of visual cues. Furthermore, the aquatic medium contains many dissolved compounds. Thus, many aquatic animals have acute chemical senses, specially those species with nocturnal activity or habitats with high water turbidity, which can help them navigate and find conspecifics and prey or avoid enemies.

The Senegalese sole is a benthic nocturnal flatfish, which typically spends most of its time half-buried in sand with only its eyes and upper nostril exposed to open water, and feeds on benthic organisms, such as crustaceans and polychaetes, which live in the sand. During metamorphosis, the left nostril remains on the left (lower) side of the head, unlike the left eye which migrates to the right side of the head. Therefore, these fish have two olfactory epithelia exposed to two distinct environments; the upper nostril samples open water whilst the lower nostril samples interstitial water. It has been shown that the two olfactory epithelia of sole have different sensitivities to a number of different compounds suggesting that there is a functional difference between the roles that the two nostrils play. This functional asymmetry has never been described before in a vertebrate; this makes the sole a highly attractive model to study the mechanisms involved in olfactory detection and discrimination. Thus, the central challenge of this study was to understand the functional importance of differential detection by the two olfactory epithelia and to further our knowledge of the neuronal mechanisms involved in olfactory perception in the sole. To establish a possible relation between differential olfactory detection and the functional importance of the

odorants, odorants related to food search and intra-specific interactions in sole were identified, and the signal transduction pathways involved in the detection of the identified compounds in both olfactory epithelia were investigated. Given that sole often lives in estuaries, where salinity can change rapidly, we also evaluated the olfactory sensitivity to changes in environmental [Na^+] and [Ca^{2+}] and the effects of changes of salinity on olfactory sensitivity.

The food stimuli used in chemical identification studies were macerates of the polychaete *Hediste diversicolor* and water containing compounds released by living worms. Both samples were fractionated by molecular weight filtration followed by C18 solid-phase extraction and the olfactory potency of the resultant fractions was assessed by the electro-olfactogram (EOG). Most of the odorants detected by sole in the ragworm macerate were low molecular weight (<500Da) relatively hydrophilic compounds. Amino acids, in general, have these chemical features and fish are, in general, sensitive to L-amino acids. Thus, the amino acid content of the macerate was analysed by gas chromatography coupled to mass spectrometry (GC-MS). The most abundant amino acids present in the macerate were glycine and L-aspartic acid; although sole is sensitive to glycine, acidic amino acids such as aspartic acid are weak odorants. The next most abundant amino acids were L-histidine, L-proline, L-alanine and L-asparagine. Sole is insensitive to L-histidine and L-proline; however, L-alanine and L-asparagine are potent odorants. To evaluate the relative contribution of amino acids to the olfactory potency of ragworm macerate, the olfactory activity of an artificial mixture of amino acids at the same concentration as measured in the macerate was evaluated. Our results confirmed that, in the macerate, the vast majority of the olfactory potency is likely to be due to the presence of amino acids.

Odorants released by living ragworms are also substances of low molecular weight (<500 Da). However, in contrast to the macerate, the majority of the odorants are relatively hydrophobic; EOG responses to C18 solid-phase extract of worm-conditioned water showed that this fraction is more potent than that of the macerate. Furthermore, it is more potent at the lower olfactory epithelium than the upper. This supports the hypothesis of specialization of the lower epithelium in food detection. The extract was then fractionated by reverse phase high performance liquid chromatography (HPLC) and the olfactory potency of each fraction was assessed on both epithelia. The first two fractions contained the majority of the odorants; LC-MS of these fractions suggested 1-methyl-L-tryptophan as the active compound. Similar to the worm-conditioned water extract, 1-methyl-L-tryptophan was more potent at the lower epithelium than the upper. Cross-adaption with 1-methyl-L-tryptophan inhibited the olfactory responses to the worm-conditioned water extract and this inhibition was greater at the lower epithelium than the upper. Together, these results strongly suggest 1-methyl-L-tryptophan to be an important food-related odorant for sole. However, in addition to 1-methyl-L-tryptophan, there are other odorant(s) in the extract of worm-conditioned water. These odorant(s) contribute more to the total olfactory potency in the upper epithelium than in the lower. Cross-adaptation using 1-methyl-L-tryptophan as the adapting solution reduced the olfactory responses to L-cysteine, L-phenylalanine and glycine equally in both epithelia. Conversely, using L-cysteine, L-phenylalanine and glycine as adapting solutions, the olfactory response to 1-methyl-L-tryptophan was highly inhibited in the upper epithelium but, at the lower epithelium, the inhibition was never higher than 50%. This suggests that receptors of the lower epithelium are more specific, and have a

higher affinity, for 1-methyl-L-tryptophan. These results are consistent with, and give support to, the functional specialisation of the lower olfactory epithelium in prey detection. These studies were also important in that they showed that the odorants present in the ragworm macerate are different from those released into the water by living worms. Given that substances released by ragworms are more likely to be involved in food searching than those in the macerate, future studies aiming to identify food-related odorants (to be used, for example, as feeding stimulants in aquaculture) should be done with prey-conditioned water instead of macerate.

In contrast to freshwater fish, pheromone mediated interactions in marine fish are not well understood; the substances involved and their biological roles have been little studied. Bile, faeces and mucus are good candidates as sources of odorants used as pheromones in marine species. Electrophysiological studies showed that, in sole, such body fluids (mucus, bile and intestinal fluid) are more potent in the upper olfactory epithelium than in the lower. Furthermore, solid-phase extraction fractions of these fluids were all more effective stimulating the upper epithelium than the lower. These results are consistent with the upper epithelium being more involved in pheromone detection than the lower.

The C18 solid phase extract of intestinal fluid was fractionated by HPLC and the olfactory potency, as well as the total bile acid content of each fraction, was measured. The vast majority of bile acids was found in only one fraction. This fraction was the only one differentially detected by the two epithelia. LC-MS showed that the main bile acids in this fraction are taurocholic acid (TCH) and taurolithocholic acid (TLC) plus a minor third, unidentified, bile acid of 544.1 Da molecular mass. These three bile salts were also present in the bile fluid.

However, in sole-conditioned water, TCH was the only bile salt released in sufficient quantities to be measured. An artificial mixture of bile acids simulating the bile acid contents of intestinal fluid evoked similar amplitude EOGs to the HPLC fraction of intestinal fluid in which bile acids were detected. Thus, the third, unidentified, bile acid does not seem to make a significant contribution to the olfactory potency of intestinal fluid. Furthermore, the sole has olfactory sensitivity to TCH but the sensitivity to TLC is low. Thus, it is likely that TCH is responsible for the differential potency of the C18 extract of intestinal fluid on the two epithelia. This gives further support to the hypothesis that the olfactory detection of bile acids may be involved in intra-specific interactions and that the upper epithelium, in contrast to the lower epithelium, is involved in this function.

Cross-adaptation showed that using L-cysteine, L-phenylalanine or cholic acid (CHO) as adapting odorants decreases the olfactory responses to TCH mainly in the lower olfactory epithelium. Not even the structurally similar CHO was able to inhibit the response to TCH in the upper epithelium by more than 60%. This suggests that the response to TCH in the upper olfactory epithelium is due to specific receptors which respond preferentially to TCH.

To evaluate which intracellular transduction pathways are involved in olfactory detection of different classes of odorants, pharmacological studies were carried out with two different drugs that selectively inhibit adenylate cyclase (AC) and phospholipase C (PLC; SQ-22736 and U73122 respectively). Our results showed that olfactory sensitivity to L-cysteine is mediated mainly by the PLC/IP_3 pathway in both olfactory epithelia. The olfactory sensitivity of the lower olfactory epithelium to both 1-methyl-L-tryptophan and L-phenylalanine is due mainly to PLC/IP_3 pathway; however at the upper epithelium the AC/cAMP

pathway also contributes. In addition, our results suggest the contribution of (a) non-PLC/non-AC dependent pathway(s) in the lower olfactory epithelium. This suggests that the olfactory receptors involved in the detection of L-phenylalanine and 1-methyl-L-tryptophan are different between upper and lower epithelia. This is in agreement with the cross-adaptation studies which suggest the existence of specific receptors on the lower nostril responding preferentially to 1-methyl-L-tryptophan. Studies carried out in fish adapted to different salinities (35‰ and 10‰) showed that the olfactory sensitivity of the upper olfactory epithelium of sole to L-cysteine, L-phenylalanine and glycine is not affected by external salinity. However, the absence of Na^+ greatly inhibits olfactory responses to these amino acids in fish adapted to 35‰ seawater but not in those adapted to 10‰ seawater. This suggests that, although the olfactory sensitivity to amino acids does not vary with changes in environmental salinity, the maintenance of olfactory sensitivity in low salinity requires some adaptation of the transduction mechanism. The absence of Ca^{2+} did not affect the olfactory sensitivity to amino acids at either salinity. This suggests that the olfactory pathway involved in amino acid detection is independent of external Ca^{2+}.

Olfactory sensitivity to bile acids in the sole seems to depend on both AC/cAMP and PLC/IP_3 pathways. In the upper epithelium the effects of either drug were approximately equal. Given that the upper epithelium of the sole is more sensitive to bile acids, this suggests that, in contrast to the amino acid odorants, the AC/cAMP and the PLC/IP_3 transduction pathway are equally important to the olfactory detection of bile acids. However the effect of both drugs differs between the two olfactory epithelia. The AC/cAMP pathway contributes more to the olfactory activity of bile acids in the upper epithelium, whereas

PLC/IP_3 pathway contributes more to the olfactory sensitivity of the lower olfactory epithelium. This suggests the existence of different receptors between both epithelia and is in agreement with the existence of specific receptors on the upper nostril responding to bile acids (suggested by cross-adaptation studies). Furthermore, there is strong evidence suggesting the involvement of a non-PLC/non-AC dependent pathway as well as a cross-talk between the AC/cAMP and PLC/IP_3 pathways in the bile acid detection. However, the mechanisms involved are unknown.

Sole has olfactory sensitivity to changes in environmental [Na^+] and [Ca^{2+}] *via* apparently independent mechanisms. The concentration/response curve of olfactory responses to changes in [Ca^{2+}] is sigmoidal, suggesting the contribution of a receptor-mediated mechanism whereas the concentration/response curve to changes in [Na^+] does not reach a maximum, suggesting that responses to Na^+ are channel-mediated. Sole has olfactory sensitivity to decreases in environmental [Ca^{2+}] but to increases in environmental [Na^+]. Adaptation to low salinity decreases olfactory sensitivity to Ca^{2+} and increases sensitivity to Na^+. In addition, decreasing environmental [Ca^{2+}] increases olfactory sensitivity to Na^+ whereas decreasing environmental [Na^+] decreases responses to Ca^{2+}. These results suggest that differences in the olfactory sensitivity of fish kept at 35‰ and those adapt to 10‰ sea water are not due to adaptation of the olfactory system to low salinity, but rather to the fact that [Ca^{2+}] modulates olfactory responses to Na^+ and environmental [Na^+] modulates responses to Ca^{2+}. Thus decreasing environmental salinity decreases [Ca^{2+}] and [Na^+], in consequence, the olfactory sensitivity to Ca^{2+} decreases and sensitivity to Na^+ increases. This suggests that, in sole, Ca^{2+}

and Na^+ are detected by distinct and separate cellular mechanisms. However, there seems to be a degree of overlap between the two mechanisms.

The work included on this thesis shows that the upper and lower olfactory epithelia of the sole are much more than mere detectors of olfactory information. Olfactory discrimination starts at the level of the epithelium and the mechanisms involved are more complex than initially believed. Nevertheless, our original hypothesis that the two epithelia have different biological functions is well supported. Further work will clarify how the sole integrates the different olfactory input from the two epithelia.

II. Resumo

O ambiente aquático é frequentemente escuro e turvo, tal leva a que muitos peixes e outros organismos não utilizem a visão como principal sentido de orientação. No entanto, este ambiente é geralmente rico em diferentes compostos químicos que se encontram dissolvidos na água, o que faz com que muitos organismos tenham os sentidos químico-sensoriais muito desenvolvidos, especialmente espécies nocturnas e/ou que habitam águas de elevada turbidez.

O linguado Senegalês é um peixe bentónico pertencente ao grupo dos peixes chatos (Pleuronectiformes). Tal como a maioria dos peixes deste grupo o linguado passa grande parte do tempo parcialmente enterrado na areia, ficando apenas com os olhos e a narina superior expostos à coluna de água. As principais presas desta espécie são organismos bentónicos que vivem enterrados na areia, tais como poliquetas e crustáceos. Durante a metamorfose, o olho esquerdo migra para o lado superior (direito), no entanto, a narina esquerda não migra permanecendo no lado inferior (esquerdo). Em consequência, os dois epitélios olfactivos do linguado encontram-se expostos a diferentes ambientes; a narina superior amostra a coluna de água, enquanto a narina inferior amostra a água intersticial. Alguns odorantes são detectados de forma diferencial pelos dois epitélios olfactivos, sugerindo a existência de diferenças funcionais no papel que cada uma das narinas desempenha; um fenómeno nunca antes descrito em vertebrados. Esta singularidade faz do linguado um modelo bastante atractivo para o estudo dos mecanismos envolvidos na detecção e descriminação olfactiva. Assim sendo, o principal desafio deste trabalho foi compreender a importância funcional da detecção olfactiva diferencial e alargar o conhecimento referente aos mecanismos de detecção olfactiva no linguado Senegalês. Numa tentativa de estabelecer uma

relação entre a diferencial sensibilidade olfactiva e a função dos odorantes envolvidos neste fenómeno, foi feita a identificação química de compostos olfactivamente activos presentes em estímulos relacionados com a detecção e localização de presas e com possíveis interacções intra-específicas. Em adição, foram investigadas as vias de transdução envolvidas na detecção dos compostos identificados. Uma vez que o linguado é frequentemente encontrado em ambientes de salinidade relativamente baixa, foi também avaliada a sensibilidade olfactiva a diferenças nas concentrações de Ca^{2+} e Na^{+}, assim como o efeito da salinidade na acuidade olfactiva nesta espécie.

Na poliqueta *Hediste diversicolor*, presa comum do linguado, foram identificados odorantes no seu macerado e na água condicionada por este anelídeo. Ambas as amostras foram fraccionadas por filtração baseada no peso molecular e extracção por fase sólida (colunas C18). A potência olfactiva das fracções resultantes foi avaliada através do electro-olfactograma (EOG). A maioria dos odorantes detectados no macerado são compostos relativamente hidrofílicos de baixo peso molecular (<500Da). Os aminoácidos, em geral, têm estas características químicas e é sabido que os peixes são olfactivamente sensíveis a L-aminoácidos. Perante a possível contribuição de aminoácidos para a potência olfactiva do macerado, o seu conteúdo em aminoácidos foi avaliado por cromatografia gasosa associada a espectrometria de massa (GC-MS). Os aminoácidos mais abundantes no macerado são a glicina e o L-ácido aspártico; a glicina é um estímulo olfactivo forte, no entanto, o linguado é pouco sensível ao L-ácido aspartico. Os restantes aminoácidos mais abundantes são L-histidina, L-prolina, L-alanina e L-asparagina. O sistema olfactivo do linguado não é sensível à L-histidina e L-prolina, no entanto, a potência olfactiva da L-alanina e L-

asparagina é elevada. Numa perspectiva de avaliar a contribuição relativa dos aminoácidos para a potência olfactiva do macerado, foi preparada uma mistura artificial de aminoácidos de composição idêntica à detectada na amostra, e a correspondente potência olfactiva foi avaliada por EOG. Os resultados mostraram que no macerado os aminoácidos são os responsáveis pela grande maioria da potência olfactiva desta amostra.

À semelhança do macerado, os odorantes presentes na água condicionada por poliquetas são compostos de baixo peso molecular (<500 Da). No entanto, em contraste com o macerado, a maioria dos odorantes são substâncias relativamente hidrofóbicas; a potência olfactiva do extracto das colunas de extracção por fase sólida (C18) é muito superior à registada para a fracção equivalente do macerado. Em adição, o extracto da amostra de água condicionada por poliquetas é mais potente no epitélio olfactivo inferior do que no superior. Estas evidências suportam a prévia hipótese de especialização do epitélio olfactivo inferior na detecção e localização de possíveis presas.

O extracto da amostra de água condicionada por poliquetas foi posteriormente fraccionado através de cromatografia líquida de alta eficiência (HPLC) e a potência olfactiva das fracções resultantes foi avaliada em ambas as narinas. Verificou-se que a maioria dos odorantes estava presente nas duas primeiras fracções cromatográficas. Análise por cromatografia líquida associada a espectrometria de massa (LC-MS) sugeriu que o principal composto presente nestas fracções é o 1-metil-L-triptofano. Á semelhança do extracto da água condicionada por poliquetas, o 1-metil-L-triptofano apresenta maior potência olfactiva no epitélio inferior do que no superior. Em adição, estudos de adaptação cruzada, em que ambos os epitélios foram adaptados com 1-metil-L-triptofano,

demonstraram que este composto inibe as respostas olfactivas ao eluato da água condicionada por poliquetas, sendo o efeito inibitório maior no epitélio inferior. Estes resultados sugerem que, no linguado, o 1-metil-L-triptofano é um estímulo olfactivo relacionado com a detecção de alimento. No entanto, no extracto da água condicionada por poliquetas existem outros odorantes, os quais contribuem mais para a potência olfactiva total no epitélio superior do que no inferior.

A adaptação de ambos os epitélios olfactivos com 1-metil-L-triptofano reduz a sensibilidade a L-cisteína, glicina e L-fenilalanina de forma idêntica nos dois epitélios. Em contraste, a sensibilidade olfactiva ao 1-metil-L-triptofano é bastante mais inibida por adaptação com L-cisteina, glicina e L-fenilalanina no epitélio superior do que no inferior; no epitélio inferior as respostas nunca foram inibidas mais do que 50%. Estes resultados sugerem que os receptores do epitélio inferior são mais específicos e têm maior afinidade para 1-metil-L-triptofano do que os do epitélio superior; o que está de acordo com hipótese de especialização do epitélio olfactivo inferior do linguado na detecção de odores das respectivas presas. Outra importante elacção que pode ser tirada destes resultados é que, os odorantes presentes no macerado são diferentes dos libertados pelos poliquetas vivos. Em ambiente natural, é mais provável que os peixes detectem os compostos libertados do que os presentes macerado. Assim sendo, futuros estudos que visem a identificação química de estímulos relacionados com comportamento alimentar deverão utilizar águas condicionadas pelas presas em vez do macerado das mesmas.

Em contraste com os peixes de água doce, o actual conhecimento referente a interacções mediadas por feromonas em peixes marinhos é muito limitado. As possíveis substâncias envolvidas e correspondente importância biológica têm sido

pouco estudados. A bílis, as fezes e o muco são potenciais candidatos a fonte de odores utilizados como feromonas em espécies marinhas. Estudos de electrofisiologia demonstraram que no linguado, os referidos fluidos corporais (muco, fluido biliar e intestinal) constituem fortes odorantes e são preferencialmente detectados pelo epitélio olfactivo superior. Em adição, todas as fracções resultantes de extracção por fase sólida destes fluidos constituem estímulos olfactivos mais potentes no epitélio superior do que no inferior. Estes resultados estão em concordância com a possível função do epitélio olfactivo superior na detecção de feromonas, em contraste com o epitélio inferior.

O extracto (C18) da amostra de fluido intestinal foi fraccionado por HPLC. Posteriormente foi avaliada a potência olfactiva e a quantidade total de ácidos biliares de cada uma das fracções resultantes. Verificou-se que a grande maioria dos ácidos biliares estavam presentes numa só fracção. Esta fracção foi também a única a ser diferenciadamente detectada pelos dois epitélios. Estudos de LC-MS demonstraram que os principais ácidos biliares presentes são o ácido taurocólico (TCH), o ácido taurolitocólico (TLC) e ainda um ácido biliar não identificado de peso molecular 544.1 Da. Estes três ácidos biliares foram também detectados no fluido biliar. No entanto nas amostras de água condicionada por linguados o único ácido biliar presente em quantidade suficiente para ser quantificado foi o TCH. Uma mistura artificial de ácidos biliares de constituição idêntica à do fluido intestinal teve actividade olfactiva idêntica à da fracção de HPLC do fluido intestinal, onde a maioria dos ácidos biliares foram detectados. Assim sendo, a contribuição do ácido biliar não identificado para a potência olfactiva do fluido intestinal não parece ser muito importante. Em adição, o linguado é pouco sensível ao TLC, contrastando com a elevada a potência olfactiva do TCH,

sugerindo que o TCH é o principal responsável pela detecção diferencial do extracto C18 do fluido intestinal. Estes resultados suportam as hipóteses de os ácidos biliares estarem envolvidos em interacções intra-específicas e de o epitélio olfactivo superior dos linguados, em contraste com o inferior, ser responsável pela detecção de possíveis feromonas.

Estudos de adaptação cruzada demonstraram que; utilizando L-cisteína, L-fenilalanina ou ácido cólico (CHO) como solução de adaptação a sensibilidade olfactiva ao TCH é inibida especialmente no epitélio inferior. Nem o CHO que é estruturalmente semelhante ao TCH, conseguiu reduzir as respostas olfactivas na narina superior mais do que 60%. Estes resultados demonstram que as respostas ao TCH no epitélio olfactivo superior são mediadas por receptores específicos que respondem preferencialmente ao TCH.

Numa perspectiva de avaliar quais as vias de transducção envolvidas na detecção olfactiva de diferentes classes de odorantes, foram efectuados estudos farmacológicos, nos quais se recorreu à utilização de drogas inibidoras das vias mediadas pela adenilciclase (AC) e pela fosfolipase C (PLC; SQ-22536 e U73122, respectivamente). Os resultados obtidos sugerem que, a sensibilidade olfactiva à L-cisteína é mediada maioritariamente pela via da PLC em ambos os epitélios. Em adição, a sensibilidade olfactiva à L-fenilalanina e 1-metil-L-triptofano no epitélio inferior é igualmente mediada, na sua maioria, pela via da PLC; no entanto, no epitélio inferior, a via da AC também contribui para a sensibilidade olfactiva. Em adição, os resultados sugerem a contribuição de uma via independente da PLC ou AC na sensibilidade olfactiva do epitélio inferior. Estes resultados sugerem que, os receptores envolvidos na detecção olfactiva da L-fenilalanina e do 1-metil-L-triptofano diferem entre epitélios olfactivos,

suportando a existência de receptores específicos para o 1-metil-L-triptofano, sugerida pelos estudos de adaptação cruzada.

Estudos efectuados com peixes adaptados a diferentes salinidades (35‰ e 10‰) demonstraram que, a sensibilidade olfactiva a L-cisteína, L-fenilalanina e glicina é idêntica em peixes mantidos a 35‰ de salinidade e peixes adaptados a 10‰. No entanto, a ausência de Na^+ na água inibe fortemente as respostas olfactivas aos referidos aminoácidos em peixes mantidos a 35‰ de salinidade, mas não em peixes adaptados à salinidade de 10‰. Estes resultados sugerem que, apesar da sensibilidade olfactiva do linguado não variar em função de alterações da salinidade, a manutenção da sensibilidade olfactiva a baixas salinidades requer uma adaptação prévia. A ausência de Ca^{2+} na água não teve qualquer influência na sensibilidade olfactiva aos aminoácidos, tanto em peixes mantidos a 35‰ como em peixes adaptados a 10‰ de salinidade. Tal sugere que, as vias envolvidas na detecção de aminoácidos em linguado não dependem do Ca^{2+} no ambiente.

A sensibilidade olfactiva do linguado a ácidos biliares parece ser mediada por ambas as vias da AC/cAMP e PLC/IP_3. No epitélio olfactivo superior o efeito de ambos is inibidores destas vias foi idêntico. Tendo em conta que o epitélio superior é mais sensível aos ácidos biliares do que o inferior, podemos considerar que (ao contrário dos amino ácidos) a detecção olfactiva dos ácidos biliares é mediada em igual proporção por ambas as vias. No entanto, o efeito de ambas as drogas diferem entre epitélios. A via da AC/cAMP contribui mais para a actividade olfactiva dos ácidos biliares na narina superior, enquanto a via da PLC/IP_3 contribui mais para a actividade olfactiva do epitélio inferior. Estes resultados sugerem a existência de receptores diferentes em ambos os epitélios e estão de acordo com a existência de receptores específicos para ácidos biliares

(sugerido pelos estudos de adaptação cruzada). Em adição, existem fortes evidências para a contribuição de uma via independente da AC a da PLC em ambos os epitélios, assim como da existência de uma possível interacção entre ambas as vias. No entanto os mecanismos que poderão estar envolvidos nestes processos não são conhecidos.

O linguado é olfactivamente sensível a variações na concentração de Na^+ e Ca^{2+}, no entanto, as respostas a ambos os catiões parecem ser independentes. A curva de concentração/resposta ao Ca^{2+} é sigmoidal, sugerindo a existência de receptores; a curva concentração/resposta ao Na^+ nunca atinge um máximo, sugerindo que as respostas ao Na^+ são devidas à abertura de canais. O linguado é olfactivamente sensível à diminuição da concentração de Ca^{2+} e ao aumento da concentração de Na^+. A adaptação dos peixes a baixa salinidade diminui as respostas olfactivas ao Ca^{2+} e aumenta as respostas ao Na^+. Em adição, a diminuição da concentração de Na^+ na água diminui as respostas ao Ca^{2+} e a diminuição da concentração de Ca^{2+} na água leva a um aumento das respostas ao Na^+. Estes resultados sugerem que, as diferentes sensibilidades olfactivas ao Na^+ e Ca^{2+} dos peixes mantidos a diferentes salinidades não são devidas a uma adaptação dos epitélios olfactivos, mas sim ao facto de a concentração de Ca^{2+} na água modular as respostas ao Na^+ e a concentração de Na^+ modular as respostas ao Ca^{2+}. Estes resultados sugerem que, a detecção de alterações na concentração de Ca^{2+} e Na^+ no ambiente é mediada por mecanismos distintos e separados, no entanto existe algum grau de dependência entre eles.

Este estudo demonstrou que, os epitélios olfactivos do linguado representam bastante mais do que simples centros de recepção da informação olfactiva. A discriminação olfactiva começa ao nível do epitélio e os mecanismos envolvidos

são bastante mais complexos do que aquilo que se pensava inicialmente. Os resultados apresentados dão forte suporte à possível especialização funcional entre os dois epitélios olfactivos do linguado. Investigação futura deverá clarificar como é que a detecção diferencial da informação olfactiva é integrada no cérebro.

III. Structure of the Thesis

The present thesis is organised into four chapters. The first chapter (General Introduction) attempts to review the available scientific literature on the sense of olfaction and its importance to the life of fishes, providing the background against which the aims of this thesis are set. The second chapter (Experimental Work) starts with a brief introduction to the main techniques used (electrophysiology, solid phase extraction, chromatography and mass spectrometry), followed by a section describing the research carried out in the Senegalese sole presented in the format of scientific papers. The six scientific papers presented are grouped into four themes: i) Chemical identification of food-related odorants (papers I and II); ii) Chemical identification of con-specific derived odorants (papers III and IV); iii) Transduction pathways in olfactory receptor neurones (Paper V); and iv) Olfactory sensitivity to environmental inorganic cations (paper VI). The Third chapter (General Discussion) attempts to integrate the conclusions of each paper and discuss such findings in a wider perspective. The final chapter (Concluding Remarks and Perspectives) summarises the main conclusions that could be taken from the current work and indicates the directions that could be taken in future studies.

List of papers in the Experimental Work chapter:

I – Velez, Z., Hubbard, P. C., Hardege, J. D., Barata, E. N. and Canário, A.V.M. (2007). The contribution of amino acids to the odour of a prey species in the Senegalese sole (*Solea senegalensis*). *Aquaculture* **265**, 336-342

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II - Velez, Z., Hubbard, P. C., Hardege, J. D., Welham, W., Barata, E. N. and Canário, A.V.M. (2008). Evidence that 1-methyl-L-tryptophan is a food-related odorant for the Senegalese sole (*Solea senegalensis*). Patent applied for.

III - Velez, Z., Hubbard, P. C., Barata, E. N. and Canário, A.V.M. (2007). Differential detection of conspecific-derived odorants by the two olfactory epithelia of the Senegalese sole (*Solea senegalensis*). *General Comparative Endocrinology* **153**, 418-425.

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IV - Velez, Z., Hubbard, P. C., Barata, E. N. and Canário, A.V.M.. Identification, release and olfactory detection of bile salts in the intestinal fluid of the Senegalese sole (*Solea senegalensis*. To be submitted to *Journal of Comparative Physiology*

V - Velez, Z., Hubbard, P. C., Barata, E. N. and Canário, A.V.M. Olfactory transduction pathways in the Senegalese sole, *Solea senegalensis*. To be submitted to *Chemical Senses*.

VI - Velez, Z., Hubbard, P. C., Barata, E. N. and Canário, A.V.M. Adaptation to reduced salinity affects the olfactory sensitivity of Senegalese sole (Solea senegalensis) to Ca^{2+} and Na^{+} but not amino acids. To be submitted to *Journal of Experimental Biology*

General Introduction

I - General Introduction

Sensory systems perceive important features of the internal and external environments; chemical sensory systems are probably the most ancient of such mechanisms. In vertebrates, olfaction and taste are the main chemo-sensory systems which provide the ability to detect and discriminate structurally diverse molecules. Understanding how olfactory stimuli translate into perception is a problem of daunting complexity. For comprehensive descriptions of neuronal olfactory mechanisms, analysis must extend from the more primitive and simpler chemo-sensory systems of invertebrates (e.g. *Caenorhabditis elegans*) to the most complex systems of vertebrates. Hopefully, this will allow understanding how elemental principles relate to the function of the whole.

Fish have often been used as models in studies of olfaction; this is due to their easily accessible olfactory system and their high sensitivity. Teleosts have served as important models for the understanding of fundamental and general principles of olfactory physiology. However, most of studies on fish olfaction have been done in freshwater species; little is known about marine teleosts. The current study was developed in a marine flatfish, the Senegalese sole (*Solea senegalensis*). Flatfishes represent an interesting and diverse order of marine, estuarine, and freshwater fish with many species and genera (reviewed in Munroe, 2005). They are easily recognised anatomically in that post-metamorphic individuals have both eyes on the same side of the head. All flatfishes begin life as pelagic, bilaterally symmetrical fish. During larval development, however, they undergo metamorphosis which involves the migration of various internal and external organs (reviewed in Munroe, 2005). The Senegalese sole is a member of

the family Soleidae. As with other members of this family, during metamorphosis the left olfactory organ does not migrate to the upper (right) side. Thus, the right olfactory organ is located on the pigmented upper side, while the left olfactory organ faces the substratum (Rodríguez-Gómez, *et al.*, 2001). Consequently, each olfactory epithelium samples distinct environments; the upper nostril samples open water while the lower nostril samples interstitial water. Furthermore, the upper and lower olfactory epithelia of sole have different sensitivities to different odorants (Velez, *et al.*, 2005). Taken together this evidence suggests that the two olfactory epithelia of sole are specialised in detecting odorants with distinct ecological roles. The main objective of the current work was to understand the functional importance of differential detection and to further our knowledge of the neuronal mechanisms involved in olfactory perception in the sole.

The current chapter attempts to review the available scientific literature on olfaction in fish. To do this, it is organized into four different themes: i) diversity of fish odorants and their function, ii) anatomy of the olfactory system, iii) odorant coding and transduction, and iv) the Senegalese sole. The first section attempts to give an overall view of the current knowledge on the chemical nature of compounds that act as odorants in the aquatic environment. The second section reviews the general anatomic organization of the olfactory system of fish, and how anatomical adaptations translate into a more efficient odour perception. This is followed by a review of the current knowledge of the mechanisms involved in odorant detection, transduction and coding of olfactory information; how stimuli detected by the olfactory epithelium translate into perception. The last part of this chapter introduces the Senegalese sole, its biology and ecology, and gives special

emphasis on the unique characteristic of this fish that makes it a very interesting model for the study of olfaction.

1.1 - Diversity of Fish Odorants and their function

In the aquatic environment, chemical senses detect compounds dissolved in the surrounding water. Thus, known fish odorants are mostly small molecules with high water solubility (reviewed in Laberge, Hara, 2001; Zeiske, *et al.*, 1992). Chemical signals, unlike visual, mechanical, sound or electrical signals, last beyond the moment of production; the viscosity of the aquatic medium allows many different dissolved compounds to remain long after other types of stimuli have faded (reviewed in Laberge, Hara, 2001; Stacey, Sorensen, 2006; Zeiske, *et al.*, 1992). The distribution pattern of molecules in water is determined by the distance to the source, diffusion characteristics of the substance and the degree of turbulence (reviewed in Stacey, Sorensen, 2006; Zeiske, *et al.*, 1992). Odorants can be defined as individual chemicals detected by olfactory receptor neurones whereas odours may be complex mixtures of different types of chemical, and the responses evoked (physiological or behavioural) may depend upon the environmental context, physiological status, past experience or imprinting. The main odorants detected by fish derive broadly from three main sources: conspecifics, heterospecifics and the environment. Detection of conspecific odours (*e.g.*, pheromones) is important in fish reproduction (reviewed in Stacey, Sorensen, 2002; Stacey, Sorensen, 2006) and in other types of social interactions such as alarm reactions (Chivers, *et al.*, 1999; Chivers, *et al.*, 2007). Odours from heterospecifics are thought to be involved in fish feeding behaviour (Caprio,

Derby, 2008; De Groot, 1971; Hara, 1993) and in recognition of predators (Chivers, *et al.*, 2000; Chivers, *et al.*, 1999; Pettersson, *et al.*, 2000). Other odorants in the environment, possibly of various sources and chemical nature, are related to habitat recognition, which is particularly important for migratory and/or estuarine species whose habitats can vary greatly in chemical composition. The homing behaviour during the migration of eels (Lamothe, *et al.*, 2000) and salmon (Dittman, Quinn, 1996; Yamamoto, Ueda, 2007) provide two such examples.

1.1.1-Inter-specific interactions

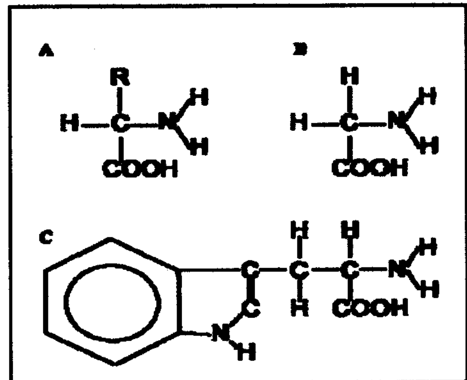

Figure 1.1 –Chemical stuctures of (A) generalized amino acid (R, side chain), (B) glycine (the simplest amino acid) and (C) tryptophan.

<u>Feeding</u> - Localization of food is of obvious importance. In fish, the olfactory system plays a key role in such process. Many identified food-related odorants in freshwater fishes are low molecular-weight organic molecules such as amines (Rolen, *et al.*, 2003), amino acids, nucleotides, nucleosides and organic acids (reviewed in Hara, 1994). However, most attention has focused on amino acids. Olfactory sensitivity to amino acids is a widespread phenomenon in fish (reviewed in Hara, 1994). Amino acids contain both an amine and carboxyl functional group attached to the same carbon (α–carbon) and the various alpha amino acids differ in the side chain (R group) attached to the α–carbon (Fig 1.1 A). Amino acids can vary in size depending on the R group, from just one hydrogen atom in glycine to a large heterocyclic group in tryptophan, and a range of other structures (Figure 1.1 B and

C). Amino acids exist naturally in a zwitterion state, with the carboxyl group ionized and the α-amino group protonated. The simplest member of this group is glycine, in which the saturated carbon atom is unsubstituted, rendering it optically inactive. The other amino acids are optically active and exist as both 'D' and 'L' stereo isomers (Nelson, Cox, 200). Naturally occurring amino acids are mainly L-isomers. Substituents on the saturated carbon atom vary from alkyl groups to aromatic amines and alcohols. There are also acidic and basic side chains as well as thiol chains that can be oxidized to dithiol linkages. Thus, side chains can be polar, non-polar or effectively neutral. Fish are able to discriminate well between similar amino acids (Hara, 2005). Amino acid detection requires several different receptors and individual receptors are relatively specific; their activation requires the presence of some molecular features and the absence of others (Fuss, Korsching, 2001). Generally, L-amino acids containing short branches and uncharged side chains are the most effective olfactory stimuli. L-proline is probably the only L-amino acid which shows little or no olfactory potency in fish (reviewed in Hara, 1994).

Other types of compound reported as being involved in feeding are betaine, quaternary ammonium compounds, nucleotides, nucleosides, organic acids (Carr, *et al.*, 1996), some opines or imino acids such as arcamine and strombine (Carr, *et al.*, 1996) and some polyamines such as spermine, spermidine, putrescine, cadaverine and histamine (Michel, *et al.*, 2003). The olfactory potency of such compounds and their ecological importance is less well studied; it may be that these compounds are more important in taste (reviewed in Hara, 1994).

Predator recognition - Fish are able to recognise potential predators using olfactory cues and can even differentiate between those that have recently fed on conspecifics; the predator becomes chemically 'labelled' (Brown, Zachar, 2002). Minnows (*Pimephales promelas*) are able to differentiate between predatory pike (*Esox lucius*) that have recently fed on conspecifics and those fed on unrelated species (Mathis, Smith, 1993a; Mathis, Smith, 1993b). However, recognition of predators is not necessarily an innate skill since past-experience and learning seem to be involved (Brown, Godin, 1999; Leduc, *et al.*, 2007). Nevertheless, newly-hatched Atlantic salmon (*Salmo salar*) seem to have innate ability for predator recognition (Hawkins, *et al.*, 2004). Although is generally accepted that fish can recognise predators *via* olfaction, the chemical identity of odorants involved is not known.

1.1.2 - Intra-specific interactions

Fish release various metabolites across the gills and *via* the urine, faeces and skin mucus. Many of such compounds act as odorants in conspecifics and give information about the sender's sex, physiological and/or social status and even relatedness to the receiver. Where this sensitivity has evolved to the benefit of the receiver, with little obvious gain for the sender, this is termed "chemical spying" (reviewed in Stacey, Sorensen, 2002; Stacey, Sorensen, 2006). However, in some cases, the sender can develop specialized organs or behaviours, which increase the efficiency of transmission of chemical messages to intended receivers with the result that both sender and receiver benefit; this is considered true chemical communication. The chemicals that convey these messages are termed

"pheromone signals" functionally different from passively released "pheromone cues" (reviewed in Stacey, Sorensen, 2006).

A pheromone has been defined as a substance, or a mixture of substances, which is released by an individual and which evokes a specific and adaptive reproductive response in conspecifics, the expression of which does not require specific learning (Sorensen, Stacey, 1999). Two types of pheromonal signalling are recognized; releaser pheromones and primer pheromones (cited in Brennan, Zufall, 2006). Releaser pheromones elicit rapid responses in conspecifics, whereas primer pheromones alter the physiology of conspecifics after a delay of hours or days, in most cases through the neuroendocrine and endocrine systems (Liley, 1982).

Fish use pheromones in a number of different ways. Most of the work done on pheromones in fish has focused on reproduction, where pheromones identified to date are sex hormones and/or their metabolites such as sex-steroids and prostaglandins (reviewed in Stacey, Sorensen, 2002). Maturation inducing steroids seem to play an important role as pheromones in some fish. The most common maturation inducing steroids in females are 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S). Electro-olfactogram (EOG) studies indicate that maturation inducing steroids and related 21-carbon steroids are detected by many Cypriniforms, Characiforms, and Siluriforms as well as Atlantic salmon (reviewed in Stacey, Sorensen, 2006). Prostaglandins, a class of fatty acids associated with a variety of autocrine and paracrine functions in vertebrates, including reproduction, are potent olfactory stimulants for many fish (reviewed in Stacey, Sorensen, 2006).

The goldfish (*Carassius auratus*) has been an important model in the studies of steroids and prostaglandins as pheromones in fish (reviewed in Stacey, Sorensen, 2006). Goldfish ovulate in the spring in response to a surge in gonadotropin (GtH) triggered by rising temperature, aquatic vegetation and pheromones. Postvitellogenic females exposed to these factors exhibit a dramatic surge of luteinising hormone that induces 17,20β-P synthesis and subsequent final oocyte maturation. During the surge and completion of spawning, females sequentially release a preovulatory steroid hormonal pheromone and a postovulatory prostaglandin-based hormonal pheromone. The preovulatory pheromone is a dynamic mixture whose multiple effects on males change with shifting ratios of its three principal components (17, 20β-P, its 20β-P sulphated metabolites and androstenedione). The main function of the preovulatory pheromone is to stimulate increased sperm production and mobility and to evoke male-male competition. The postovulatory pheromone (prostaglandin $F_{2\alpha}$ and 15-keto-prostaglandin $F_{2\alpha}$) induces spawning behaviour in males - pursuing and nudging the ovulating female - and activates mechanisms that increase release of milt (reviewed in Stacey, Sorensen, 2006).

Bile acids are a class of compound that may also act as pheromones. Bile acids are steroidal compounds produced by the vertebrate biliary system that function as detergents to solubilise fats (Sorensen, Caprio, 1998). They are synthesized from cholesterol in the liver and stored in the gall bladder. All bile acids consist of two parts: a rigid steroid nucleus and a short aliphatic side chain containing the acidic group. The steroid nucleus of bile acids has the saturated tetracyclic hydrocarbon perhydrocyclopentanophenanthrene, containing three six-carbon rings and one five-carbon ring (Figure 1.2). In higher vertebrates, the nucleus of bile acid molecules is curved because the A and B rings are in a *cis*-fused configuration. It has been suggested that the most recent mammalian bile acids have a 5β-configuration with hydroxyl groups at 3α, 7α and 12α (reviewed in Mukhopadhyay, Maitra, 2004). There are two major classes of bile acids depending on the length of the side chain: C_{27} and C_{24} bile acids. In vertebrates, C_{24} bile acids constitute a major part of the bile fluid, and they are generally conjugated to glycine or taurine (reviewed in Mukhopadhyay, Maitra, 2004). In teleosts, the main bile acids are sulphated bile alcohols (mainly 5α-cyprinol, 5-chima-erol) and C_{24} bile acids (cholic acid, chenodeoxycholic acid, deoxycholic acid and heamulcholic acid). C_{24} bile acids are mainly taurine amidated or/and sulphated, although glycine-amidated bile acids have been found in rainbow trout (Denton, *et al.*, 1974). In trout, cholic acid is the main component and constitutes over 85% of the total in bile. Chenodeoxycholic acid accounted for 14% or less

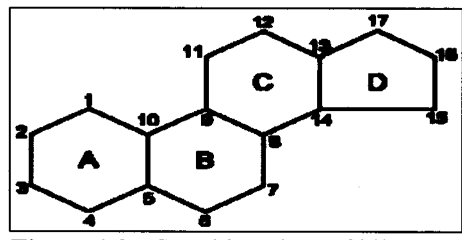

Figure 1.2 - Steroid nucleus of bile acids containing three six-carbon rigs (A, B and C) and one five-carbon ring (D).

and the 3α, 12α-7-keto- and 7α, 12α-3-keto-5β-cholanoates for 1% or less (Denton, *et al.*, 1974). In addition, cysteinolic bile acids were found in the bile of red seabream (Une, *et al.*, 1991). Thus, fish have a great variety of bile acids that may be further metabolised into different products. Their ubiquitous occurrence in vertebrates combined with their wide variation in chemical nature and olfactory potency are clearly compatible with a pheromonal role.

The possibility that bile acids may act as pheromones was first raised by Døving *et al.* (1980), who showed that juvenile Artic charr (*Salvelinus alpinus*) release bile acids in a range that can be detected by conspecifics. Since then, bile acids have been described as olfactory stimuli in many species (Baker, *et al.*, 2006; Frade, *et al.*, 2002; Lo, *et al.*, 1994; Rolen, Caprio, 2007; Siefkes, Li, 2004; Yun, *et al.*, 2003; Zhang, *et al.*, 2001). However, only in the case of sea lamprey (*Petromyzon marinus*) has a mixture of species-specific bile acids been identified with clearly defined pheromonal roles (Li, *et al.*, 2002; Sorensen, *et al.*, 2005).

Pheromones are also involved in other type of social interactions such as alarm reactions, social hierarchies and shoaling. Specialised cells in the skin of ostariophysan fishes called "club" cells release an alarm pheromone when damaged, warning other fish of impending danger. This substance has been tentatively identified as hypoxanthine-3-N-oxide (Brown, *et al.*, 2001). The behavioural effect of this alarm pheromones is mediated by the olfactory system (Ide, *et al.*, 2003). A behavioural alarm reaction was shown by juvenile matrinxa (*Brycon cephalus*) exposed to conspecific skin extract. Complete sectioning of the olfactory tracts, as well as bilateral sections of the olfactory sub-tracts (medial or

lateral), stopped the alarm reaction, confirming the importance of olfaction in this response (Ide, *et al.*, 2003).

Behavioural discrimination of siblings or closely-related conspecifics has been shown in organisms of various taxonomic groups including fish (Olsén, *et al.*, 2002; Willse, *et al.*, 2006; Willse, *et al.*, 2005). Cichlids often show parental care, and it would be advantageous for the parents to discriminate between their own offspring and unrelated fry. The cichlid *Hemichromis bimaculatus* seems to be capable of discriminating between odours of its own brood and those of unknown conspecifics (reviewed in Olsén, 1999). Stickleback (*Gasterosteus aculeatus*) fry raised in captivity could discriminate between siblings and unfamiliar or familiar non-siblings. In addition, fish raised alone from the egg and isolated from other individuals preferred chemical cues from siblings (reviewed in Olsén, 1999). Arctic char can discriminate between odours of siblings and non-siblings from the same population. They are more attracted to water conditioned by unfamiliar siblings than that by unfamiliar non-siblings (Olsén, *et al.*, 1998). This recognition is probably learned since charr reared in isolation from the egg until 15 months are unable to discriminate between siblings and non-siblings, while their siblings reared in groups have this ability even after two months of isolation (Olsén, *et al.*, 1998). Kin recognition has also been suggested to occur in poecilids; females preferentially cannibalized conspecific fry of other females in preference to their own (reviewed in Olsén, 1999). Social behaviour in vertebrates is influenced by the highly polymorphic genes of the major histocompatibility complex (MHC; reviewed in Spehr, *et al.*, 2006). MCH molecules determine immunological identity at the tissue level (reviewed in Brennan, Zufall, 2006). In

addition to their established role in the immune response, MHC genes are thought to give rise to odour signals that carry information about individuality (reviewed in Spehr, *et al.*, 2006). However, the cellular and molecular mechanisms by which this occurs, and the molecular nature of these chemo-signals remains unclear.

A "disturbance pheromone" released by stressed fish that may alert conspecifics without the requisite of physical damage to the sender has also been suggested (Jordão, Volpato, 2000). Other pheromones may have dual functions: male-derived pheromones may attract females but deter rival males.

1.1.3 - Environmental recognition

Fishes have the ability to recognize specific habitats such as natal waters or specific spawning sites. In salmonids for example, of which sockeye salmon is the most studied, sexually mature adults in the ocean travel thousands of kilometres back to their home rivers to spawn (Hinch, *et al.*, 2006). The Atlantic cod (*Gadus morhua*) is another example as they range widely in the ocean but each year return to specific spawning grounds (Green, Wroblewski, 2000; Rawson, Rose, 2000). Also, reef fish larvae, despite pelagic dispersal, are capable of returning to their home reef (Gerlach, *et al.*, 2006). How fish find such specific habitats remains an unsolved question. Many studies suggest that fish predominantly use olfactory cues during homing (Dittman, Quinn, 1996; Gerlach, *et al.*, 2006; Mitamura, *et al.*, 2005; Shoji, *et al.*, 2000; Yamamoto, Ueda, 2007). However, the odorants involved remain unknown. Amino acids, bile acids and inorganic cations are good candidates suggested to be involved in homing of salmonids (Shoji, *et al.*, 2000). Increasing evidence suggest that fish have olfactory sensitivity to inorganic

cations, mainly calcium and sodium. Sockeye salmon are able to detect changes in calcium levels in the water and may use calcium concentration as one of the olfactory cues by which they recognise their natal rivers (Bodznick, 1978). The gilthead seabream, (*Sparus aurata*) is also able to detect changes in environmental [Ca^{2+}], suggesting that there are other reasons than homing for a fish to monitor environmental calcium concentration (Hubbard, *et al.*, 2000). The olfactory sensitivity of marine fish to changes in environmental ion concentrations may be important in the maintenance of a constant extracellular [Ca^{2+}] or to warn the fish that it is reaching the limit of its salinity tolerance (Hubbard, *et al.*, 2000). The gilthead seabream is predominantly marine; nevertheless, it can survive in brackish water as low as 5‰, and thus can osmoregulate in a hypo-osmotic environment. However, the [Ca^{2+}] of this dilute sea water is very close to the plasma free [Ca^{2+}] of this species (Hubbard, *et al.*, 2000). It seems possible, therefore, that it is not the low osmolality of diluted sea water *per se* that limits the penetration of this species into freshwater environments, but its inability to maintain its plasma [Ca^{2+}] in a low Ca^{2+} medium (Hubbard, *et al.*, 2000). This olfactory sensitivity to changes in environmental [Ca^{2+}] may be connected to the need to maintain extracellular [Ca^{2+}] within physiological limits. The detection of decreasing levels of calcium may activate the appropriate endocrinological/physiological mechanisms to maintain plasma Ca^{2+} concentration and/or manifest the appropriate behavioural response (*e.g.* avoidance of low [Ca2+] water; Hubbard, *et al.*, 2000). However, olfactory sensitivity to changes in environmental [Ca^{2+}] is not restricted to migratory species or marine fish that regularly penetrate estuaries. The goldfish, a

stenohaline, non-migratory freshwater cyprinid, also has olfactory sensitivity to changes in external [Ca^{2+}] within the range of concentration that is likely to encounter in nature (Figure 1.2.2; Hubbard, *et al.*, 2002). Fish olfactory sensitivity to changes in external [Na^{+}] have been also reported, but with a much higher threshold of detection; the olfactory sensitivity to changes in external [Ca^{2+}] and [Na^{+}] seem to be mediated by distinct and separate cellular mechanisms (Hubbard, *et al.*, 2002). Studies carried on a higher number of species are necessary to confirm whether olfactory sensitivity to changes in environmental ions is a widespread phenomenon in fish and understand the functional and ecological significance of this phenomenon.

1.2 - Anatomy of the olfactory system

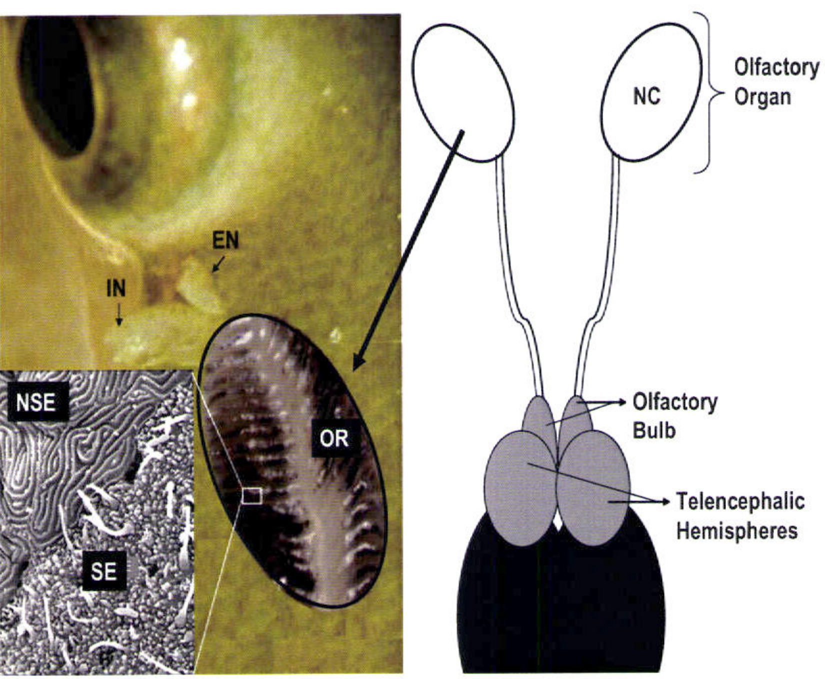

Figure 1.3 - Olfactory system in fish (dorsal view). Abbreviations; (NC) nasal chamber, (EN) exhalant nostril, (IN) inhalant nostril, (OR) olfactory rosette, (NSE) non-sensory epithelium and (SE) sensory epithelium.

The most peripheral part of the olfactory system is the olfactory organ, wherein lies the olfactory epithelium. The epithelium is separated into two regions: sensory and non-sensory. Odorants are detected by olfactory sensory neurons located within the sensory region of the epithelium. The axons of olfactory neurons form the olfactory nerve, which projects directly to the bulb; information from the bulb is conveyed through the olfactory tract to the telencephalic hemispheres (Figure 1.3).

1.2.1 - The Olfactory Organ

Each olfactory organ commonly consists of a cavity (the nasal or olfactory chamber) which is connected to the exterior through two openings, the anterior (inhalant) and the posterior (exhalent) nostril. The nasal bridge separates the inhalant and the exhalent nostril, (Figure 1.4; reviewed in Kasumyan, 2004; Zeiske, *et al.*, 1992; Zielinski, Hara, 2006). Generally, fish have two olfactory organs, one on each side of the head, rostral to the eyes (ditrematous type). The olfactory epithelium is located on the bottom of the nasal chamber. Depending on different ecological habitats and life-styles, this general scheme of the olfactory organ of fishes varies greatly. For example, cyclostomes (hagfish and lampreys) are monorhinal; the olfactory organ represents an unpaired structure; thus water enters and leaves the nasal cavity

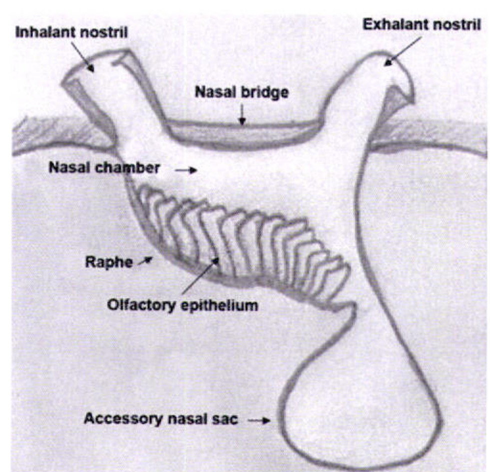

Figure 1.4-Diagrammatic median section of the olfactory organ of a generalized fish (adapted from Zeiske, *et al.*, 1992).

through a single nostril located on the dorsal surface of the head (reviewed in Kasumyan, 2004; Zielinski, Hara, 2006). Another interesting variation is that, in some fish, the olfactory and the oral cavity are linked (*e.g.* chimaera, dipnoans, some teleosts and in fish of the family Echelidae and Ochichthyidae; reviewed in Kasumyan, 2004). Usually, the olfactory organs are located on the dorsal surface, one on each side of the head, rostral to the eyes. However, there are some exceptions; in the family Soleidae, the lower (left) olfactory epithelium and associated structures do not migrate to the upper side during metamorphosis, as does the left eye, but remain on the lower side. Thus, these fish have the right olfactory organ on the dorsal side, close to the eyes and the left olfactory organ on the ventral side. Another exception are the elasmobranches (sharks and rays) in which the olfactory organs are in cartilaginous capsules situated on the ventral side of the head (reviewed in Kasumyan, 2004; Zeiske, *et al.*, 1992).

The structural diversity of the olfactory organ is evident in many other characteristics, such as the morphology of the inhalant and exhalent nostrils and the presence and size of the nasal bridge. In sharks, the nasal bridge is absent, the lateral and the medial edges of the olfactory opening have a well-developed skin outgrowths directed toward each other. The size of these outgrowths is so large that, in many species of sharks and rays, they cover a special nasal-oral groove, going from the nasal to oral cavity and providing a connection between the two (reviewed in Kasumyan, 2004). Typically, the inhalant nostril is situated at the end of a tubule of varying length, but it can also be funnel-shaped or just a simple opening. For example, in carp, cod, salmon and many other species, the nostrils are well defined, close to each other and the nasal bridge bears a large ridge,

forming a funnel that directs water into the nasal cavity. Some eels, catfishes, snakeheads (bottom dwelling and inactive, burrowing in the substrate or hiding among stones) have nostrils in the shape of long or short tubes. In some cases, these tubes are separated from each other and have an inflow valve in the anterior tube and an outflow valve in the posterior (reviewed in Kasumyan, 2004). In certain species, such as in *Bedotia geayi*, the openings leading to the olfactory organ are very small and difficult to discern. In some tetraodontiform fish, nostrils are lacking and the olfactory organ is located within bifid or solid tentacle-like structure outgrowths on the head (reviewed in Kasumyan, 2004; reviewed in Zeiske, *et al.*, 1992).

Some groups have sexual dimorphism of the olfactory organ. In Myctophiformes and Lophiformes, the olfactory organ is poorly developed in females, whereas they are very large in males. This is thought to be an adaptation enabling the male to find females (reviewed in Zeiske, *et al.*, 1992).

Some teleosts have, associated with the olfactory organ, accessory nasal structures (reviewed in Zielinski, Hara, 2006). These accessory nasal structures assist in pumping water over the olfactory epithelium, an event that has been compared to "sniffing" (Nevitt, 1991). Another function of olfactory sacs is the production of mucus in the epithelium of the accessory sacs. The number of accessory sacs varies between one and four, and they are named depending on the bones of the skull to which they are attached (*e.g.* lacrymal, ethmoidal, suborbital, supraorbital; reviewed in Kasumyan, 2004).

Olfactory sampling in fish has been thought to be a relatively involuntary behaviour. However, evidence suggests that some species may use coughing as a

mechanism of olfactory sampling (Nevitt, 1991). The functional importance of coughing is not clear; it may be used to clean the gills and also to eject particulate mater from the olfactory chamber (Nevitt, 1991). However, many species are known to cough in clean, well-aerated water (Nevitt, 1991). It was suggested that coughing represents a behaviour analogous to sniffing in certain air-breathing organisms (Nevitt, 1991). During coughing, water is rapidly sucked into the olfactory sac, in addition coughing rates increase significantly over background activity when flounders are presented with attractive food odorants (Nevitt, 1991).

1.2.2 - The Olfactory Rosette

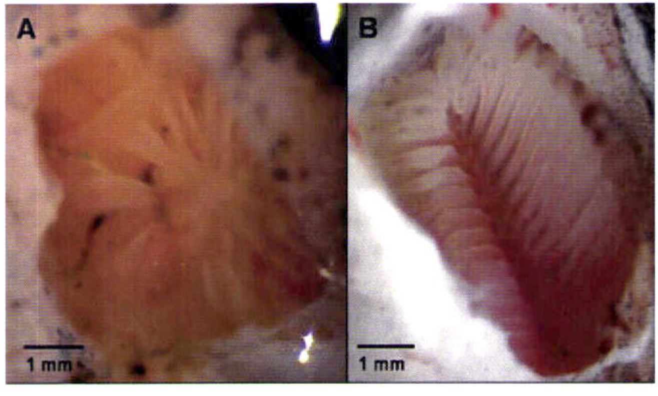

Figure 1.5 - Example of olfactory rosettes of A) *Oreochromis mossambicus* and B) *Anguilla anguilla*.

In most fish, the olfactory epithelium is located on multilamellar mucosal folds that form a flower-like arrangement, called the olfactory rosette (Figure 1.5). The arrangement, shape and degree of development of the lamellae vary considerably from species to species. In most cases, the lamellae radiate from a central ridge, the 'raphe', arising rostro-caudally from the floor of the nasal cavity (reviewed in Hara, 1994). In some fish the lamellae are arranged in a circle (Figure 1.5 A), a semicircle, lie parallel to one-another (Figure 1.5 B) or form an irregular pattern. There is a marked variability in the number of olfactory lamellae between species. For example, in cyprinids, sturgeons, salmonids, percids, and several other groups of fish, the

number of lamellae can reach several dozen. In some species, like *Holopagus guentheri* from the family Lutjanidae, the number of olfactory lamellae reaches 230 (reviewed in Kasumyan, 2004). In contrast, in other species rosettes may have only few lamellae (*e.g.* rainbow fish, fifteen-spined stickleback) or even be totally absent (*e.g.* mosquito fish, guppy, and garfish). In fish with a symmetrical body shape, rosettes on both the left and right olfactory organ have the same number of lamellae. However, in Pleuronectiformes the olfactory organs located at the eyed and blind sides of the body differ in size and number of lamellae in the olfactory rosette (reviewed in Kasumyan, 2004). The basic function of multi-lamellar olfactory epithelium is to increase the surface area for odorant-receptor interaction. Some fish have secondary lamellae on the surface of basic olfactory lamellae (*e.g.* salmonids and sturgeons). Secondary lamellae may be oriented parallel or perpendicular to the base of primary lamella and, in some cases, along the diagonal. In sharks, there are also tertiary lamellae. Like the primary lamella, the basic function of secondary and tertiary lamellae is to increase the area of the olfactory epithelium (reviewed in Kasumyan, 2004).

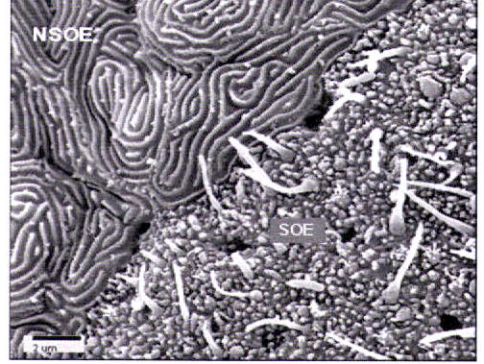

Figure 1.6 - Scanning electron micrograph of the upper olfactory epithelium of *Solea senegalensis*. Abbreviations: NSOE, non sensory olfactory epithelium; SOE, sensory olfactory epithelium.

1.2.3 - The Olfactory Epithelium

Like the olfactory organ, the olfactory epithelium also shows great variability. Generally, the epithelium is separated into two regions; sensory and non-sensory (Figure 1.6). Both are covered with a mucous layer and rest on a basal lamina

and the underlying lamina propria (supporting loose connective tissue; reviewed in Zeiske, *et al.*, 1992). Receptor cells occur in the sensory epithelium in areas covering the side surface of the lamellae. They are absent on ridges of the lamellae, and on the walls of the olfactory cavity. The sensory olfactory epithelium alternate with the non-sensory epithelium. The distribution of receptor cell differs between species. Four different types of distribution of the sensory epithelium within the olfactory lamellae have been described: I) continuous; II) large-zonal; III) irregular; IV) small-zonal (Figure 1.7; reviewed in Kasumyan, 2004).

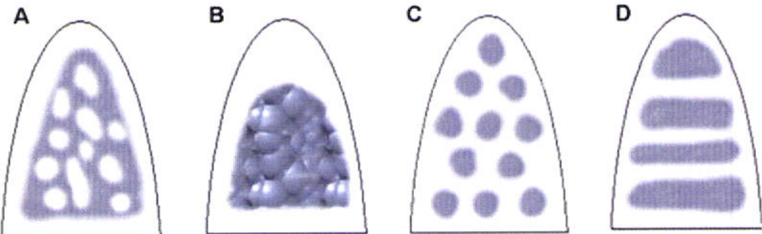

Figure 1.7 - Types of the distribution of the sensory olfactory epithelium on olfactory lamellae: (a) irregular; (b) continuous; (c) small-zonal and (d) large-zonal.

Non-sensory epithelium - The non-sensory epithelium has ciliated and non-ciliated areas. The basic components of the non-sensory epithelium are the epithelial cells; the general structure of this area is identical to that of the free surface of fish epidermis in that it has micro-ridges arranged in fingerprint-like patterns (reviewed in Zeiske, *et al.*, 1992). Non-sensory ciliated cells extend from the basal lamina to the epithelial surface, they are columnar and have multiple kinocilia sprouting from the apex; these kinocilia are motile and serve to irrigate the olfactory organ (reviewed in Kasumyan, 2004; Sorensen, Caprio, 1998). Kinocilia of the ciliated non-sensory cells move synchronously; this is important

to provide the movement of water that, in turn, carries the signal to the receptor cells. Other abundant cells on the non-sensory olfactory epithelium are goblet cells, which produce and release mucus. The mucous layer completely covers the sensory and non-sensory areas of the olfactory epithelium. The olfactory mucus includes mucopolysaccharides, proteins, lipids and different ions (reviewed in Kasumyan, 2004). It is thought that mucus plays an important role in chemoreception; putative odorants enter the mucus, interact with its components, and only then reach the receptor areas of the olfactory epithelium. Mucus creates optimal conditions for interactions between the odorants and receptor proteins (reviewed in Kasumyan, 2004) and also protects the olfactory epithelium against chemical, mechanical and pathogenic damage. Mucus production increases significantly when various chemical agents contaminate the water, or when the temperature increases.

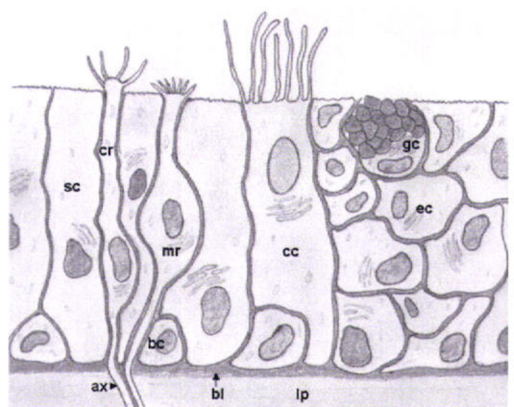

Figure 1.8 - Illustrative Diagram of the olfactory epithelium of teleosts.
Abbreviations: ax, axon; bc, basal cell; bl, basal lamina; cc, ciliated non-sensory cell; cr, ciliated receptor cell; ec, epithelial cell; gc, goblet cell; lp, lamina propria; mr, microvillous receptor cell; sc, supporting cell.

Sensory epithelium - The sensory epithelium consists of three main cell types: receptor cells, basal cells and supporting cells (Figure 1.8).

Supporting cells are tall bottle-shaped cells that surround the receptor cells. Two different types of supporting cells have been described; cells with a free surface not bearing any specialized structures, and ciliated cells that have numerous kinocilia on the surface. It is unclear whether

these ciliated supporting cells are identical to the ciliated cells previously described in the non-sensory areas of the olfactory epithelium (reviewed in Hansen, Zielinski, 2005; Kasumyan, 2004).

Basal cells are undifferentiated cells located in the deepest layer of the olfactory epithelium and have no cytoplasmic processes reaching the free surface (reviewed in Zeiske, *et al.*, 1992). They are small cells of different shape that occupy the space between the feet of the supporting cells and the proximal portion of the olfactory receptor neurons (reviewed in Hansen, Zielinski, 2005). Their nuclei are large in relation to cell size and heterochromatin is distributed in a chessboard pattern as in fully differentiated olfactory receptor neurons (reviewed in Hansen, Zielinski, 2005). It is believed that new cells of the olfactory epithelium (receptor, supporting and mucus cells) develop and differentiate from the basal cells. Receptor cells are renewed every seven to ten days; the capacity for constant renewal is an important adaptation, allowing the fish to obtain olfactory information after chemical or physical damage.

The first olfactory neurons described were the ciliated and microvilli receptor cells. Recently, a third type of olfactory receptor neuron has been discovered; the crypt cell (Hansen, Zeiske, 1998; Hansen, Finger, 2000). These cells have now been reported in several species and may represent a conserved trait (reviewed inHamdani, Døving, 2006; Hansen, Finger, 2000). Crypt cells are oval-shaped and bear apical microvilli as well as occult cilia extending into a crypt at the apex of the cell. A special type of supporting cell surrounds crypt cells. Either one or two of these cells wrap around a crypt olfactory receptor neuron (reviewed in Hansen, Zielinski, 2005). They also bear numerous microvilli at their apical rim. When two

supporting cells are present they inter-digitate with each other. Mitochondria in crypt supporting cells are larger than those in common supporting cells (reviewed in Hansen, Zielinski, 2005).

Ciliated receptor cells are bipolar nerve cells with a peripherally directed dendrite, which terminates rounded protuberance called the olfactory knob. Each knob is the dendritic process of one neuron, and it gives rise to extended olfactory cilia, up to 10 cm long, which are immotile and are thought to contain the olfactory receptor proteins (reviewed in Hansen, Zielinski, 2005). The dendrite of the ciliated olfactory receptor neurons is thin (~1μm diameter), and the cell body is located in the lower third region of the olfactory epithelium. The number of cilia varies considerably according to species, size and age, usually from 3 to 10 (reviewed in Hansen, Zielinski, 2005).

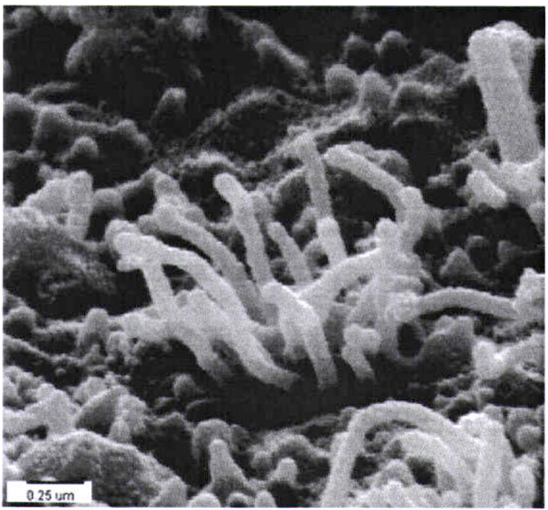

Figure 1.9 - Scanning electron micrograph of microvillous receptor neurones in the olfactory epithelium of the Sole, *Solea senegalensis*.

Microvillous receptor cells (Fig. 1.9) have a flattened free surface and bear up to 80 microvilli, which are up to 5 μm long. The dendrites are wider than in the ciliated receptor neurons (~5μm) and the cell body is found in the centre of the olfactory epithelium (reviewed in Zielinski, Hara, 2006). The knobs of microvillous receptor neurons are often less pronounced than those of ciliated neurons (reviewed in Hansen, Zielinski, 2005).

1.2.4 - Spatial distribution of olfactory receptor cells

Evidence suggests that, in fish, the different types of olfactory receptor neurons are uniformly distributed within the sensory epithelium. Immunocytochemical studies of trout olfactory receptor neurons showed that subsets of receptor neurons, identified by unique lectin-binding properties, are widely distributed and intermingled with the other receptor neurons in the olfactory mucosa and nerve (Riddle, *et al.*, 1993). This was confirmed by labelling rainbow trout olfactory fibres with different neurotracers (Riddle, Oakey, 1991; Yasuhiro, Finger, 1999). A similar uniform distribution pattern appears to be present in channel catfish (Chang, Caprio, 1996; Morita, Finger, 1999), snakehead fish, *Channa punctatus* (Mandal, *et al.*, 2005), round goby, *Neogobius melanostomus* (Belanger, *et al.*, 2003), and *Fugu rubriques* (Asano-Miyoshi, *et al.*, 2000).

1.2.5 - Specificity of the different types of odorant receptor cells

During the past 20 years, physiological, morphological, immunochemical, and molecular studies have investigated the possible link between olfactory receptor morphology and odorant specificity. However, it remains unclear whether each receptor cell type (microvillous, ciliated and crypt) detect specific types of odorants. Different types of receptor neurons seem to be sensitive to specific classes of odorant (Hansen, *et al.*, 2003; Lipschitz, Michel, 2002; Sato, Suzuki, 2001; Thommesen, 1982); however, this may differ between different species. Cross-adaptation studies showed the existence of relatively independent olfactory receptor neurons for acidic, basic, neutral amino acids (Caprio, Byrd, 1984;

Valentincic, *et al.*, 2000a) and bile acids (Li, Sorensen, 1997). Furthermore, evidence suggests that microvillar cells detect mainly amino acids. In char (*Salmo alpinus* L.), responses to amino acids appeared to be correlated with high density of microvillar cells (Thommesen, 1982). Electrophysiological and anatomical studies carried in three salmonid species showed that the central parts of the olfactory lamella (rich in microvillous receptors cells) respond mainly to amino acids (Thommesen, 1983). Additional evidence that microvillous neurons respond to amino acids comes from studies on zebrafish (Friedrich, Korsching, 1998; Lipschitz, Michel, 2002), goldfish (Speca, *et al.*, 1999), channel catfish (Hansen, *et al.*, 2003), crucian carp (Hamdani, *et al.*, 2001a) and rainbow trout (Sato, Suzuki, 2001). Thus, evidence seems to suggest that microvillous receptor neurons respond to amino acids. However, the chemical nature of odorants in ciliated cells is less clear. Ciliated cells were reported to respond to bile acids in a number of species (Thommesen, 1982; Thommesen, 1983), however they also seem to respond to other classes of compound. Sato and Suzuki (2001) showed that ciliated neurons respond to amino acids, conspecific urine and a steroid (etiocholan-3α-ol-17-one glucuronide). In a marine fish, the cabinza grunt (*Isacia conceptionis*), patch-clamp recording showed that ciliated neurons respond to amino acids (Schmachtenberg, Bacigalupo, 2004). In the channel catfish (*Ictalurus punctatus*), these cells respond to bile acids and amino acids (Hansen, *et al.*, 2003). Thus, the available evidence seem to suggest that microvillous receptor neurons respond preferentially to amino acids whereas ciliated neurons are less specific responding to a wider range of compounds. However, definitive conclusions cannot yet be drawn.

1.2.6 - The Olfactory Nerve

In fish, as in other vertebrates, olfactory receptor neurons are the only neurons whose axons carry sensory information directly to the brain. The dendritic endings of the olfactory receptor neurons are immersed in the mucus of the nasal cavity, the cell bodies lie within the olfactory epithelium and the proximal part of the perikaryon tapers to form an axon (reviewed in Zielinski, Hara, 2006). The axons pass through the basement membrane and group together in the submucosa to form the olfactory nerve (cranial nerve I). The olfactory nerve projects directly to the olfactory bulb where it synapses with dendrites of second-order olfactory neurons (mitral cells; reviewed in Zeiske, *et al.*, 1992).

1.2.7 - The Olfactory Bulb

The olfactory bulb of fish is less distinctly laminated than that of mammals. It is generally divided into four concentric layers, the olfactory nerve layer, the glomerular layer, the mitral cell layer and the internal cell layer (Korsching, *et al.*, 1997). The olfactory glomeruli are roughly spherical masses of neuropile containing glutaminergic synapses between sensory neurons axons and the dendrites of bulbar projection neurons and interneurons (reviewed in Zielinski, Hara, 2006). Moreover, not all olfactory axons synapse onto glomeruli; a subset of axons terminates in so-called aglomerular plexuses, which contain smaller and less well separated terminal specialisations.

The mitral cell layer contains the somata of secondary olfactory neurons, whose axons project to the telencephalon. These large projection neurons include both mitral and ruffed cells (reviewed in Zielinski, Hara, 2006). The mitral cells

are distributed diffusely between the olfactory nerve layer and the internal cell layer. Unlike mammals, mitral cells of fish commonly project from more than one glomerulus. The ruffed cells, unique to fish, are specialised projection neurons that apparently do not receive direct input from olfactory neurons. These cells are much less common than the mitral cells and have a lamellar ruffle located along the proximal axonal segment. They are located in the glomerular layer and internal cell layer in zebrafish, and may be present in all fish species, but with slight differences in the position of the ruff (reviewed in Zielinski, Hara, 2006).

The deeper portion of the fish olfactory bulb is the internal cell layer in which many cells of smaller size are densely packed. The internal cell layer contains many inhibitory inter-neurons called granule cells. These cells lack axons and send dendritic processes into the mitral cell layer where they make reciprocal synaptic connections with the dendrites, somata and initial segments of the axons of mitral cells. These reciprocal synapses are made up of an excitatory portion, from mitral to granule cell and an inhibitory portion from granule to mitral (reviewed in Korsching, *et al.*, 1997).

The olfactory bulb also contains centrifugal fibbers extending from the telencephalic hemisphere and terminating mainly in the internal cell layer, probably onto granule cells. In addition, there are terminal nerve fibres which originate in ganglion cells (multipolar or bipolar) associated with the olfactory nerve or the rostro-medial part of the olfactory bulb (reviewed in Whitlock, Westerfield, 1998; reviewed in Zielinski, Hara, 2006). The output neurons from the olfactory bulb then projects through the olfactory tracts to telencephalic territories (Zielinski, Hara, 2006).

Although there are important differences between the olfactory systems of fish and mammals, the stereotypical pattern of glomeruli organization appears to be a general feature of vertebrates (Baier, *et al.*, 1994; Korsching, *et al.*, 1997). Zebrafish receptor neurons terminate within the olfactory bulb in about 80 different glomeruli which are arranged in a stereotyped pattern (Baier, *et al.*, 1994). It was suggested that the stereotypy of the pattern found in zebrafish olfactory bulb is reminiscent of the insect olfactory system (Baier, *et al.*, 1994).

1.3 - Odorant coding and transduction

The first step of olfaction is the detection of odorants by the olfactory receptor neurons. Intracellular transduction pathways then translate this into changes of membrane voltage and generation of action potentials. The action potentials propagate along the axons of the receptor neurons and thus the signals are transmitted to the olfactory bulb, where different odours are coded as different combinations of activated glomeruli. Information from the olfactory bulb is then conveyed to the telencephalic hemispheres. How the nervous system recognizes and distinguishes between a vast range of odorants is an intriguing question. Although our knowledge of the neuronal mechanisms involved in olfactory perception and codification remains limited, evidence suggests that odorant coding occurs at several different levels within the olfactory system. Firstly, different odorants may bind to different membrane receptor proteins. It is generally assumed that one receptor neuron expresses only one type of odorant receptor and that different classes of odorants may activate different olfactory receptor proteins.

Furthermore, different signal transduction mechanisms are involved, depending on the type of odorant, and more than one mechanism of olfactory transduction may be operational in the same neuron. A single olfactory receptor neuron may respond to one or more odorants and may respond either with excitation or with suppression depending on the odorant (Ache, Zhainazarov, 1995; Hatt, Ache, 1994; Schmiedel-Jakob, *et al.*, 1990). All this evidence suggests that the mechanisms involved in odour quality coding may start early at the level of odorant detection.

Other possible level of odorant coding is at the olfactory bulb where receptor neurons, randomly distributed over the sensory epithelial surface, project to a particular glomerulus. The information from the olfactory bulb travels to the telencephalic hemispheres through the olfactory tracts. Each olfactory tract seems to be specialized to carry different functional olfactory information, suggesting olfactory coding at the level of the olfactory tracts. The last proposed level of olfactory coding is the telencephalon, but knowledge remains limited.

1.3.1 - Odorant receptors

The first step required in olfaction is the binding of an odorant to the olfactory receptor proteins in the plasma membrane of receptor neurons. The genes that code olfactory receptors were first described in 1991 (Buck, Axel, 1991). Seventeen years later, the process of receptor-gene discovery continues. Because the large gene families involved in odour detection are highly diverse, new receptor genes are identified almost daily by genome sequencing projects.

There are two main types of olfactory receptor proteins, the vomeronasal receptors type (VR-type) and the olfactory receptor type (OR-type). In mammals, it is commonly assumed that the OR-type (found in the olfactory epithelium) deals with common (non-pheromonal) odorants whereas VR-type (found in different cell types within the vomeronasal organ) are specialized for detecting pheromones (Alekseyenko, *et al.*, 2006; Korsching, 2004). Fish, however, do not have a vomeronasal system; both OR-type and VR-type receptors are present in the main olfactory epithelium.

In the mid 1990s, two classes of VR-type were identified; the V1R and V2R families. A new class of olfactory receptors, trace amine-associated receptors, was recently discovered in the olfactory epithelium of mice (Liberles, Buck, 2006). Genes encoding such receptors were also found in humans and fish. At least three mouse trace amine-associated receptors recognise volatile amines found in urine; one detects a compound linked to stress, whereas the other two detect compounds enriched in male *versus* female urine (one of which is reportedly a pheromone; Liberles, Buck, 2006). So far, ligands identified for trace amine-associated receptors are all associated with the detection of social cues suggesting that pheromone detection may not be restricted to the vomeronasal organ. In fish, the OR-type and also the V2R-type receptor genes have been identified in several species; zebrafish (Barth, *et al.*, 1996), catfish (Hansen, *et al.*, 2005; Ngai, *et al.*, 1993), goldfish (Cao, *et al.*, 1998 ; Hansen, *et al.*, 2005), *Fugu* (Asano-Miyoshi, *et al.*, 2000), carp, Atlantic salmon (Wickens, *et al.*, 2001), and medaka fish (Kondo, *et al.*, 2002). A single V1R-type gene is expressed in fish, and the apical

localization of V1R cells suggests that such cells are crypt or microvillous receptor neurons (Asano-Miyoshi, *et al.*, 2000; Pfister, Rodriguez, 2005).

Since fish lack a vomeronasal organ, it is difficult to establish a clear distribution pattern for OR-type and VR-type receptors; apparently, both types are uniformly distributed within the olfactory epithelium (Asano-Miyoshi, *et al.*, 2000). However, in zebrafish, putative olfactory receptor genes are expressed in different concentric domains in the lamellae suggesting some organization in the distribution of the olfactory receptors (Weth, *et al.*, 1996). Furthermore, in goldfish, OR-type receptors are homogenously distributed across the entire epithelium in ciliated neurons, whereas V2R-type receptors are expressed in microvillous neurons that are mainly located along the dorsal margin of the lamellae and near the midline raphe (Hansen, *et al.*, 2004).

The expression pattern of olfactory receptor genes varies between different classes of organism and remains a controversial issue. The nematode *Caenorhabditis elegans* does not have a distinct olfactory system; even so, it can detect different chemicals in the environment. Each chemo-sensory neuron in *C. elegans* expresses many different chemoreceptor genes (L'Etoile, Bargmann, 2000). In *Drosophila*, most of the approximately 2,600 olfactory neurons express two olfactory receptor genes; one specific to the cell type and a second ubiquitous gene known as *Or83b*, whose protein product dimerises with the receptor and mediates its transport to the cilia (Vosshall, *et al.*, 1999). In vertebrates, the olfactory neurons seem to follow an apparently strict expression pattern of one odorant-receptor gene being expressed *per cell* (Serizawa, *et al.*, 2005). After one functional receptor is expressed, expression of other receptor genes is suppressed

(Serizawa, *et al.*, 2005). However, a recent study showed that the one receptor-one neuron rule is not always applicable; *in situ* hybridization studies demonstrated the co-expression of different olfactory receptors in a small population of receptor neurons in the zebrafish (Sato, *et al.*, 2007).

Much evidence suggests that olfactory gene expression is not strictly stochastic, but rather it can depend on development, sex, physiological status and the environment. The expression of certain chemoreceptor genes in *C. elegans* is regulated by pheromones and food, allowing the animal to adjust its chemical sensitivity to the environment (cited in Bargmann, 2006). In the malaria mosquito, the expression of some receptor genes is down-regulated after a blood meal, suggesting that insects, like nematodes, may use gene-expression changes to alter sensitivity (cited in Bargmann, 2006). Fish olfactory receptors may differ between the sexes and may be induced at different times during development (Barth, *et al.*, 1996; Lastein, *et al.*, 2006). In zebrafish, specific receptor expression occurs asynchronously in the developing olfactory placode (Barth, *et al.*, 1996).

1.3.2 - Mechanisms of Olfactory Signal Transduction

The binding of an odorant to the receptor protein leads to the activation of intracellular signal transduction pathways that modify the frequency of action potentials travelling to the olfactory bulb. In fish, as in other animals, G proteins (heterotrimeric) mediate olfactory transduction. The G-protein-coupled receptor family has a highly conserved molecular architecture consisting of seven hydrophobic transmembrane regions linked by three extracellular loops and three intracellular loops (cited in Metpally, Sowdhamini, 2005; Simon, *et al.*, 1991).

With respect to ligand-binding, odorant receptors are similar to rhodopsin and related G-protein coupled receptors (reviewed in Bargmann, 2006). Rhodopsin-like G-protein coupled receptors exist in one of two main conformations: an inactive conformation, and an active conformation that interacts with an intracellular heterotrimeric G protein. The transition between these conformations occurs through the movement of various membrane-spanning domains. This movement is centred around a small molecule - the odorant - that interacts with several domains simultaneously. Although a subset of membrane-spanning region is most frequently associated with ligand binding, different agonists of the same G-protein coupled receptor do not necessarily bind to the same site(s) within the receptor. G-protein coupled receptor agonists stabilize the active form of the receptor, whereas antagonists can block agonist binding and inverse agonists stabilize the inactive conformation. Partial agonists can stabilize sub-conformations of the active state, allowing different ligands to have different effects. Thus the efficacy of a G-protein coupled receptor agonist does not depend on a single functional group or feature, or even necessarily on its affinity for the receptor binding site, but rather on its ability to stabilize the active state and/or destabilize the inactive state (reviewed in Bargmann, 2006). When agonists stabilize the active state of the receptor, conformational changes in the receptor allow it to couple to the G-protein. G-proteins consist of three subunits: an alpha subunit (the 'active' portion), and beta and gamma subunits that regulate the alpha subunit, although some studies suggest that beta and gamma subunits may also have regulatory effects on certain types of adenylate cyclase (Tang, Gilman, 1991; Wu, *et al.*, 1993). In the inactive state, G proteins bind guanosine di-phosphate

(GDP). When a ligand stabilizes the active state of a G-protein-coupled receptor, the G protein releases the GDP, binds a molecule of guanosine tri-phosphate (GTP) and becomes active. In this state, the alpha subunit separates from the other subunits and binds to an effector protein (*e.g.* adenylate cyclase, phospholipase C and phosphodiesterase; cited in Germann, Stanfield, 2002) and modulates its activity (Figure 1.10). This results in changes in the intracellular concentration of second messengers (cAMP, IP_3 and cGMP) and consequent modulation of enzyme activity (*e.g.* phosphorylases) or alterations in the ion-permeability of the plasma membrane. The properties of odorant receptors are likely to follow the rules described above for rhodopsin and related G-protein coupled receptors. Different types of G-proteins regulate different second messenger pathways.

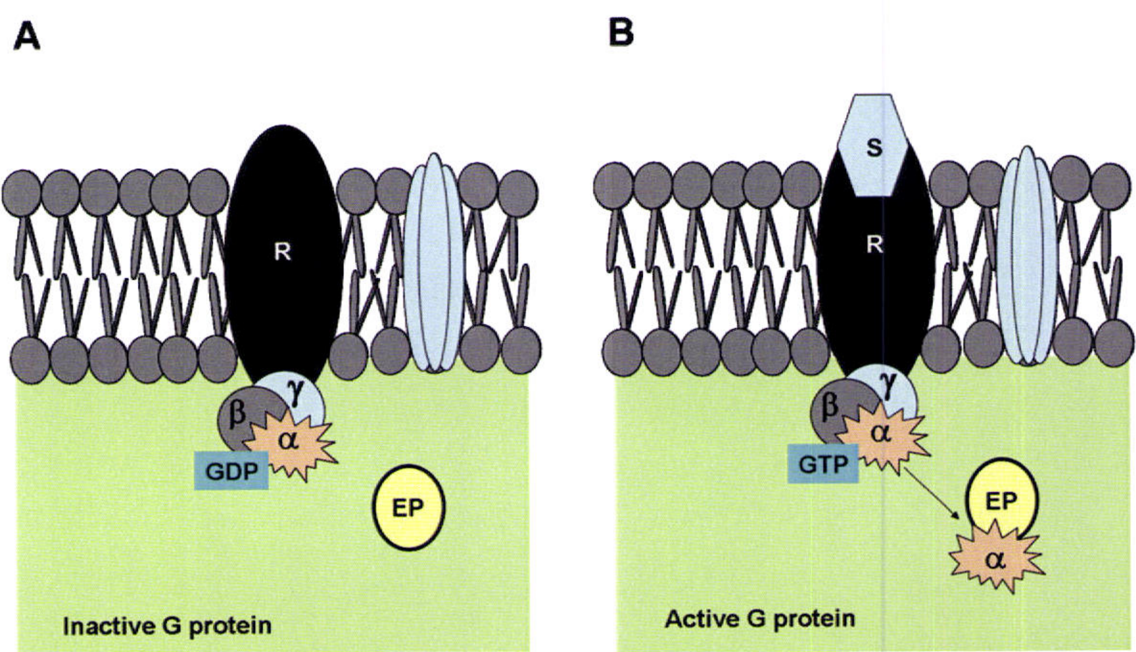

Figure 1.10 - General mechanism of G-protein activation. (R) olfactory receptor protein; (S) receptor ligand; (EP) effector protein.

In mammals, it is generally accepted that olfactory transduction occurs *via* the adenylate cyclase (AC) pathway (Chen, *et al.*, 2000; Wong, *et al.*, 2000); whether other pathways are involved - the dual second messenger model - remains unclear. However, in fish, the available literature suggests the contribution of both AC/cAMP and PLC/IP_3 pathways, for example in carp (Gdovskii, Ruzhinskaya, 2001), goldfish (Sorensen, Sato, 2005), channel catfish (Hansen, *et al.*, 2003) and Atlantic salmon (Lo, *et al.*, 1993; Lo, *et al.*, 1994).

In addition to AC/cAMP and PLC/IP_3, other pathways have been suggested to be involved. An increase of cyclic guanosine monophosphate (cGMP) on odorant exposure occurs in rats (Ingl, Ronnett, 1995) and salamanders (Leinders-Zufall, *et al.*, 1996; Zufall, Leinders-Zufall, 1997) raising the possibility that cGMP may act as second messenger. Furthermore, some evidence suggests the existence of inhibitory responses (Vogler, Schild, 1999). In invertebrates, it is thought that cAMP mediates inhibitory responses by opening a cAMP-gated K^+ channel (Ache, Zhainazarov, 1995). A single odorant can elicit an increase of firing frequency in one cell and/or reduce it in other cell and the same cell can be excited or inhibited depending on the odorant (Ache, Zhainazarov, 1995; Hatt, Ache, 1994; Schmiedel-Jakob, *et al.*, 1990). This is exciting because it suggests that peripheral olfactory transduction plays a role in odour quality coding. Even though the mechanisms involved in olfactory signal transduction and the extent to which different pathways operate in the same cell are yet to be resolved, it is becoming clear that intracellular signalling in the olfactory receptor neurons is sufficiently complex to allow the cells to serve as more than simple peripheral selectivity filters.

Adenosine 3',5'-cyclic monophosphate-mediated signalling - An odorant acting *via* increasing AC/cAMP, binds to its receptor and activates a specific G protein subtype (Golf; Jones, Reed, 1989) which, in turn, activates AC type III (Figure 3.1.2; Bakalyar, Reed, 1990). One adenylate cyclase molecule is capable of producing about a thousand molecules of cAMP per second. This then opens cyclic nucleotide-gated channels which are permeable to monovalent (mainly Na^+ and K^+) and divalent cations (including Ca^{2+}). Calcium reduces the permeability for other ions while passing through the pore so that, under physiological conditions, the current through these channels is almost exclusively carried by Ca^{2+} (Schild, Restrepo, 1998). Three cAMP molecules are required to open one channel, but hundreds of thousands of ions can pass through an open channel (reviewed in Firestein, 2001).

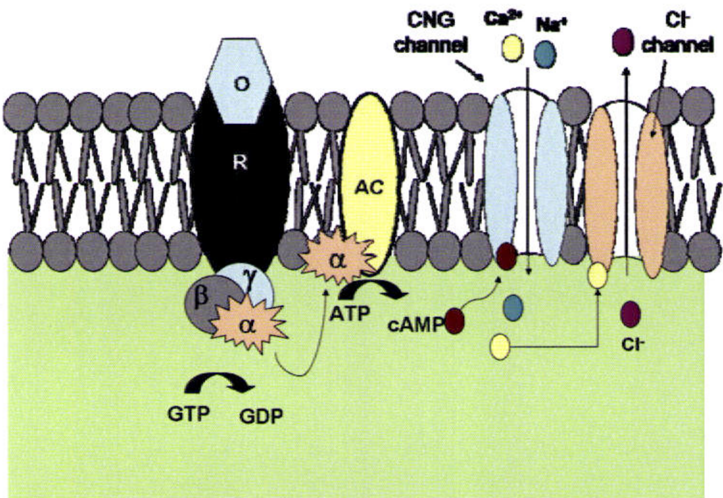

Figure 1.11 - Olfactory transduction via adenylate cyclase. (AC) adenylate cyclase; (R) receptor; (CNG channel) cyclic nucleotide gated channel; (O) odorant.

In addition, calcium ions activate other ion channels (Ca^{2+} activated Cl^- channels) permeable to the negatively charged chloride ion (Kleene, Gesteland, 1991; Sato, Suzuki, 2000). Normally, Cl^- ions mediate inhibitory responses due to

their concentration gradient. However, olfactory receptor neurons maintain an unusually high intracellular [Cl^-] (presumably by the action of a membrane pump) such that there is a Cl^- efflux when these channels are activated (Figure 1.11; Kaneko, *et al.*, 2004). As a result, the opening of the Ca^{2+} activated Cl^- channels leads not only to amplification of the odour-induced current, but also enhances the stability of the transduction current in a wide variety of extracellular ionic environments. A study with rainbow trout showed that Ca^{2+} activated Cl^- conductance plays an important role in fish olfactory transduction. Furthermore, the authors suggested that this mechanism allows fish to adapt to various ionic environments (Sato, Suzuki, 2000). In freshwater, ionic concentrations are low and the influx of cations (including Ca^{2+}) is small. However, increased intracellular [Ca^{2+}] due to Ca^{2+} influx activates Cl^- channels, resulting in the generation of a depolarizing receptor current sufficient for action potential generation. In seawater, however, the concentrations of Na^+, Ca^{2+} and other cations are high and the efflux of Cl^- in fresh water is replaced by influxes of Na^+ and Ca^{2+} (Sato, Suzuki, 2000). In this way, fish are able to smell in a wide range of salinities; such a mechanism may be especially important in species that move between sea- and freshwater.

Inositol triphosphate-mediated signalling - The identity of the G-protein(s) that couple(s) odorant receptors to IP_3 formation is not firmly established. There is a variety of classes of PLC enzymes and each of these occurs in a number of isoforms. Isoforms of the PLC β class were identified in the olfactory epithelium of lobsters (Abogadie, *et al.*, 1995; Xu, McClintock, 1999), rodents (Bruch, *et al.*,

1995; Elsaesser, *et al.*, 2005) and fish (Elsaesser, *et al.*, 2005). In addition, members of the G_q class of G-protein α subunits were found to activate PLC β in a variety of systems (Hepler, *et al.*, 1993; Lee, *et al.*, 1992; Wu, *et al.*, 1993; Wu, *et al.*, 1992a; Wu, *et al.*, 1992b). A number of laboratories have found immunoreactivity to G_q in olfactory cilia (Abogadie, *et al.*, 1995; Menco, *et al.*, 1994), antibodies raised against G_q and G_o inhibited IP_3-mediated whole-cell responses of cultured Caribbean spiny lobster (*Panulirus argus*) olfactory neurons (Fadool, *et al.*, 1995). This suggests that PLC β, together with G_q and G_o, are involved in IP_3-mediated olfactory transduction.

The IP_3-mediated olfactory transduction pathway is much less understood than the cAMP-mediated pathway. The exact mechanisms involved in action potential generation are yet unknown. A number of studies showed that PLC stimulation in olfactory receptors neurons causes increases in intracellular [IP_3] (Lo, *et al.*, 1994; Restrepo, *et al.*, 1993; Restrepo, *et al.*, 1990) and [Ca^{2+}] (Cadiou, *et al.*, 2000; Liu, *et al.*, 2006; Schild, *et al.*, 1995). Also, PLC activation leads to the occurrence of inwards cations currents in different systems (Cadiou, *et al.*, 2000; Liu, *et al.*, 2006; Okada, *et al.*, 1994; Restrepo, *et al.*, 1990; Schild, *et al.*, 1995) and IP_3 activated channels are present in the apical plasma membrane of receptor neurons (Cadiou, *et al.*, 2000; Hatt, Ache, 1994; Munger, *et al.*, 2000; Okada, *et al.*, 1994; Restrepo, *et al.*, 1990). Together, these findings suggest that odorants activate PLC, which then activates membrane cation channels. Consequently, Ca^{2+} and other cations enter the cell causing depolarisation and action potential generation. However, further studies are required in order to draw

definitive conclusions on the mechanisms involved in IP_3 mediated olfactory transduction.

Cyclic guanosine monophosphate signalling - Cyclic guanosine monophosphate (cGMP) is well established as the main second messenger in signal transduction in the retina. Gaseous second messengers, such as nitric oxide and carbon monoxide, produce cGMP and activate cyclic nucleotide-gated channels. In mice, a subset of olfactory neurons expresses cGMP-stimulated phosphodiesterase (PDE2; Juilfs, *et al.*, 1997). Furthermore, odorants increase cGMP levels in olfactory tissues of rats (Breer, *et al.*, 1992; Verma, *et al.*, 1993). When compared with the odorant-induced increase in cAMP and IP_3 levels, the rise in cGMP levels occurs with a slower, more sustained, time-course. This delayed response suggests that cGMP may not be involved in initial signalling events, but rather in longer-term events such as desensitization (Leinders-Zufall, *et al.*, 1996) or the activation of neuronal activity–dependent transcription (Moon, *et al.*, 1998). Thus, there is evidence of a role for cGMP in olfactory transduction but much remains unclear.

Inhibitory responses to odorants - Kurahashi *et al.* (1994) showed that odorants not only evoke an inward current in olfactory receptor cells, but can also suppress such currents. This suppression could result from a direct blockage of ion channels by odorants (Kurahashi, *et al.*, 1994). In channel catfish, a large percentage of receptor neurons respond to amino acids by decreasing firing frequency (Kang, Caprio, 1997). A similar result was found in the lobster, where odorants elicit either depolarization, mediated by opening of an IP_3-gated non-specific cation

channel, or hyperpolarization, mediated by opening of cAMP-gated K^+ channels (Boekhoff, *et al.*, 1994; Hatt, Ache, 1994; McClintock, Ache, 1989; Michel, Ache, 1992; Schmiedel-Jakob, *et al.*, 1990). Patch-clamp recordings from *Xenopus laevis* receptor neurons stimulated with different amino acids showed that each amino acid could induce both excitatory and inhibitory responses, although in different cells (Vogler, Schild, 1999).

Currently, knowledge of inhibitory responses and their functional importance is scarce. However, differential stimulation or suppression of receptor neurons by odorants could act as a mechanism for contrast enhancement; simultaneous stimulation and inhibition of different neurons by one odorant could be contrasted in the olfactory bulb in such a way that low odorant concentration could be detected at signal levels that could not be resolved from noise in a purely excitatory system (*cf* lateral inhibition in the visual system).

1.3.3 - Spatial coding within the olfactory bulb

Olfactory receptor neurons project to a particular glomerulus. Responses to amino acids, for example, occur mainly in the lateral bulb, whereas bile acids activate the medial bulb (Thommesen, 1978). In channel catfish, retrograde tracing experiments showed that ciliated receptor neurons predominantly project to the medial regions, which respond to bile acids, whereas microvillous neurons project to the dorsal surface where responses to nucleotides (posterior) and amino acids (anterior) predominate (Hansen, *et al.*, 2003). In transgenic zebrafish (which express fluorescent proteins), ciliated receptor neurons project their axons mainly to the dorsal and medial regions, whereas microvillous neurons project their axons

to the lateral region (Sato, *et al.*, 2005). Evidence for spatial coding within the olfactory bulb has been provided by studies in several species: rainbow trout (Hara, Zhang, 1996; Laberge, Hara, 2004; Riddle, Oakey, 1991; Riddle, *et al.*, 1993), brown trout (Laberge, Hara, 2004), Atlantic salmon (Hara, Zhang, 1996), crucian carp (Hamdani, Døving, 2003; Hamdani, Døving, 2006; Lastein, *et al.*, 2006), goldfish (Sorensen, *et al.*, 1991) and channel catfish (Nikonov, Caprio, 2001).

Although there are variations between different teleosts, available results suggest that food-related odorants (*e.g.* amino acids and nucleotides) are mapped onto regions of the bulb distinct from those of odorants related to social interactions (*e.g.* bile acids and pheromones). Food-related odorants are generally mapped onto the lateral part of the olfactory bulb whereas social related odorants seems to be preferentially mapped onto the medial bulb (Laberge, Hara, 2004; Nikonov, Caprio, 2001; Sorensen, *et al.*, 1991). In addition to this spacial coding of olfactory information, it was also suggested the occurrence of temporal coding within the olfactory bulb. Mitral cells, as well as local interneurons, form extensive lateral connections within the olfactory bulb. These connections involve lateral inhibition, which can refine the spatial and temporal patterns of output activity of mitral cells. Studies of presynaptic activity in different glomeruli of the zebrafish olfactory bulb showed that stimulation of the epithelium with amino acids induces complex combinatorial patterns of active glomeruli that are unique for different stimuli and concentration (Friedrich, Korsching, 1997). Quantitative analysis has shown that defined molecular features of stimuli were correlated with activity in spatially confined groups of glomeruli. This provides evidence that both

the identity and concentration of odorants are encoded by glomerular activity patterns. Furthermore, mixtures of odorants are detected differently from their individual components (Valentincic, Koce, 2000; Valentincic, *et al.*, 2000b). At some level, the brain discards information on individual constituents when a mixture is presented and individual components acquire a mixture-specific property. These mixture effects may arise from olfactory bulb interneurons as, sometimes, mitral cells that were excited by a single component were not responsive when that component was present in a mixture (Tabor, *et al.*, 2004).

1.3.4 - Functional specificity of the olfactory tracts

The axons of the mitral cells form the olfactory tract that conveys information to the telencephalic hemispheres. Axons from the mitral cells in the lateral part of the bulb travel mainly trough the lateral olfactory tract, whilst those in the medial part travel mainly through the medial tract. The neuronal activities on one side of the bulb are not influenced much by those on the opposite side, which is explained by limited dendritic fields of neurons in each part of the olfactory bulb (reviewed in Caprio, Derby, 2008).

Evidence suggests a functional division between the medial and the lateral olfactory tracts. In crucian carp, the lateral tract mediates feeding behaviour whereas the medial tract mediates reproductive and alarm behaviours (Hamdani, Døving, 2002; Hamdani, *et al.*, 2001a; Hamdani, *et al.*, 2001b; Weltzien, *et al.*, 2003). In goldfish, sectioning the medial olfactory tract reduces courtship behaviour, whilst feeding is reduced by cutting the lateral tract (Stacey, Kyle, 1983; von Rekowski, Zippel, 1993). Thus, olfactory information related to

reproduction or feeding may be processed independently through distinct “subsystems”.

Although most olfactory neurons terminate in the olfactory bulb, there is physiological and anatomical evidence for some olfactory sensory neurons terminating directly onto the telencephalon. In salmonids, putative pheromones failed to elicit any bulbar response; olfactory detection of hormonal pheromones may be processed in a manner distinct from that of amino acids and bile acids, and may possibly be mediated by extra-bulbar primary olfactory fibres bypassing the bulb (Hara, Zhang, 1998).

1.3.5 - Odorant coding within the Telencephalon

Another pertinent question is whether odotopic maps occur in higher olfactory centres (forebrain) and, if so, whether this organisation is related to odorant molecule types as suggested for in the olfactory bulb. An alternative possibility is that forebrain map is based in odorant function (*e.g.* feeding or social cues; reviewed in Caprio, Derby, 2008). Anatomical studies in rodents indicate that the neurons of single glomeruli project widely to down-stream forebrain targets and show considerable overlap (Haberly, 2001). This organizational pattern suggests that, in mammals, third-order neurons in the olfactory pathway integrate odour information arriving from multiple olfactory bulb glomeruli, possibly encoding features of odorant quality and which may also relate to the odour’s behavioural significance (Haberly, 2001). In channel catfish, the spatial mapping of different odorants in the olfactory bulb is similar to that described in the forebrain (Nikonov, *et al.*, 2005). Three classes of biologically relevant

odorants for fish are processed in distinct regions of the forebrain; feeding cues (mainly amino acids and nucleotides) are represented in lateral, pallial portions of the forebrain, equivalent to the olfactory cortex of amniote vertebrates, whereas social signals (bile acids) are represented in medial forebrain centres, possibly homologous to portions of the amygdala. Thus, in the forebrain, as in the olfactory bulb, the different odorant classes map onto different regions (Nikonov, *et al.*, 2005). Furthermore, a study aiming to determine specificity of forebrain neurons to four types of L-α-amino acids (neutral with long side-chains, neutral with short side-chains, basic and acidic), known biologically relevant odorants for teleosts, showed that group I forebrain units gave similar patterns of response as described previously for bulbar neurons (Nikonov, Caprio, 2007). This suggests that, at least for these forebrain units, no major modifications of olfactory information occur between the olfactory bulb and the forebrain. However, group II forebrain units exhibit an excitatory pattern dependent on odorant molecular structure different from that seen in bulbar units, suggesting convergence of odour information between the olfactory bulb and the forebrain.

The above section has attempted to show that, although much is known about the olfactory system of fish, there is still much to learn. In particular, it seems that the olfactory system of fish is different from – arguably more complex than - that of mammals. The following section will outline the model species of the current study and discuss why it may be a useful model in the study of olfaction in fish.

1.4 - The Senegalense Sole

The Senegalese sole (*Solea senegalensis)* is a benthic flatfish of the family Soleidae. It is distributed from the north African coastal waters and the western Mediterranean up to the Bay of Biscay (Cabral*, et al.*, 2003). The spawning season in the Atlantic coast occurs mainly in spring (May to June) and secondarily in autumn, but spawning may occur throughout the year (García-López*, et al.*, 2006). The eggs are planktonic and between 0.9-1.5 mm. Larvae are bilaterally symmetric and planktonic and, at hatching are 2.5-3.1 mm in length (López, 1999). They are carried to coastal zones by currents, where they undergo metamorphosis (López, 1999). Metamorphosis involves a 90º rotation of the body and the migration of the left eye to the right (upper) side, resulting in asymmetric benthic post-larvae (Munroe, 2005). Adults are benthonic and colonise habitats with sandy or muddy substrate in brackish lagoons or down to 100 m depth in the open sea (Gibson, 1997). The sole is gonochoric and sexual maturity is usually attained at a length of 30 cm. In culture, one year after hatching they reach the size of 16.6 ± 2.1 cm and 40.3 ± 2.5g; the maximum size is about 60 cm and 800 g (Munroe, 2005). Like other species of the family Soleidae, the Senegalese sole feeds mainly on polychaetes and molluscs (De Groot, 1971). Studies carried out in the Tagus estuary have shown that *S. senegalensis* eat polychaetes (mainly *Hediste diversicolor*), amphipods (*Corophium spp.*) and bivalve molluscs (*Scrobicularia plana*) (Cabral, 2000). Typically, these fish spend the whole day half-buried in sand with only their eyes and upper nostril exposed to open water, and carry out all activity - feeding in particular - at night. A closely related species (*Solea solea*)

is capable of finding its prey after removal of the eyes (De Groot, 1971). Thus, chemoreception seems likely to be the more important sense in food location.

1.4.1 - The olfactory organ of sole

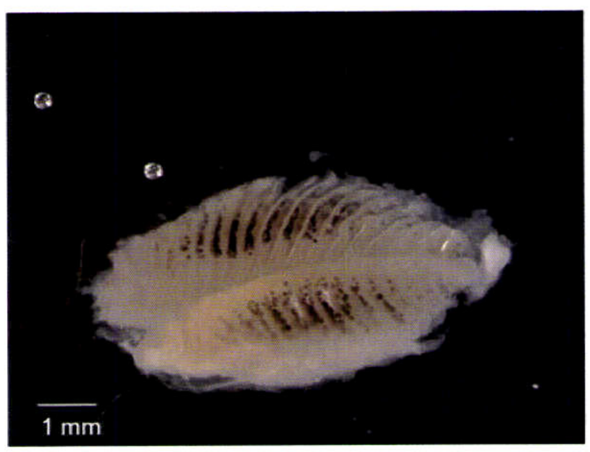

Figure 1.12 – Lower olfactory epithelium of the senegalese sole, *Solea senegalensis.*

Little is known about the olfactory system of the Senegalese sole. In the closely related *S. solea* each olfactory organ consists of a nasal chamber with olfactory lamellae, and a posterior and anterior nostril. The olfactory organs are connected to each other by means of a large unpaired infrasphenoidal sac. The respiratory movements cause changes in the volume of this sac, and this brings about water circulation in the two nasal chambers. The water enters through the anterior nostril and exits through the posterior nostril, which only opens to let the water pass (De Groot, 1971). Preliminary studies suggest that the Senegalese sole is able to control the movement of water between the two olfactory organs. Two different coloured dyes introduced into the lower and upper olfactory cavities, respectively, of anaesthetised fish, were expelled only through the exhalent nostril on the upper side when the fish recovered (Velez and Hubbard, unpublished observations). Flounders use coughing as an olfactory sampling mechanism (Nevitt, 1991); the possibility of coughing as an active olfactory sampling behaviour in the

Senegalese sole has not been investigated. However, the olfactory system of sole is anatomically similar to that of flounder so this remains a distinct possibility.

The olfactory epithelia are oval rosettes (Figure 1.12), consisting of a series of parallel lamellae with a central longitudinal raphe (Harvey, 1996). Electron microscopy studies on *S. solea* showed that, in both olfactory epithelia, two ciliated cell types and a microvillar cell are present soon after hatching and persist into adult stage. Densities of microvillar cells were significantly higher in the upper olfactory organ in two of the four regions examined while ciliated cells were significantly more dense in the lower olfactory organ in three of the four regions examined (Harvey, 1996). There are no studies on the types of receptor cell and their distribution within the olfactory lamellae and rosette on the Senegalese sole; preliminary studies showed the presence of cilliary and microvilli neurons; apparently, within each lamella, receptor cells are absent at the borders and increase in density towards the centre (Velez *et al.*, unpublished observations).

S. senegalensis is nearly always dextral, thus in post-metamorphic fish the eyes appear on the right side of the head and the left side faces towards the sediment. During metamorphosis, the left olfactory epithelium and associated structures do not migrate to the upper side, as does the eye, and so the right olfactory organ is located on the pigmented upper side, while the left olfactory organ faces the substratum (Rodríguez-Gómez, *et al.*, 2001). This effectively means that the two olfactory epithelia are exposed to two distinct environments; the upper nostril samples open water while the lower nostril samples interstitial water. This raises the possibility that the two olfactory epithelia have evolved sensitivity to different compounds and a functional difference in the roles that the

two olfactory organs play. Furthermore, the upper epithelium is slightly larger and has more lamellae than the lower epithelium (Velez, *et al.*, 2005). The fact that the main preys of sole live under the substrate and the lower olfactory nostril is in contact with the bottom raises the possibility that the lower olfactory epithelium may be specialized for food location. However, plugging the lower olfactory organ of *S. solea* has little effect on food intake, whereas blocking the upper olfactory organ prevented it (De Groot, 1971). Although this suggests that the upper olfactory epithelium is more important in food search than the lower, a possible functional difference between the two epithelia is still valid. Electrophysiological studies (Velez, *et al.*, 2005) have shown that upper and lower olfactory epithelia of *S. senegalensis* have different sensitivities to different odorants; some amino acids are better detected by the lower epithelium (*e.g.* aromatic amino acids) whilst others are better detected by the upper (*e.g.* glycine). In addition, the upper epithelium is more sensitive to conspecific body fluids (bile fluid and intestinal fluid) than the lower suggesting a difference between the roles that the two nostrils play. It is also possible that the upper and lower olfactory bulbs process olfactory input differently, even when concerning the same odorant(s); sensing a particular odorant in the interstitial water may affect the behaviour in a different way from when sensed in the open water.

1.4.2 - The Olfactory Bulbs of S. senegalensis

The most rostral part of the brain of the Senegalese sole consists of the sessile olfactory bulbs (Figure 1.13), which are coupled to the antero-ventral portion of the telencephalic lobes (Rodríguez-Gómez, *et al.*, 2000). The olfactory bulbs are

relatively large and asymmetric in shape and size, the right bulb being ovoid, whereas the left bulb is roughly spherical. The right olfactory nerve and bulb are slightly larger than the left ones (Rodríguez-Gómez, *et al.*, 2000). Thus, the asymmetric external morphology of *S. senegalensis* is correlated with asymmetry of the olfactory system and forebrain. The olfactory bulbs have four concentric cell layers, from the centre to the periphery; an internal cell layer which exhibit densely packed round and ovoid small cells, a secondary olfactory fibre layer, an external cell layer, which contains small rounded cells and larger mitral cells, and a glomerular layer almost devoid of cells (Rodríguez-Gómez, *et al.*, 2000). Just rostral to the junction of the olfactory bulbs with the ventral telencephalon lay a group of large polymorphic cell bodies distributed along the ventro-medial surface of the olfactory bulbs. These neurons, two to seven by section, could be the ganglion cells of the terminal nerve (Rodríguez-Gómez, *et al.*, 2000).

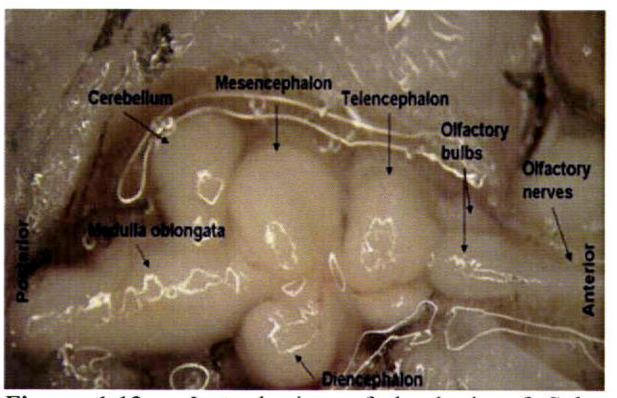

Figure 1.13 – Lateral view of the brain of *Solea senegalensis*.

1.4.3 - Odorants

Very little is known about the types of compounds to which *S. senegalensis* has olfactory sensitivity, although it has high olfactory sensitivity to amino acids and conspecific body fluids (Velez *et al.*, 2005). Behavioural studies carried out with juvenile *S. solea* that were fed either with an unflavoured casein based

particulate diet or with an identical diet flavoured with mussels showed that fish did not accept the untreated diet. However, they readily ate the diet flavoured with either mussel flesh or a mixture of pure chemicals whose composition was based on an analysis of the low molecular weight fraction of mussel flesh. The active constituent in the mixture of pure chemicals was identified as glycine betaine for fish wet weight exceeding 50g, whereas glycine betaine plus certain amino acids was required for fish about 2.5g wet weight (Mackie, *et al.*, 1980). Another study on *S. solea* larvae showed that the most potent substances evoking feeding behaviour are L-phenylalanine, L-lysine, L-asparagine, inosine 5-monophosphate and glycine betaine (Knutsen, 1992). It is known that *S. senegalensis* has high olfactory sensitivity to amino acids in general (Velez, *et al.*, 2005), but no olfactory sensitivity to glycine betaine or inosine 5-monophosphate (Velez *et al.*, unpublished observations).

1.4.4 - Senegalese sole as model in the study of olfaction

The vast majority of studies on fish olfaction were carried with freshwater species. In orther to further the current knowledge on fish olfaction, it is important to increase information on marine species. Given the apparent functional asymmetry in the olfactory system of the Senegalese sole (Velez, *et al.*, 2005), understanding of the neuronal mechanisms involved in this asymmetry, and its ecological importance, may be instrumental in explaining the organisation of modern olfactory systems. Therefore, the main objective of the current work was to explore the biological significance of this olfactory asymmetry and the neural mechanisms involved.

Based on the available evidence, our hypothesis was that the lower epithelium is specialised for food detection and/or localisation and the upper for pheromone detection. To test this, we identified some of the active compounds in samples from two different sources; i) *Hediste diversicolor* (the sole's main prey) and ii) conspecific body-fluids (probably used in intra-specific interactions). The olfactory potency of each identified compound was then assessed by electro-olfactogram (EOG) in both olfactory epithelia.

Another pertinent question is whether olfactory transduction pathways in marine fish are similar to those described for freshwater species. In the marine environment, the availability of different ions is much higher than in freshwater. Furthermore, sole often live in habitats with reduced salinities; they can be found in sandy or muddy substrates in lagoons (where the salinity can vary daily) or down to 100 m depth in the open sea. Freshwater species are, in many ways, different from marine species, so we hypothesised that the neuronal mechanisms involved in olfaction could be different. Furthermore, it is also possible that the pathways involved in the detection of different odorants differ between the two epithelia. To answer these questions, pharmacological and electrophysiological studies were carried out on both olfactory epithelia.

Thus, the central aims of this thesis were i) the identification of naturally occurring odorants derived from prey and conspecifics, ii) to establish whether these odorants are detected differentially by the two olfactory epithelia and iii) to investigate whether the transduction mechanisms may differ from freshwater fish and/or between the two epithelia.

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Chapter II

Experimental Work

II – Technical Research Work

2.1 - Introduction

The current chapter aims to outline the main methods involved in this thesis. This description starts with the techniques of chemical separation and identification (solid phase extraction, chromatography and mass spectrometry) of odorants followed by the electrophysiological approaches (electro-olfactogram and electro-encephalogram) used to assess olfactory potency. This is followed by six scientific papers grouped into four themes: i) chemical identification of food-related odorants (papers I and II); ii) identification of conspecific-derived odorants (putative pheromones) (papers III and IV); iii) transduction pathways in olfactory receptor neurones (Paper V); and iv) olfactory sensitivity to inorganic cations (paper VI). All together these six papers summarize the research work developed during this PhD.

2.1.1 Chemical identification techniques:

Solid phase extraction (SPE) is a method of sample preparation that concentrates and purifies analytes from solutions; the separation ability of SPE is based on the preferential affinity of the desired analyte(s) in a liquid for a solid stationary phase through which the sample is passed (Thurman, Mills, 1998). A typical SPE process involves four basic steps. First, the cartridge is activated with a non-polar or slightly polar solvent, which wets the surface and penetrates the bound phase. Then water or buffer of the same composition as the sample is passed trough the column followed by the sample itself. As the sample passes through the stationary phase, the analytes in the sample interact with, and some

are retained by, the sorbent while the solvent, salts, and other impurities pass through the cartridge. After the sample is loaded, the cartridge is washed with buffer or solvent to remove further impurities. Then the analyte(s) are eluted with non-polar solvents or a buffer at the appropriate pH (Thurman, Mills, 1998). The mechanisms of retention include ion exchange, normal phase and reverse phase. Ion exchange sorbents separate analytes based on electrostatic interactions between the analyte of interest and the charged groups of the stationary phase. For ion exchange to occur, both the stationary phase and the sample must be at a pH where both are charged. To elute the analytes from the sorbent, the stationary phase is washed with a solvent that neutralises the charges of either the analyte, the stationary phase, or both. Once the charge is neutralised, the electrostatic interactions between the analyte and the stationary phase no longer exist and the analyte elutes from the cartridge (Thurman, Mills, 1998).

Normal-phase sorbents consist of a stationary phase that is more polar than the solvent or sample matrix that is applied to the SPE sorbent. Thus, water is not usually a solvent in normal-phase SPE because is too polar. Normal-phase sorbents, therefore, are used in SPE when an organic solvent contains the analyte of interest. Polar interactions, such as hydrogen bonding and dipole-dipole interactions, are the primary mechanisms for solute retention (Thurman, Mills, 1998).

Reverse-phase sorbents are more hydrophobic than the sample. Reverse-phase sorbents are commonly used in SPE when aqueous samples are involved. The mechanism of interaction is through van der Waal's forces and occasionally secondary interactions such as hydrogen bonding and dipole-dipole interactions. The stationary phase of a reverse-phase SPE cartridge retains compounds of mid

to low polarity due to hydrophobic effect. The analytes can be eluted by washing the cartridge with a non-polar solvent, which disrupts the interactions of the analyte and the stationary phase (Thurman, Mills, 1998).

In this thesis, solid phase extraction of food related odorants (paper I) was carried out using C18 cartridges. Two fractions were obtained; the extract (retained compounds); with compounds relatively hydrophobic and the filtrate (compounds that passed through) containing substances relatively hydrophilic. Solid phase extraction of body fluids (paper III) was carried with C18 and C2/ENV+ cartridges. C2/ENV+ cartridges are more effective retaining relatively hydrophilic compounds than C18 (Ingvarsdóttir, *et al.*, 2002), thus each sample was first fractionated using C18 cartridges. The filtrates of C18 cartridges were then passed through C2/ENV+ cartridges. Thus, we obtained three fractions containing compounds with different degrees of hydrophobicity: i) a fraction containing compounds that were not retained by the C2/ENV+ cartridges and thus are relatively hydrophilic; ii) a fraction with compounds retained by the C18 cartridges and are, therefore, relatively hydrophobic and iii) a fraction with substances that were not retained by C18 cartridges but were retained by C2/ENV+ cartridges and are therefore of intermediate hydrophobicity.

Chromatography – The chief purpose of chromatography is to separate compounds from a mixture. Most separations depend upon the distribution of substances between two phases, which are chosen so as to obtain the maximum difference between the distribution of the desired substances and the unwanted impurities (Smith, 2004). In the current thesis, two different types of chromatography were used; gas chromatography (paper I) and liquid chromatography (paper II and IV). In liquid chromatography the sample is

applied to the column, containing a stationary material, and a liquid mobile phase is passed through the column so that the components are eluted. Liquid chromatography that uses very small packing particles and a relatively high pressure is referred to as high performance liquid chromatography (HPLC). The dominance of HPLC is due to its versatility, as it encompasses a variety of separation modes, the principal modes being adsorption, partition, ion exchange and size exclusion. In adsorption chromatography, the analytes interact with a solid stationary surface and are displaced by competition with the eluent for the active sites on the surface (Smith, 2004). In size exclusion, chromatography the resistive force for separation is the physical size of the analyte, which determines their accessibility to differently size pores in the stationary phase material (Smith, 2004). The original chromatographic method based on adsorption was the normal-phase separation in this mode the stationary phase is polar, usually silica or alumina, and the analytes are retained by the column according to their polarity by a polar-polar interaction. Thus, weakly polar analytes are readily eluted, whereas alcohols and amines are retained. The strength of the eluent is determined by its interaction with the stationary phase and, hence, its ability to compete with the analyte for the stationary phase (Smith, 2004). In contrast, in reverse-phase chromatography, these parameters are reversed; normally a non-polar stationary phase is used, consisting of a bonded hydrocarbon layer on a solid support, with a polar mobile phase. The analyte is partitioned between the mobile and stationary phases so that low-polarity analytes are more readily retained because they dissolve in the hydrocarbon layer and are less soluble in the mobile phase, whereas polar analytes favour the mobile phase and are more rapidly eluted (Smith, 2004).

Gas chromatography (GC) is a separation technique in which the mobile phase is a gas. This separation technique is based on the partition equilibrium of the analyte between a solid stationary phase and a mobile gas (usually helium). GC is widely used in analytical chemistry, although the high temperatures used make it unsuitable for high molecular weight biopolymers or proteins; heat will denature them (Marriott, 2004).

Mass spectrometry is an analytical technique that employs chemical fragmentation of the samples into charged particles (ions).The design of a mass spectrometer has three essential modules: an ion source, which transform the molecules in a sample into ionized fragments; a mass analyzer, which sorts the ions by their masses by applying electrical or magnetic fields; and a detector, which measures the value of some indicator quantity and thus provides data for calculating the abundances of each ion present (Nelson, Cox, 2000). Analytes are first ionized in a vacuum, when the newly charged molecules are introduced into an electric and/or magnetic field, and their paths through the field are a function of their mass-to-charge ratio, m/z. This measured property of the ionized species can be used to deduce mass of the analyte with high precision. Techniques for ionization have been key to determining what types of samples can be analysed by mass spectrometry. Electro-ionization and chemical ionization are used for gases and vapours. For liquid and solid biological samples, the most common techniques are electrospray ionization and matrix-assisted laser desorption/ionization (MALDI; Nelson, Cox, 2000).

An important enhancement to the mass resolving and mass determining capacities of mass spectrometry is using it in tandem with chromatographic separation techniques. A common combination is gas chromatography-mass

spectrometry (GC-MS). In this technique, a gas chromatograph is used to separate different compounds and the stream of separated compounds is fed online into the ion source (a metallic filament to which voltage is applied). This filament emits electrons which ionize the compounds. The ions can then fragment further, yielding predictable patterns. Intact ions and fragments pass into the detector of the mass spectrometer (Nelson, Cox, 2000). In the current thesis, the amino acid analysis was carried out by GC-MS (paper I). Given that amino acids are non-volatile compounds, samples had to be derivatized before GC-MS analyses. Derivatization is a technique which transforms a chemical compound into a product of a related structure. Generally a specific functional group of the analytes participates in the derivatization reaction; changes in a functional group often alters the chemical properties of compounds (Blau, Halket, 1997a). Here, derivatization of amino acids was carried out by acylation, which involves the introduction of an acyl group into a molecule with a replaceable hydrogen atom (Blau, Halket, 1997b). Acylation confers volatility onto substances such as carbohydrates or amino acids that are not volatile and normally decompose on heating; this makes it possible to analyse this class of compounds by GC. In addition, acylation changes the chemical properties of amino acids allowing the chromatographic separation which is not possible with the underivatized compound (Blau, Halket, 1997b).

Similar to GC-MS, liquid chromatography mass spectrometry (LC-MS) separates compounds chromatographically before they are introduced into the ion source and mass spectrometer. It differs from GC-MS in that the mobile phase is liquid, instead of gas (Nelson, Cox, 2000). The ionization source used in the current work and also the most common used in LC, was the electrospray

ionization source. In the electrospray ionization, molecules in solution are forced directly from the liquid to gas phase. A solution of analytes is passed through a charge needle that is kept at high electrical potential, dispersing the solution into a fine mist of charge microdroplets. The solvent, which is usually much more volatile than the analyte, rapidly evaporates and the resulting multiple charged macromolecular ions are thus introduced non-destructively into a gas phase. In the electrospray process, the ions may be formed by the addition of a proton (a hydrogen ion) and denoted $[M+H]^+$ or the removal of a proton $[M-H]^-$ (Nelson, Cox, 2000).

2.1.2 Electrophysiological techniques

There are different methods that allow the evaluation the olfactory sensitivity of fish to odorants; the two techniques used in the work described in the current thesis were the electro-olfactogram (EOG) and electroencephalogram (EEG).

Electro-olfactogram

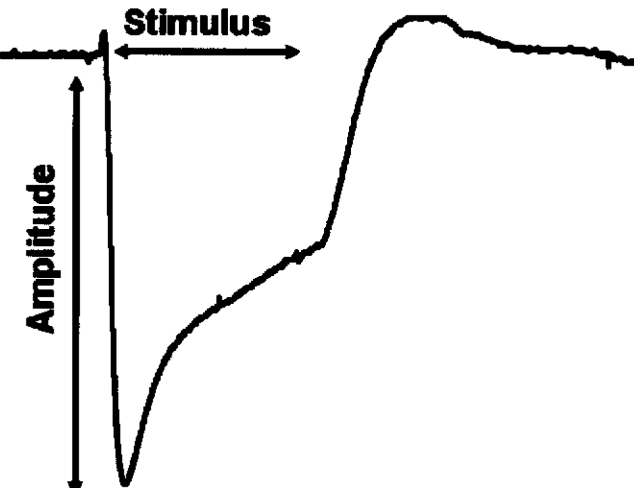

Figure 2.1 – Typical EOG record from the olfactory epithelium of the Senegalese sole using the software 'Axoscope'. The amplitude was measured as the initial negative deflection of the trace following the arrival of the stimulus at the epithelium. Note that there is a period of accommodation following this peak, even in the continued presence of the stimulus. After the stimulus finishes, the trace returns to baseline.

The EOG is a direct current (DC) 'field' voltage measured from above the olfactory epithelium. It is generally assumed to be a summation of the generator potentials of olfactory receptor neurones responding to a given stimulus (Scott, Scott-Johnson, 2002).

EOG usually gives negative wave that corresponds to a sink caused by positive charges entering cells (Figure 2.1). The main advantages of the EOG are; the large amplitude responses (can be greater than 20 mV), 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 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 from marine fish are much smaller than those from freshwater fish (in euryhaline fish, this effect can be reduced by lowering the salinity of the water; Velez, *et al.*, 2005). This also means that responses of odorants that themselves affect the conductivity of the water (*e.g.* Ca^{2+} and Na^{+}) cannot be measured by EOG (e.g. Hubbard, Canário, 2007).

Electroencephalogram (EEG)

The EEG is an alternating current (AC) extra-cellular recording method in which the electrodes are placed on the surface of, in this case, the olfactory bulb. Olfactory neurons normally show spontaneous activity (impulse discharges without chemical stimuli) and respond to stimuli, in the majority of cases, by increasing firing frequency (reviewed in Hara, 1994). This method measures the increases of neuronal activity of the neurones in the olfactory bulb. It is usually necessary to 'integrate' the response (summate the electrical activity) electronically to get an easily quantifiable measure of activity (Figure 2.2).

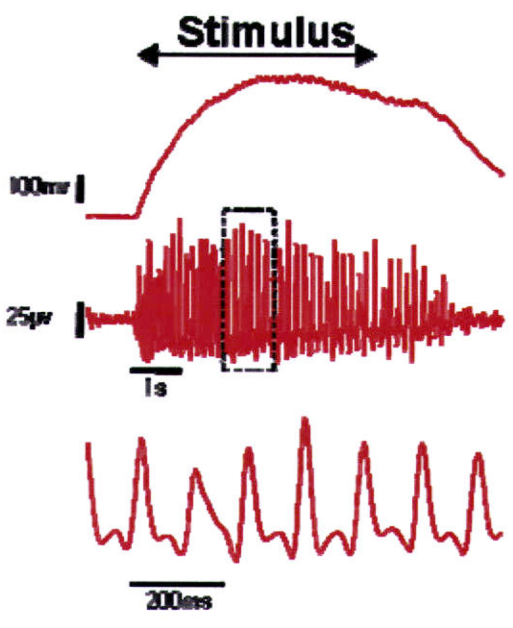

Figure 2.2 – Typical EEG record from the olfactory bulb of the Senegalese sole. The upper trace is the integrated signal, the middle trace is the raw EEG signal and the lower trace shows an expansion of the section outlined by the rectangle to show the wave-form and frequency. Horizontal bar indicated the presence of the stimulus at the epithelium.

Alternatively, action potentials can be counted and expressed as frequency (slightly more complicated). The main advantage of the EEG is the fact that the signal is independent of external salinity (i.e. can be carried out in both marine and freshwater species or whilst external concentrations of cations are being changed e.g. Hubbard*, et al.*, 2000). The main disadvantages of this technique are; as the responses are of much lower amplitude than the EOG, so this technique requires slightly more sophisticated equipment. It also often requires extensive surgery to the fish (the fish cannot recover after recording) and responses induced by a given stimulus differ in magnitude at different regions of the bulb, thus the position of the electrode may affect *relative* amplitude of responses.

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2.2.1 Chemical identification of food related odorants

Summary

Little is known about the type of chemical cues used by sole to detect prey; this is true for marine fish in general. Fish are known to have high olfactory sensitivity to amino acids and it is believed that these compounds are used as cues in food-search behaviour. In the last few years, many studies have focused on the identification of feeding stimulants in fish. Most of these studies used macerates of natural prey organisms; however, it is not known whether any of these compounds are released by the prey under natural conditions.

The objective of the work described in the current section was two-fold; to identify odorants in both whole-body macerates and water conditioned by living ragworms (one of the sole's chief prey species), and to evaluate whether these odorants are more potent at the lower olfactory epithelium.

Both whole-body macerates and substances released to the water by living ragworms were fractionated by molecular weight filtration followed by solid-phase extraction (SPE), and the olfactory potency of the resultant fractions was assessed by the EOG. In the macerate, the majority of odorants were small molecular weight compounds (<500 Da) which were not retained by C18 SPE cartridges (*i.e.* remained in the filtrate). In worm-conditioned water, the odorants were also small molecular weight compounds (<500 Da) but the majority of olfactory activity could be extracted by C18 SPE cartridges (*i.e.* were in the extract).

The amino acid concentrations of the macerate and water were determined by gas chromatography and mass spectrometry (GC–MS). C18 SPE cartridges do not retain amino acids, thus after extraction amino acids were only detected in the filtrate of both homogenate and worm-conditioned water. An artificial mixture of amino acids at the same concentrations as found in the macerate had similar olfactory potency. However,

the concentrations of amino acids in worm-conditioned water were too low to contribute greatly to its olfactory potency. The most abundant amino acid present in the macerate was glycine, followed by L-aspartic acid. The sole has olfactory sensitivity to glycine but not to acidic amino acids such as L-aspartic acid. In worm-conditioned water, L-glutamate was the most abundant amino acid. This, again, is an acidic amino acid. The next most abundant amino acids were L-phenylalanine and glycine, both with significant olfactory potency. These results suggest that, whilst the olfactory sensitivity to the macerate can be explained mostly by its amino acid content, living ragworms are releasing odorants (which are retained by the C18 cartridge) other than amino acids. Thus, the C18 extract of worm-conditioned water was fractionated by reverse-phase high-performance liquid chromatography (HPLC). The fractions were then tested for olfactory potency by EOG. Most olfactory activity was found in the first two fractions to elute from the HPLC column (0-4 minutes); these fractions were also more potent at the lower olfactory epithelium than the upper. Liquid chromatography-mass spectrometry (LC-MS) of both fractions gave a spectrum with a molecular mass of 219.4 Da and a fragment of 205.3 Da as the most abundant solute. These data are consistent with a methylated form of tryptophan. Commercially available 1-methyl-L-tryptophan had a similar HPLC retention time (2.8 minutes) and a similar LC-MS spectrum. Furthermore, the lower olfactory epithelium is more sensitive to 1-methyl-L-tryptophan than the upper. Also, cross-adaptation suggested that the olfactory receptors responding to 1-methyl-L-tryptophan are different between the two epithelia.

Taken together these results suggest that, similar to other fish species, amino acids may play an important role in food detection in sole. Glycine may be important, given that it was among the more abundant amino acids found in both homogenate and worm-conditioned water. Acidic amino acids were also found in high concentrations in both

samples. As the sole does not have olfactory sensitivity to acidic amino acids, it may be that these are more important in the gustatory response. L-phenylalanine and 1-methyl-L-tryptophan were both identified in worm-conditioned water. 1-Methyl-L-tryptophan is responsible for the majority of its olfactory potency. Furthermore, both compounds are more potent at the lower epithelium than the upper, and there are receptors on the lower nostril responding specifically to 1-methyl-L-tryptophan. These results suggest that 1-methyl-L-tryptophan may be used as a chemical cue to detect a specific prey species (ragworm) and that the lower olfactory epithelium is specialised for this.

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Aquaculture 265 (2007) 336–342

Aquaculture

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The contribution of amino acids to the odour of a prey species in the Senegalese sole (*Solea senegalensis*)

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Received 18 December 2006; received in revised form 13 February 2007; accepted 16 February 2007

Abstract

For many fish, olfaction is important in food search and consumption. Amino acids are known to elicit feeding behaviour in several species. The aim of the current study was to evaluate the contribution of amino acids to the odour of a natural prey organism of the Senegalese sole (*Solea senegalensis*). Both whole-body macerates and substances released to the water by living ragworms (*Hediste diversicolor*) were fractionated by molecular weight filtration followed by solid-phase extraction (SPE), and the olfactory activity of the resultant fractions was assessed by the electro-olfactogram (EOG) in the sole. The amino acid concentrations of the macerate and water were determined by gas chromatography and mass spectrometry (GC MS). In the macerate, the majority of odorants were small molecular weight compounds (<500 Da) which were not retained by C-18 SPE cartridges. An artificial mixture of amino acids at the same concentrations as found in the macerate had similar olfactory potency. The odorants released to the water by living ragworms were also small molecular weight compounds (<500 Da) but the majority of olfactory activity could be extracted by C-18 SPE cartridges. The concentrations of amino acids in these samples were too low to contribute greatly to its olfactory potency. These results suggest that, whilst olfactory sensitivity to amino acids may explain most of the potency of the macerate, living ragworms are releasing additional odorants other than amino acids which may be equally important in chemosensory food location in the sole.
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Keywords: Olfaction; Feeding; Amino acids; *Solea senegalensis*; *Hediste diversicolor*

1. Introduction

Many fish species, particularly those with nocturnal activity and/or from habitats with high water turbidity, rely heavily on chemo-sensory mechanisms to detect food (Hara, 1994). In different species, feeding behaviour is triggered by different chemical substances which may act as attractants *via* olfaction or taste (Hara, 1994). A range of low molecular weight compounds such as amino acids, nucleotides, quaternary ammonium compounds and organic acids have been found in tissue extracts of a range of marine animals (Carr et al., 1996). Fish, in general, have a well-defined olfactory sensitivity

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doi:10.1016/j.aquaculture.2007.02.029

to L-amino acids (Hara, 1994); this phenomenon is generally held to be involved in the location and identification of food. Although most of our current knowledge about the classes of compounds to which fish have olfactory sensitivity stems from freshwater species, several studies have shown that marine fish are equally sensitive to amino acids (e.g. Goh and Tamura, 1980; Hubbard et al., 2003a; Ishida and Kobayashi, 1992; Silver et al., 1976; Velez et al., 2005; Yacoob and Browman, 2007; Yacoob et al., 2004).

The Senegalese sole (*Solea senegalensis*), a fish of high economic value both as farmed and wild-caught (Imsland et al., 2003), is a benthic nocturnal flatfish which feeds mainly on invertebrates living in the sediment, mainly polychaetes (the ragworm *Hediste diversicolor*) and bivalves (*Scrobicularia plana*; Cabral, 2000). Other fish species do not form part of the natural diet of sole so conventional feeds, formulated with fishmeal as one of the main ingredients, are not attractive to sole (Reig et al., 2003). In the closely related sole (*S. solea*) glycine, betaine plus certain amino acids are effective in stimulating feeding behaviour and increasing food intake (Knutsen, 1992; Mackie et al., 1980). It has previously been shown that *S. senegalensis* have olfactory sensitivity to amino acids (Velez et al., 2005). However, it is not known which amino acids, or other classes of compounds, are released into the water by natural prey species of the sole; most previous work of this type has focused on extracts of whole-body macerates of prey species (e.g. Carr et al., 1996). Accordingly, the aim of this study was two-fold; to evaluate the contribution of amino acids to the odour of one of the sole's main prey species, the ragworm (*H. diversicolor*), and assess the involvement of other, non-amino acid compounds in this odour. This was done by molecular weight filtration and solid-phase extraction of both ragworm macerates and of substances released by living ragworms, followed by measurement of amino acid concentrations and assessment of the olfactory potency of these fractions by the electro-olfactogram (EOG).

2. Materials and methods

2.1. Ragworm macerate

H. diversicolor, caught in the Ria Formosa (Algarve, Portugal), were bought from local fishermen. The ragworms were homogenised in distilled water with a blender (10 g wet-weight 100 ml^{-1}) and filtered sequentially through 100 μm, 50 μm, 10 μm, and 1.2 μm (Whatman GF/C filters, VWR International Ltd, Lisbon, Portugal). The final filtrate was then ultra-filtered consecutively at 4 °C through 10 kDa Amicon Centripep-10 tubes, 3 kDa Amicon Centripep-3 tubes (Millipore Ibérica S.A., Madrid, Spain) and finally through 500 Da Amicon ultra-filtration membranes (Amicon Limited, U.K.). The fraction containing compounds smaller than 500 Da was then passed through a solid-phase extraction (SPE) C-18 cartridge (IST — International Sorbent Technology, Hengoed, U.K.). These cartridges do not retain small, relatively polar compounds such as amino acids. Extractions were carried out according to the manufacturer's instructions. Briefly, cartridges were conditioned with methanol (2 ml) and distilled water (2 ml). 10 ml samples of the homogenates (<500 Da) were then passed through the cartridges. Substances retained were eluted with 2 ml pure ethanol (eluate), evaporated (under nitrogen) and the residue was dissolved in 10 ml of 12 ppt sea water aliquot and stored at −20 °C until use. The filtrate was also aliquotted and frozen (−20 °C).

2.2. Ragworm water

H. diversicolor (100 g) were kept in paper towel moistened with artificial seawater (35 ppt) for 24 h at 4 °C; polychaetes survive well under these conditions in captivity. The paper towel was then soaked in distilled water (1000 ml), mixed thoroughly and the water, containing soluble compounds released by the ragworms, carefully decanted off. To remove any salt from this water, a sub-sample (10 ml) was freeze-dried and re-dissolved in methanol (10 ml). This was vortexed thoroughly and then centrifuged at 5000 *g* for 10 min. The supernatant was collected and evaporated under nitrogen and the residue re-dissolved in distilled water (final concentration; 0.1 g worm ml^{-1}). A water control was also prepared in exactly the same way except without addition of the ragworms. Samples and the control were then filtered according to molecular weight using 500 Da Amicon ultra-filtration membranes (Amicon Limited, U.K.), as described above. The fraction containing compounds smaller than 500 Da was then passed through a C-18 cartridge (IST — International Sorbent Technology) as above. The eluate and filtrate were stored at −20 °C.

2.3. Measurement of amino acids

The fractions of ragworm macerate and water containing compounds less than 500 Da (*i.e.* before being

passed through the SPE cartridges) were derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) plus trimethylchlorosilane (TMCS) according to the procedure described by Deng et al. (2005). Using this approach we were able to derivatize 24 amino acids. Briefly, samples (200 μl) and the internal standard (L-norvaline; 20 mg/ml) in aqueous solution were placed into a 2 ml screw-cap vial and lyophilized. When completely dry, 500 μl of acetonitrile and 500 μl of MBSTFA+TMCS (99:1, v/v) were added. The reaction was preformed under microwave irradiation at a power of 750 W for 60 s. After cooling to room temperature, samples (1 μl) were injected on the GC–MS.

2.4. Gas chromatography/mass spectrometry

The GC–MS system used was an Agilent 6890N gas chromatograph equipped with an Agilent 7683 series injector and an Agilent 5973 inert mass-selective detector (Agilent technologies UK Ltd, West Lothian, U.K.). The capillary column used for simple chromatography was a 30 m × 0.25 mm I.D. fused-silica column (Agilent HP-5MS, 0.25 μm film thickness). Helium, at a flow-rate of 0.8 ml min^{-1}, was used as a carrier gas. The oven initial temperature was 80 °C, followed by an increase to 150 °C at 10 °C min^{-1} and then to 300 °C at 15 °C min^{-1} and this final temperature was maintained for 5 min. The temperature of the injection port was set at 250 °C and that of the detector was set at 280 °C. Split injection mode was used with a split rate of 20:1. The mass-selective detector was operated in electron impact (EI) mode at 70 eV of activation energy. To confirm the mass fragment of the derivatives, data were obtained in the full scan mode with a scan range from m/z 50 to 550. Data were collected and analysed using a personal computer with MSD ChemStation software (Agilent technologies UK Ltd, West Lothian, UK).

2.5. Amino acid quantification

To construct calibration curves, amino acids at various concentrations (100 μg ml^{-1}, 40 μg ml^{-1}, 10 μg ml^{-1} and 5 μg ml^{-1}) were prepared and analysed using the same derivatization and GC–MS analytical procedure used for the samples. The calibration curves were obtained by plotting the peak area ratio between the derivatives of amino acids and that of L-norvaline (internal standard). For measuring limits of quantification and detection, standards were serially diluted and processed according to the procedure described above.

2.6. Recording the electro-olfactogram (EOG)

S. senegalensis (hereafter 'sole') were obtained from the experimental station of Ramalhete (University of the Algarve). Fish were grown according to the procedure described by Dinis et al. (1999) and juveniles were fed daily on commercial pellets (AQUASOJA 2–3.5 mm, Sorgal SA, Portugal). At the time of experiments animals were between 100 and 300 g. The EOG was recorded as previously described (Velez et al., 2005). Briefly, soles were adapted gradually to dilute seawater (12 ppt) over several days. This increases the amplitude of the recorded EOG (due to the reduced conductivity of lower-salinity water). Soles regularly penetrate estuaries for feeding and recording from salmonids suggests that olfactory sensitivity to amino acids is unaffected by changes in external salinity (Shoji et al., 1994). Prior to recording, the fish were anaesthetised and placed on a padded surface (with a slight backward tilt); aerated water was pumped over the gills. The upper olfactory rosette was exposed by cutting the overlying skin and musculature. The recording electrode was placed at a position that resulted in the largest response to the "standard" stimulus (10^{-3} M L-cysteine) and the reference electrode was placed lightly on the skin of the head nearby. All stimuli were dissolved directly in seawater of 12 ppt. At least 1 min was allowed between successive stimuli. Different stimuli were given in a varied order, but individual odorants were presented in order of increasing concentration. EOGs were similar in amplitude and form to our previous study (Velez et al., 2005) and typical of fish EOGs in general.

2.7. Data treatment and statistical analysis

The amplitude of the initial 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). Standard and blank responses were recorded at regular intervals throughout the recording period. Differences in sensitivity to given odorants were assessed as previously described (Hubbard et al., 2003b). A P value of less than 0.05 was taken to be statistically significant.

3. Results

3.1. Ragworm macerate

The ragworm macerate proved to be a potent olfactory stimulus for sole, evoking robust olfactory responses down to a dilution of 1:1000 (Fig. 1A). Although the

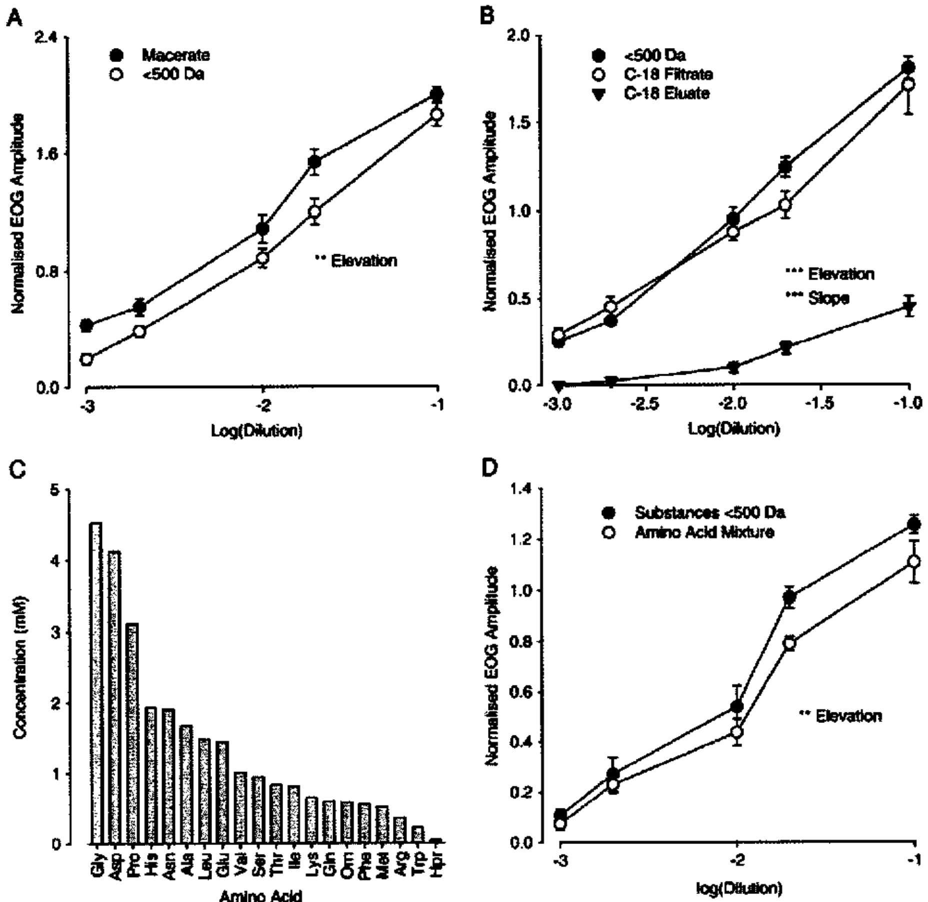

Fig. 1. Olfactory responses of the Senegalese sole (*Solea senegalensis*) to a homogenate of *H. diversicolor*. A. Semi-logarithmic plot of normalised EOG amplitude recorded to the original homogenate (filled circles) and the fraction containing compounds smaller than 500 Da (open circles). Data are shown as mean±S.E.M. ($n=7$). B. Semi-logarithmic plot of normalised EOG amplitude recorded to original homogenate (<500 Da; filled circles) and respective C-18 SPE filtrate (open circles) and C-18 eluate (triangles). Data are shown as mean±S.E.M. ($n=6$). No significant differences were found between the responses to the original sample (<500 Da) and filtrate. C. Histogram showing the amino acid concentrations found in the homogenate. Essential amino acids are designated by their conventional three-letter abbreviations. Orn: L-ornithine; Hpr: hydroxyl L-proline. D. Semi-logarithmic plot of normalised EOG amplitude recorded in responses to homogenate of *H. diversicolor* (<500 Da; filled circles) and an artificial mixture of amino acids at the same concentration as measured in the homogenate (open circles). Data are shown as mean±S.E.M. ($n=6$). $^{**}P<0.01$, $^{***}P<0.001$.

fraction containing substances smaller than 500 Da was significantly less potent, it was evident that this fraction contained the majority of the olfactory activity (93% at a dilution of 1:10). The olfactory potency of the C-18 filtrate, however, was statistically equal to that of the original sample; much less olfactory activity (25% at a dilution of 1:10) was contained in the eluate (Fig. 1B). The concentrations of amino acids measured in the C-18 filtrate are shown in Fig. 1C. The most abundant amino acids were glycine, L-proline and L-aspartic acid. An artificial mixture of amino acids based on these concentrations was only slightly less potent (88% at 1:10) than the original macerate (Fig. 1D).

3.2. Substances released by living ragworms

The substances released by living ragworms evoked lower amplitude EOGs than the macerate, only giving

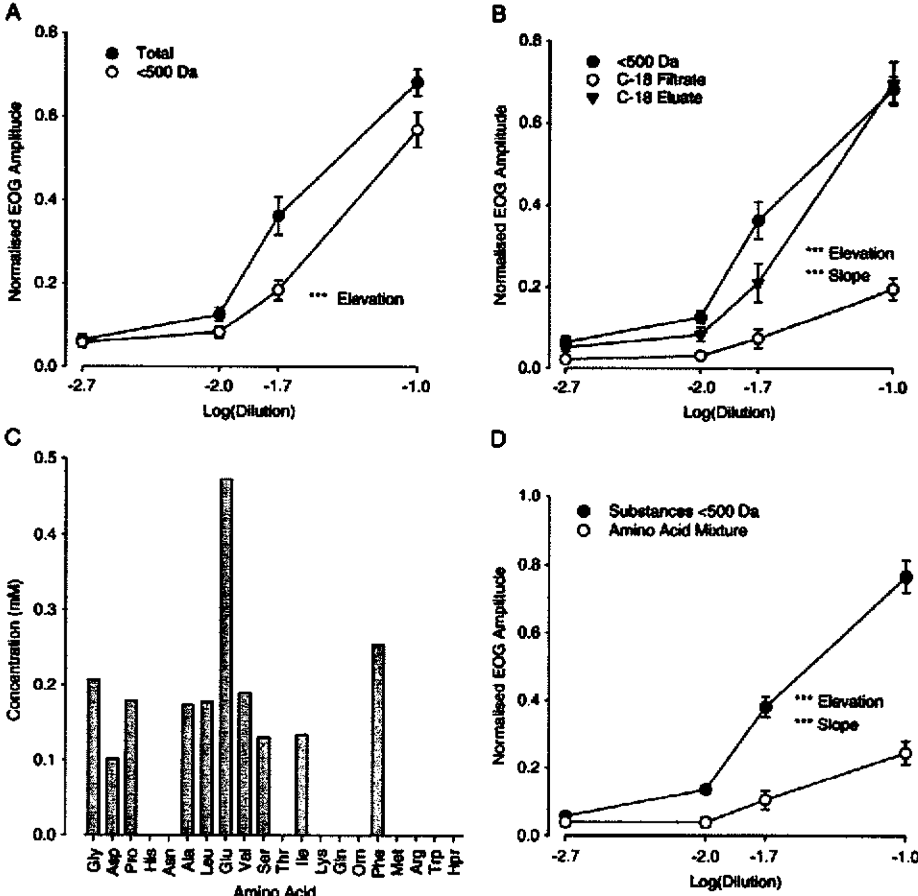

Fig. 2. Olfactory responses of the Senegalese sole (*Solea senegalensis*) to compounds released by living *H. diversicolor*. A. Semi-logarithmic plot of normalised EOG amplitude recorded in response to original sample (filled circles) and the fraction containing compounds smaller than 500 Da (open circles). Data are shown as mean ± S.E.M. (n = 6). B. Semi-logarithmic plot of normalised EOG amplitude recorded in response to original sample (< 500 Da; filled circles) and its respective C-18 SPE filtrate (open circles) and C-18 SPE eluate (triangles). Data are shown as mean ± S.E.M. (n = 6). No significant differences were found between the responses to the original sample and C-18 eluate. C. Histogram showing the amino acid concentrations in the sample containing substances released by living ragworms. Essential amino acids are designated by their conventional three-letter abbreviations. Orn: L-ornithine; Hpr: hydroxyl L-proline. Amino acids without bars were not detected. D. Semi-logarithmic plot of normalised EOG amplitude recorded in response to original sample of substances realised by living worms (< 500 Da; filled circles) and an artificial mixture of amino acids at the same concentration as in the original sample (open circles). Data are shown as mean ± S.E.M. (n = 6). ***P < 0.001.

significant responses down to a dilution of 1:100 (Fig. 2A). Again, although the potency of the sample containing substances less than 500 Da was significantly lower than the original, it was clear that the majority (83% at 1:10) of olfactory activity was contained in this fraction. In contrast to the macerate, however, the olfactory potency of the C-18 eluate was statistically equal to that of the original sample (Fig. 2B); the filtrate contained much less activity (29% at 1:10). The concentrations of amino acids in the C-18 filtrate are shown in Fig. 2C; the most abundant amino acid released by living ragworms was L-glutamic acid. Significant amounts of L-phenylalanine were also released by living ragworms; this amino acid was only a relatively minor component of the macerate. Glycine and L-aspartic acid, the most abundant amino acids in the macerate, were relatively minor components. In contrast to the macerate, the olfactory potency of the

artificial mixture of amino acids at the same concentration as measured in the C-18 filtrate was significantly lower (32% at 1:10) than that of the original sample (Fig. 2D).

4. Discussion

The current study shows that most odorants detected by sole released by, or contained within, ragworms (one of its main prey organisms) are compounds of <500 Da molecular weight. In the macerate, most of the olfactory potency is likely to be due to the presence of amino acids; the C-18 filtrate, the fraction where amino acids are likely to be present, had nearly as much activity as the 'total' (<500 Da), as did the artificial mixture of amino acids at the same concentrations as measured in the macerate. The most abundant amino acid present in the macerate was glycine. Although L-aspartic acid was present at almost equal concentrations, soles have little olfactory sensitivity to acidic amino acids (Velez et al., 2005) and it is not a major component of macerates of marine fish, crustaceans or molluscs (Carr et al., 1996). Other marine fish, such as cod and gilthead seabream, also seem to have relatively poor olfactory sensitivity to acidic amino acids (Hubbard et al., 2003a; Velez et al., 2005; Yacoob et al., 2004). The next most abundant amino acids were L-histidine, L-proline, L-alanine and L-asparagine. Of these, the latter two are potent odorants for the sole; L-histidine is only marginally potent and the olfactory potency of L-proline is essentially zero (Velez et al., 2005 and unpublished observations).

In contrast, of the substances released by living ragworms, amino acids contributed only in a minor way to the overall olfactory potency. Of the amino acids measured, L-glutamate was the most abundant. This, again, is an acidic amino acid that is not a potent odorant for sole. The next most abundant amino acids were L-phenylalanine and glycine, both with olfactory potency. Interestingly, the former is an aromatic amino acid; the lower olfactory epithelium of the sole is significantly more sensitive to aromatic amino acids than the upper one (Velez et al., 2005). This supports the hypothesis that the lower olfactory epithelium of sole is specialized for prey detection. The artificial mixture of amino acids, however, was much less potent than the original suggesting that ragworms are releasing significant amounts of non-amino acid odorants. This is consistent with the fact that the majority of the olfactory potency of the substances released by living ragworms was retained by C-18 cartridges. These cartridges do not retain amino acids well. Combined, these results suggest that the odours contained in the ragworm macerate and ragworm water are quantitatively and qualitatively different; major components of one are substantially less abundant in the other. Under natural conditions, of course, the sole feeds on intact, living ragworms. Preliminary observations showed that naïve soles were attracted to, and tried to eat, paper that had been in contact with ragworms whilst the worms themselves were ignored (data not shown).

In aquaculture, the use of feeding stimulant supplementation can enhance the acceptance of artificial food leading to an increase of growth rate. In the last few years many studies have focused on the identification of feeding stimulants in fish (e.g. Burrells et al., 2001; Carr et al., 1996; Kubitza et al., 1997; Mackie et al., 1980; Papatryphon and Soares, 2000; Reig et al., 2003). Most of these studies were preformed using macerates of natural prey organisms; however, it is not known whether any of these compounds are released by the prey under natural conditions. The current study shows that the compounds released into the environment by ragworms may be substantially different from those present in their body tissues. The chemical identity of these substances is currently under investigation; they may also play an important role in food location. Amino acids are well known to often elicit feeding behaviour (Hara, 1994). The amino acids profiles of the macerate and substances released into the water were also markedly different and, unsurprisingly, present at much higher concentrations in the macerate. The amino acids present in the macerate can explain most of the olfactory potency of the original sample whereas the amino acids released to the water can only explain a fraction of the total activity. It is relevant, however, that glycine was relatively abundant in both. Soles have well-developed olfactory sensitivity to glycine (Velez et al., 2005) and it has been reported to have attractant properties (Knutsen, 1992; Polat and Beklevik, 1999). However, the contribution of any of these non-amino acid odorants in the stimulation of searching and/or feeding behaviour is not yet clear. It is possible that substances released by living ragworms may act as (olfactory) attractants whereas substances, including amino acids, released by damaged prey (after having been bitten) may be more important in the gustatory response.

In conclusion, the current study suggests that, although amino acids may be important odorants for the sole, other non-amino acid compounds, released by natural prey, are also detected. In future studies directed at identifying the chemical nature of these compounds, the possibility that prey organisms may be releasing odorants into the water that are not abundant in tissue extracts should be considered.

Acknowledgements

This work was supported by grants POCTI/CVT/38831/2001 and SFRH/BD/16242/2004 from the Fundação para a Ciência e a Tecnologia (Portugal), funded by the European Union Fundo Social Europeu/Fundo Europeu para desenvolvimento Regional and the Ministério da Ciência e do Ensino Superior. Thanks are due to the Aquaculture Group, CCMar, for supplying the soles used in this study.

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Evidence that 1-methyl-L-tryptophan is a food-related odorant for the Senegalese sole (*Solea senegalensis*)

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Abstract

Nocturnal animals often rely heavily on olfactory cues to locate their food. This is especially true for fish whose prey live buried in the substrate. Previous work has shown that Senegalese sole (*Solea senegalensis*) have high olfactory sensitivity to substances released by living ragworms (*Hediste diversicolor*) - one of its major prey species - and which, therefore, may be food-related cues. The aim of the current study was to identify these substances. Ragworm-conditioned water was extracted with C18 solid-phase cartridges and the eluate fractionated by reverse-phase high-performance liquid chromatography (HPLC). The fractions were then tested for olfactory activity in the sole by the electro-olfactogram (EOG). Most olfactory activity was found in the first two fractions to elute from the HPLC column (0-4 min). Liquid chromatography-mass spectrometry (LC-MS) of both fractions revealed a base peak with a molecular mass of 219.4 Da and a fragment of 205.3 Da. These data are consistent with a methylated form of tryptophan; synthetic 1-methyl-L-tryptophan had a similar HPLC retention time (2.8 min) and similar LC-MS spectra. Furthermore, sole showed olfactory sensitivity to 1-methyl-L-tryptophan with the lower (left) epithelium being more sensitive than the upper (right). Cross-adaptation experiments (using EOG recording) suggested that the olfactory receptors responding to 1-methyl-L-tryptophan are different in the lower epithelium from the upper. These results suggest that ragworms release 1-methyl-L-tryptophan, or one of its isomers, and that sole may use this chemical cue to locate and/or identify one of their main prey species. This may help the formation of artificial feeds that are more attractive to sole.

Introduction

The aquatic environment is often dark or turbid. Many fish, therefore, rely on chemo-sensory systems to locate and identify food, rather than vision (Zielinski and Hara, 2006). The Senegalese sole (*Solea senegalensis*; hereafter 'sole') is a highly prized food-fish of increasing value to the aquaculture industry (Imsland *et al.*, 2003). It is a nocturnal flatfish (Bayarri *et al.*, 2004) which feeds on polychaetes and bivalves that live buried in the substratum (Cabral, 2000). As such, it is highly likely that it relies heavily on olfaction to locate its prey. Nevertheless, little information is available about the type of chemical cues it uses; this is true for marine fish in general. Fish are known to have high olfactory sensitivity to amino acids and it is believed that these compounds are used as cues in food-search behaviour (Hara, 1994). However, we have recently shown that one of the sole's chief prey species (Cabral, 2000), the polychaete *Hediste diversicolor* (hereafter 'ragworm'), releases substances other than essential amino acids which the sole is able to smell (Velez *et al.*, 2007b). Furthermore, we have shown that the upper (right) olfactory epithelium has different sensitivities to some odorants from the lower (Velez *et al.*, 2005; Velez *et al.*, 2007a). This suggests that there may be a functional asymmetry in the roles that the two epithelia play; the upper sampling the water column may be more important in chemical communication, whilst the lower sampling the bottom may be more specialised for food detection.

Therefore, the aim of the current study was two-fold. Firstly, using a combination of chromatographic techniques and electrophysiological recording from the olfactory system of the sole, we aimed to identify non-essential amino-acid odorants released by the ragworm. Secondly, if these odorants are involved in food location or identification, we predicted that the lower epithelium would be more sensitive to them

than the upper. This was tested by comparing the sensitivities of the upper and lower epithelia to one identified odorant.

Materials and Methods

Ragworm-conditioned water

H. diversicolor (total weight 100 g) were collected from the River Humber estuary (Hull, U.K.) and kept in a 200 l tank with re-circulating artificial seawater (14 ‰) for four months at 10 °C; polychaetes survive well under these conditions. Water (5 l) was collected and filtered through 1.2 µm filter (Whatman GF/C filters). It was then passed through C-18 solid phase extraction glass cartridges (500 mg, IST – International Sorbent Technology; Hengoed, UK) as previous described (Velez *et al.*, 2007b). Retained substances were eluted with methanol (50 ml), evaporated under vacuum, re-dissolved in 5 ml methanol and stored at -20°C until use.

High Performance Liquid Chromatography (HPLC)

Fractionation was carried out using a HPLC system (Agilent 1100 series, Agilent Technologies, South Queensferry, West Lothian EH30 9TG, UK) consisting of a quaternary pump, a degassing device, an auto-sampling injector, an automatic sample collector, a column oven with a cooling device (keeping the column at 28 °C) and a diode array detector scanning from 200 nm to 300 nm. The column used was an Ascentis C18 column (25 cm x 4.6 mm, particle size 5 µm; Sigma-Aldrich, UK). The mobile phase was distilled water during the first 5 min followed by a linear gradient of water/methanol during 30 min and continuous 100% methanol during the last 5 min. The flow rate was 1 $ml.min^{-1}$. Samples were run over 40 min and fractions collected every two min. Data were collected and analysed with the software Agilent ChemoStation for LS and LS-MS system. The olfactory potency of each fraction was later evaluated by recording of the electro-olfactogram (EOG) (see below).

Liquid Chromatography-Mass Spectrometry

The HPLC conditions were identical to that previously described above. LC-MS analysis was carried out using a Thermo-Finnigan LCQ Classic ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA) and a binary liquid chromatography (LC) pump (Series 200, Perkin Elmer, UK). The mass spectrometer was operated in positive ion electrospray mode spray voltage of 4 kV. Nitrogen gas flows of 60 arbitrary units (sheath flow) and 20 arbitrary units (auxiliary flow) and a capillary temperature of 270°C were employed to produce stable spray conditions. Data were collected in the full-scan mode, over the range m/z 100 to 1200. Xcalibur software (Thermo Scientific, UK) was used to process the mass spectral data and produce total ion chromatograms for the separation.

Compounds were identified by comparing the retention times and the mass spectra with standards, where possible. Additional MS-MS data was obtained for unknown peaks. For MS-MS data the molecular species was isolated with a 1 Da mass window and collisions with He gas at a normalised collision energy of 50% to 65% were used to induce fragmentation.

Recording the Electo-Olfactogram (EOG)

Sole (100-300 g) were obtained from local aquaculture facilities (IPIMAR, Olhão, Portugal). Fish were grown according to procedure described by Dinis *et al.* (1999) and were fed daily on commercial pellets (AQUASOJA 2-3.5 mm, Sorgal SA, Portugal). The EOG was recorded as previously described (Velez *et al.*, 2005). To reduce the electrical shunting of seawater, sole were gradually adapted to diluted seawater (12 ‰). Briefly, the fish were anaesthetised (100 $mg.l^{-1}$ 3-aminobenzoic acid ethyl ester; Sigma-Aldrich) followed by intra-peritoneal injection of 300 $\mu l.100\ g^{-1}$ body weight Saffan™

(Schering-Plough Animal Health, Welwyn Garden City, U.K.); aerated water (12 ‰) was pumped over the gills *via* a tube placed in the mouth. The EOG was recorded using a Grass CP122 A.C./D.C. strain-gauge amplifier (Grass Technologies, Astro-Med, West Warwick, RI, USA) and glass micropipettes filled with 3 M NaCl in 1% agar. The recording electrode was placed at a position that gave the largest response to the 'standard' stimulus (10^{-3} M L-cysteine) and the reference electrode was placed lightly on the skin of the head nearby. The signal was digitised (DigiData 1322A, Molecular Devices, Inc., Foster City, CA, USA) and stored on a computer running Axoscope™ software (version 9.1, Molecular Devices, Inc.). All stimuli were dissolved directly in 12 ‰ seawater. At least one minute was allowed between successive stimuli. Different stimuli were given in a varied order, but individual odorants were presented in order of increasing concentration. Except for the cross-adaptation studies, responses were recorded from both olfactory epithelia of the same fish.

Cross-Adaptation

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

Data Analysis and Statistics

The amplitude of the initial peak of the EOG was measured in millivolts and was blank-subtracted (amplitude of EOG in response to water treated in the same way as stimulus solutions, but without the addition of odorant). For comparison between the upper and lower epithelia, the amplitudes were normalized to the amplitude of response to a 'standard' stimulus (10^{-3} M L-cysteine) recorded from the same epithelium (upper or lower, respectively) and similarly blank-subtracted. Standard and blank responses were recorded at regular intervals. When only one concentration was tested, such as the HPLC fractions, normalised responses of the two epithelia were compared using Student's *t*-test. For the concentration/response curves, differences in sensitivity between the upper and lower epithelia were assessed by linear regression of log-transformed data (Hubbard *et al.*, 2003) and comparing both the slopes and elevations of the regressions (Zar, 1996). Unless otherwise stated, data are presented as mean ± standard error of the mean (SEM). A P value of less than 0.05 was taken to be statistically significant.

Results

Fractionation of Olfactory Activity

The C18 eluate of ragworm-conditioned water was more potent on the lower epithelium than the upper (Fig. 1). The majority of the olfactory activity was contained in HPLC fractions 1 and 2 (0-4 min). In the upper epithelium, around 60 % of the total olfactory activity was contained in these two fractions; in the lower epithelium, they contained around 90%. Both fractions were more potent on the lower epithelium than the upper. Fractions collected between 16-20 min (fractions 9 and 10) and 24-30 min (fractions 13-15) also contained some activity; however, the sensitivity of both epithelia to these fractions was equal and responses were less than those to the first two fractions. The later three fractions were pooled and analysed by LC-MS and LC-MS-MS (see below). A similar C18 eluate of water conditioned by a congener polychaete (*Diopatra neapolitana*) had no olfactory potency (data not shown).

Putative Chemical Identification

LC-MS of fractions 1 and 2 showed one main peak with a retention time of approximately 2.8 min and an apparent molecular peak at m/z 219.4 (Fig. 2A). MS-MS of this peak gave one fragment with an apparent molecular peak at m/z 205.3 (Fig. 2B); the difference between these two is 14, the molecular weight of a methyl group. Furthermore, 205.3 is the molecular weight of tryptophan. This suggests that the compound is a methylated form of tryptophan. Injection of commercially available 1-methyl-L-tryptophan (Sigma-Aldrich) showed a retention time and mass spectrum identical to that of fraction 1 and 2 (data not shown).

The pool of fractions 13-15 gave three peaks with retention times of 25.9, 26.8 and 27.9 min and apparent molecular masses of 453.4, 453.4 and 566.5 m/z, respectively. MS-MS spectra suggested small peptides (four amino acids) including L-leucine and/or L-isoleucine and L-cysteine residues (data not shown).

Olfactory Sensitivity to 1-Methyl-L-Tryptophan

Both upper and lower olfactory epithelia responded to 1-methyl-L-tryptophan; however, the lower was more sensitive than the upper (Fig. 3A); in either case, the estimated threshold of detection was below 10^{-6} M. Cross-adaptation of the olfactory epithelia with 10^{-3} M 1-methyl-L-tryptophan decreased the amplitude of response to the C18 eluate of ragworm-conditioned water (concentrated x10) in both epithelia (Fig. 3B). This inhibition was greater in the lower epithelium (83 %) than the upper (57 %).

Cross-Adaptation

Cross-adaptation is a method used to assess the specificity of the receptor mechanisms for two given odorants; the responses to one odorant (A) in the continual presence and absence of another odorant (B) are compared. If the two odorants share the same receptor mechanism, then the presence of odorant B will reduce the response to odorant A. If, however, the two odorants act at completely independent receptor mechanisms, then the response to odorant A will be the same whether odorant B is present or not. Using 1-methyl-L-tryptophan (10^{-3} M) as the adapting solution reduced the olfactory responses to L-cysteine, L-phenylalanine and glycine equally in both epithelia (Fig. 4A). Responses to L-cysteine were reduced by 53 % in the upper epithelium and 56 % in the lower. Responses to glycine were reduced by 52 % in the upper epithelium and 65 % in the lower. The responses to L-phenylalanine, however, were reduced to below 10

% of the un-adapted response. Conversely, using L-cysteine as adapting solution the olfactory response to 1-methyl-L-tryptophan was nearly abolished in the upper epithelium (inhibited by 96 %) but, at the lower epithelium, the inhibition was only around 46% (Fig. 4B). With glycine as the adapting solution the response to 1-methyl-L-tryptophan was reduced by 75% in the upper epithelium but only 28% in the lower. Finally, cross-adaptation with L-phenylalanine reduced the response to 1-methyl-L-tryptophan by 70 % in the upper epithelium but only 47 % in the lower.

Discussion

The current study shows that living ragworms release 1-methyl-L-tryptophan, or a related compound, into the water and that sole are able to smell it. It is probable, that sole use this odorant in order to locate, and/or identify, its prey. This hypothesis needs to be tested by behavioural experiments. Nevertheless, the lower olfactory epithelium was more sensitive to this compound, probably involving different olfactory receptors. This is consistent with a functional specialisation of the lower olfactory epithelium to detect chemical cues released by prey.

We have previously shown that living ragworms release a mixture of essential amino acids into the water, mainly L-glutamate, L-phenylalanine and glycine (Velez *et al.*, 2007b). Of these, the aromatic amino-acid L-phenylalanine is detected better by the lower epithelium, glycine by the upper and L-glutamate not detected well by either (Velez *et al.*, 2005). As such, there is no clear match between the amino acids released by a given prey species and the olfactory sensitivity of the sole. Thus, prey are likely to be identified by a mixture of different odorants, and 1-methyl-L-tryptophan may play a role in this. Significantly, a congener polychaete (*Diopatra neapolitana*) apparently does not release 1-methyl-L-tryptophan; these worms do not feature in the sole's natural diet (Cabral, 2000). Whether 1-methyl-L-tryptophan is released by other marine invertebrates and/or is detected by other fish that feed on ragworms remains to be tested. Also, other isomers of methyl-tryptophan exist (*e.g.* 5-methyl-L-tryptophan) that were not analysed by LC-MS-MS or tested by EOG. Nevertheless, cross-adaptation showed that the olfactory responses to the natural odour were markedly reduced by pre-exposure to 1-methyl-L-tryptophan; this effect was greater in the lower epithelium than the upper. This suggests that, even if different isomers are released by the ragworm,

they are detected by the same olfactory receptors in the sole, but possibly by different receptors in the upper and lower epithelia. Furthermore, the odorant receptors of the lower epithelium are apparently more specific, and have a higher affinity, for 1-methyl-L-tryptophan. The structurally similar l-phenylalanine was unable to reduce responses by more than 50 %. The exact nature of these receptors and their transduction pathways are currently under investigation.

Although the main aim of the current study was the identification - and not the quantification - of odorants released by ragworms, comparison of the responses to the C18 eluate with the concentration/response curve to 1-methyl-L-tryptophan suggests a concentration of about 10^{-4} M in the original water. Even if the ragworms were kept at artificially high densities in the laboratory, this still represents a substantial rate of release. Nevertheless, population densities of ragworms can reach 2000-4000m^{-2} (Cardoso *et al.*, 2007; Durou *et al.*, 2007); it would be interesting to measure levels of 1-methyl-L-tryptophan in such environments. The reason why ragworms release 1-methyl-L-tryptophan is not known. It may be because this compound inhibits an enzyme of tryptophan catabolism, indoleamine 2,3-dioxygenase (IDO) (Terness *et al.*, 2002). Some invertebrates, such as gastropods, have a IDO-like myoglobin (Weber and Vinogradov, 2001); however, as far as we are aware, this myoglobin has never been described in neriid polychaetes. Alternatively, many inter-tidal or estuarine invertebrates (such as ragworms) release amino-acid derivatives as a response to lowered environmental salinity; release of 1-methyl-L-tryptophan may be involved in this (*e.g.* Hoeger and Abe, 2004). It is known that L-tryptophan and derivatives are important in the synthesis of serotonin, and that this neurotransmitter is present in ragworms (Heuer and Loesel, 2008); 1-methyl-L-tryptophan may simply be an inactive metabolite..

The identities and biological roles of the odorants, possibly peptides, eluting later in the HPLC run are worthy of investigation. One of the pheromones released by ragworms is a peptide (Ram *et al.*, 1999) and sole have a limited olfactory sensitivity to this (data not shown). Evidence for olfactory sensitivity to peptides in teleosts is scarce (Hara, 1994). However, most of these studies have been carried out in freshwater fish; the intriguing possibility that sole are able to smell small peptides remains to be tested.

The current study suggests that 1-methyl-L-tryptophan is a food-related odorant for the sole. Furthermore, the lower olfactory epithelium is specialised for the detection of this compound. As far as the authors are aware, 1-methyl-L-tryptophan has not been previously described as an odorant in fish. The possible behavioural consequences of detecting this odorant remain to be investigated, as does how widespread olfactory sensitivity to it is among teleosts. This will help in formulating artificial feeds that are more palatable to aquaculture species.

Acknowledgements

Financial support from the Portuguese National Science Foundation and the European Union Social Funds for Regional Development is gratefully acknowledged (grants POCTI/CVT/38831/2001 and SFRH/BD/16242/2004).

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Figure Legends

Figure 1. Electro-olfactogram (EOG) responses evoked by HPLC fractions (1-20) of the C18 eluate of ragworm-conditioned water (total) in the upper (black bars) and lower (white bars) olfactory epithelia of the sole. Both the total water and fractions were applied at 10 times their original concentration in ragworm-conditioned water. Note that both the total and fractions 1 and 2 evoke larger amplitude responses in the lower epithelium. Data are shown as mean ± S.E.M. (n=7). * $P<0.05$, ** $P<0.01$.

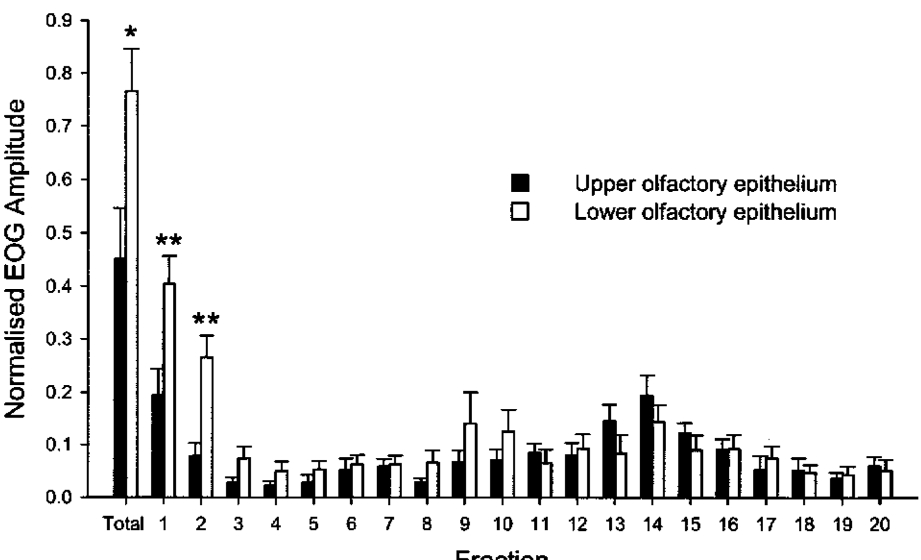
Figure 2. (A) Mass spectrum the most abundant peak detected in HPLC fraction 1 (0-2 min) of the C18 eluate of ragworm-conditioned water. (B) MS-MS spectrum of the apparent molecular peak of (m/z) 219.4 detected in the same fraction.

Figure 3. Olfactory response of sole to 1-methyl-L-tryptophan. (A) Semi-logarithmic plot of normalised EOG amplitude recorded from the upper (filled circles) and lower olfactory epithelia (open circles) of sole. Data are shown as mean ± S.E.M. (n=7). (B) Inhibitory effect of cross-adaptation to 10^{-3} M 1-methyl-L-tryptophan on the response to the C18 eluate of ragworm-conditioned water (10 times original concentration), recorded from the upper (black bar) or lower olfactory epithelium (white bar). Data are shown as mean ± S.E.M. (n=6). ** $P<0.01$, *** $P<0.001$.

Figure 4. (A) Effect of cross-adaptation to 10^{-3} M 1-methyl-L-tryptophan on the olfactory response evoked by 10^{-3} M L-cysteine, L-phenylalanine and glycine, recorded from the upper (black bars) and lower epithelium (white bars) of sole. Data are shown as mean ± S.E.M. (n=6). (B) Effect of cross-adaptation to 10^{-3} M L-cysteine, glycine

and L-phenylalanine on the olfactory response to 10^{-3} M 1-methyl-L-tryptophan, recorded from the upper (black bars) or lower epithelium (white bars) of sole. Data are shown as mean ± S.E.M. (n=6). ** $P<0.005$.

Figure 1.



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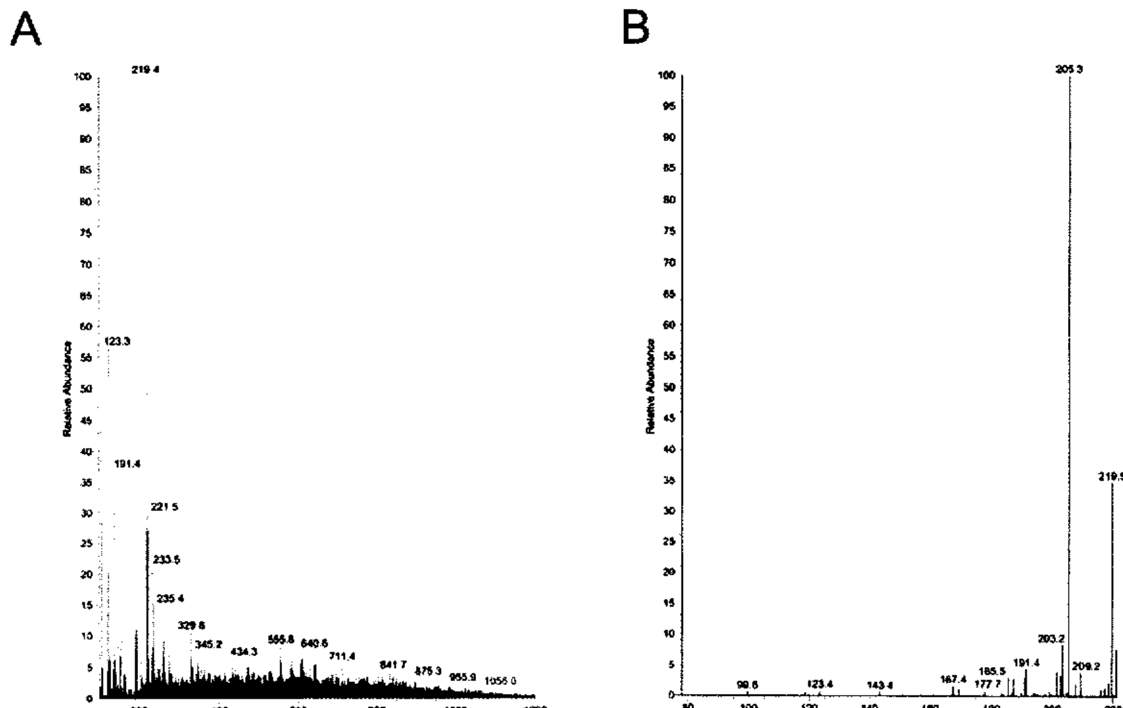


Figure 3A.

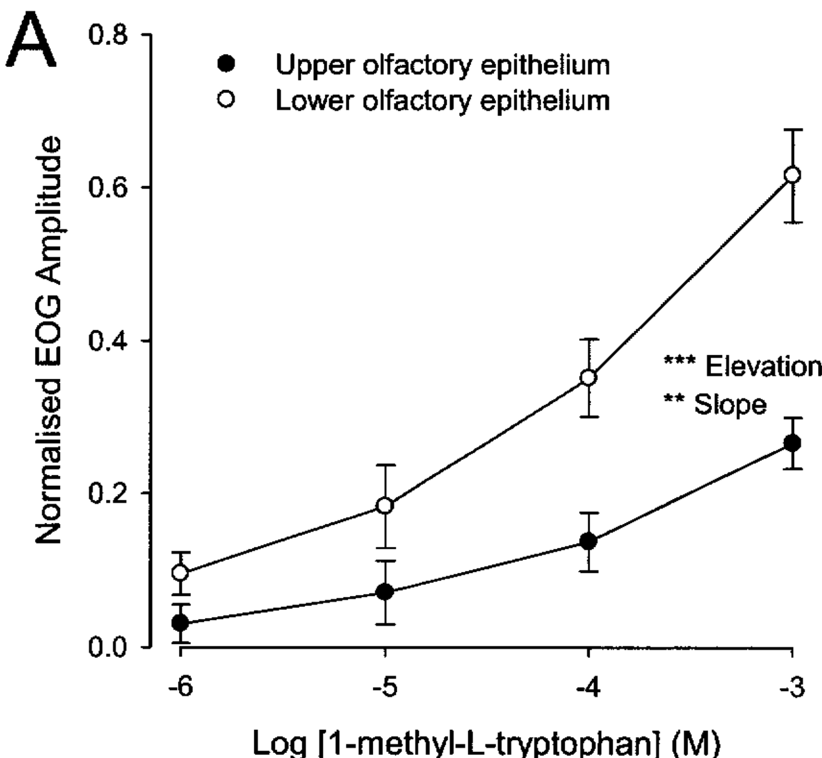


Figure 3B.

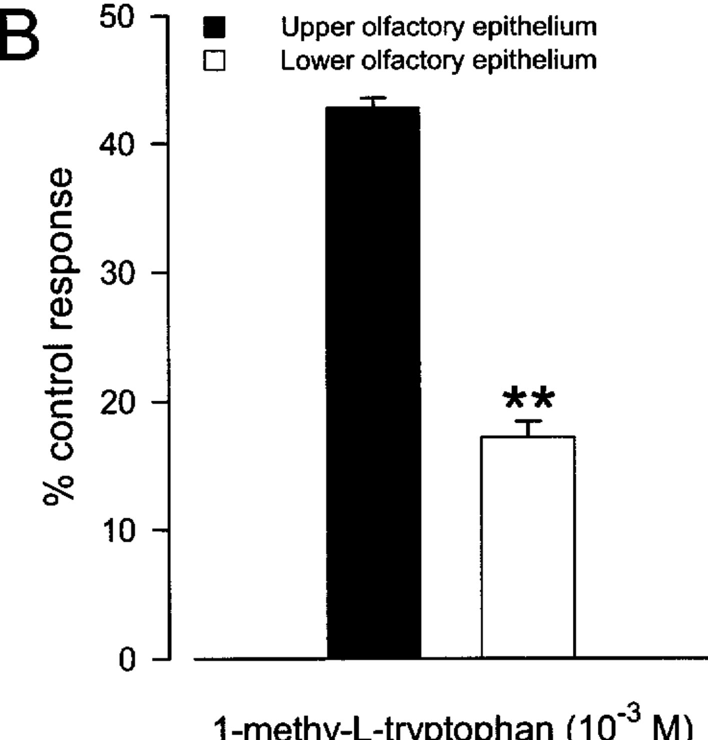


Figure 4A.

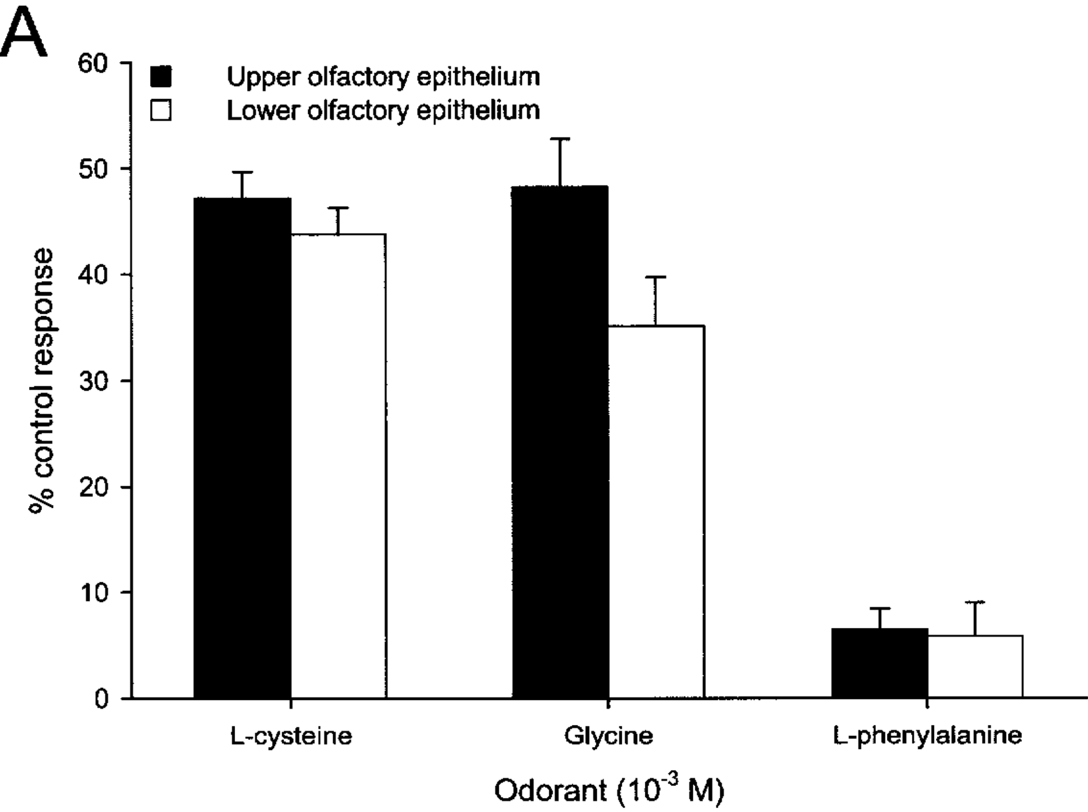
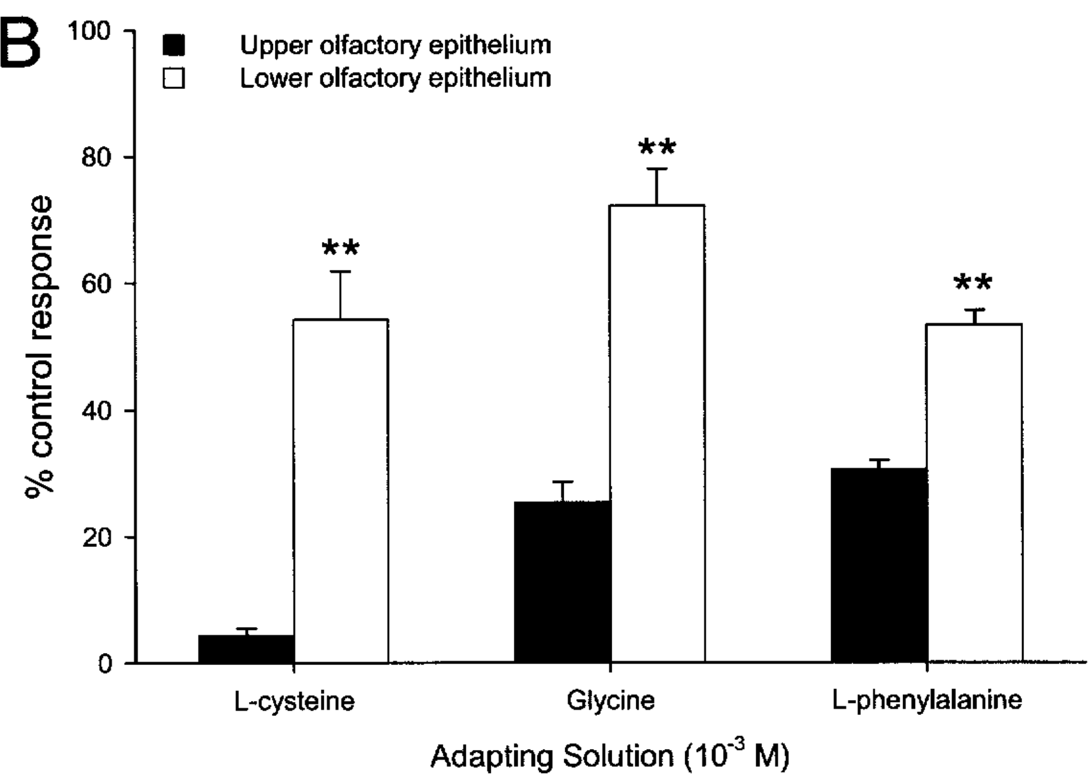


Figure 4B.



2.2.2 Chemical identification of conspecific-derived odorants

Summary

In contrast to freshwater fish, the odorants involved in intra-specific interactions in marine fish are not well understood. According to the literature, in fish, conspecific-derived odorants (putative pheromones) are released *via* the urine, faeces, mucus, gonadal fluids and across the gills. Marine fish produce and release urine at a much lower rate than freshwater fish. Therefore, urine may be less important in chemical signalling in marine fish. A recent study showed that sole has olfactory sensitivity to intestinal and bile fluid; these stimuli are more potent at the upper epithelium than the lower. In the faeces, it is likely that a proportion of the olfactory potency is due to bile acids; fish, in general, have a high olfactory sensitivity to bile acids. However, the exact reason(s) that teleosts have evolved such sensitivity is not yet clear, although it may well be involved in intra-specific interactions; only in the sea lamprey (*Petromyzon marinus* L.) have pheromonal roles for bile acids been described. Given that skin mucus is in intimate contact with the surrounding water, and that contents of the mucus may reflect the endocrine status of the fish, odorants contained in the mucus may also be involved in intra-specific interactions. The sole is known to produce large quantities of mucus; thus bile, faeces and mucus are possible concentrated sources of odorants involved in intra-specific interactions in this species.

The olfactory potency of conspecific body fluids (bile, intestinal fluid and mucus) was assessed in both epithelia and the crude solid-phase fractions of these fluids with olfactory potency, including those differentially detected by the two epithelia, were identified. The upper epithelium was significantly more sensitive to conspecific bile fluid, intestinal fluid and mucus than the lower nostril. Crude fractionation of these samples (solid-phase extraction with C18 and C2/ENV+

cartridges) revealed that olfactory activity in each body fluid was likely due to a mixture of compounds. In each case, the upper olfactory epithelium was significantly more sensitive than the lower. To evaluate whether bile acids could be involved in the differential detection of body fluids, the olfactory sensitivity to a range of C_{24} and C_{27} bile acids was accessed in both olfactory epithelia. The sensitivity to bile acids was greater in the upper epithelium, suggesting that bile acids are involved in the differential detection of body fluids.

To clarify the role of olfactory sensitivity to bile acids in fish, it is necessary not only to assess the sensitivity to different bile acids but also to identify those bile acids released in quantities sufficient to be detected by con-specifics and/or potential predators and prey. Thus, by combining electrophysiological with chemical identification studies, we have evaluated the bile acid content of bile fluid, intestinal fluid and sole conditioned-water and their contribution to the olfactory activity of these samples.

Intestinal fluid was fractionated by HPLC. The olfactory potency of each fraction was evaluated by EOG in both upper and lower olfactory epithelium. The total bile acid content of each HPLC fraction was assessed using an enzymatic approach. The fraction of intestinal fluid containing most bile acids was also that which evoked larger EOG responses on the upper olfactory epithelium than the lower. Thus, it is likely that bile salts are responsible for the differential potency of intestinal fluid on the two epithelia.

Using LC-MS, the bile acids present in the bile fluid, intestinal fluid and sole-conditioned water were identified and quantified. The main bile acids produced by the sole are taurocholic acid and taurolithocholic acid plus a minor, unidentified, bile acid of 544.1 Da molecular mass. These three bile salts were also present in

the intestinal fluid. However, in sole-conditioned water, taurocholic acid was the only bile salt released in sufficient quantities to be measured. An artificial mixture of bile acids simulating the bile acid content of intestinal fluid evoked EOGs of similar amplitude to those of the HPLC fraction containing the bile acids. Thus, the unidentified bile acid does not seem to make a significant contribution to the olfactory potency of intestinal fluid. Furthermore, the sole has high olfactory sensitivity to taurocholic acid. However, olfactory sensitivity to taurolithocholic acid is much lower. It is therefore, likely that taurocholic acid is responsible for the differential potency of intestinal fluid at the two epithelia.

Taken together, these results show that the olfactory potency of con-specific body fluids is due to a mixture of compounds. In intestinal fluid and bile fluid part of the total olfactory activity is due to bile acids. The main bile acids detected in both body fluids were taurocholic acid and taurolithocholic acid. Taurocholic acid is likely to be mainly responsible for the contribution of bile acids to the olfactory activity of intestinal fluid and also for the differential detection of this sample. Furthermore, taurocholic acid is released *via* the faeces to the water in sufficient quantities to be detected by the olfactory system of con-specifics, especially by the upper (right) olfactory epithelium. This gives support to the hypothesis that the olfactory detection of bile acids may be involved in intra-specific interactions and that the upper epithelium is specialised for this function.

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General and Comparative Endocrinology 153 (2007) 418–425

GENERAL AND COMPARATIVE
ENDOCRINOLOGY

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Differential detection of conspecific-derived odorants by the two olfactory epithelia of the Senegalese sole (*Solea senegalensis*)

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Received 13 September 2006; revised 15 February 2007; accepted 17 February 2007
Available online 28 February 2007

Abstract

The two olfactory epithelia of members of the family Soleidae sample two distinct water sources; the upper (right) side is in contact with the open water column whilst the lower (left) side is in contact with interstitial water. To evaluate whether there are differences in the sensitivities, and therefore functional roles, of the two epithelia the olfactory activity of conspecific-derived odorants was assessed in both using the electro-olfactogram (EOG). The upper nostril was significantly more sensitive to conspecific bile fluid, intestinal fluid and mucus than the lower nostril. Crude fractionation of these samples (solid-phase extraction with C18 and C2/ENV+ cartridges) revealed that olfactory activity in each body fluid was likely due to a mixture of compounds. In each case, the upper olfactory epithelium was significantly more sensitive than the lower. Similarly, olfactory sensitivity to a range of C_{24} and C_{27} bile acids was greater in the upper epithelium. These results suggest that intra-specific chemical communication is mediated mainly, if not entirely, by the upper olfactory epithelium. The odorants involved, and their functional roles, remain to be established.
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Keywords: Olfaction; Asymmetry; Chemical communication; Odorant; Flatfish; Bile; Skin mucus; Faeces; Pheromone

1. Introduction

In contrast with freshwater fish, chemical communication in marine fish is not well understood; the substances involved and their biological roles have been little studied. This is especially true for flatfish. The Senegalese sole (*Solea senegalensis*) is a marine flatfish which spends most of its time half-buried in the substrate with only its eyes and upper nostril exposed. It has a large olfactory cavity containing a highly developed, foliate olfactory epithelium (De Groot, 1971), the lower epithelium being slightly smaller than the upper (in common with other flatfish; Kasumyan, 2004). Although 'reversed' (left-handed) sole are rare, it is always the lower epithelium that is smaller (rather than always the left). Amongst teleosts, this structural asymmetry seems to be unique to flatfishes (Kasumyan, 2004). The external asymmetric morphology of *S. senegalensis* is correlated with an asymmetry in the forebrain; the right (upper) olfactory nerve and bulb are slightly larger than the left (lower) ones (Rodríguez-Gómez et al., 2000). Under normal conditions, the two olfactory epithelia are exposed to two distinct odour sources; the upper nostril is in contact with the water column whilst the lower nostril samples interstitial water. Previous work has shown that the two olfactory epithelia have differential sensitivities to some amino acids (Velez et al., 2005), whereas other amino acids and food-related odorants (unpublished observations) are detected equally well. This suggests that there is a functional difference in the roles that the two nostrils play.

In fish, intra-specific chemical communication (including pheromones) may be mediated by substances released *via* the urine (Almeida et al., 2005; Appelt and Sorensen, 1999; Miranda et al., 2005; Moore et al., 1994; Vermeirssen et al., 1997; Yambe et al., 1999; Yambe and Yamazaki, 2000), faeces (Frade et al., 2002; Hubbard et al., 2003a;

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0016-6480/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2007.02.016

Miranda et al., 2005; Vermeirssen and Scott, 2001; Zhang et al., 2001), mucus (Huertas et al., 2007; Saglio, 1982; Stabell and Selset, 1980), gonadal fluids (Hubbard et al., 2003a; Olsén et al., 2001; Resink et al., 1989a; Resink et al., 1989b) and across the gills (Vermeirssen and Scott, 1996). In teleosts, it is the olfactory system that detects pheromones; the thresholds of detection for pheromonal steroids and prostaglandins released in the urine and/or ovarian fluid of the goldfish (*Carassius auratus*), for example, are as low as 10^{-12} M (reviewed in Sorensen and Stacey, 2004; Stacey and Sorensen, 2002). However, the majority of these studies have been carried out on freshwater fish; marine fish produce and release urine at a much lower rate (e.g., Fletcher, 1990). Indeed, the urine of the gilthead seabream is much less potent as a source of odorants than the intestinal fluid (Hubbard et al., 2003a). Subsequently, urine may be less important in chemical signalling in marine fish compared to freshwater fish. In the faeces, it is likely that a proportion of the olfactory potency is due to bile acids; fish, in general, have a high olfactory sensitivity to bile acids (e.g., Døving et al., 1980; Hara, 1994). However, the exact reason(s) that teleosts have evolved such sensitivity is not yet clear, although it may well be involved in intra-specific chemical communication (Zhang et al., 2001). Only in the sea lamprey (*Petromyzon marinus* L.) have pheromonal roles of released bile acids been described (Li et al., 2002; Sorensen et al., 2005).

Given that skin mucus is in intimate contact with the surrounding water, and that contents of the mucus may reflect the endocrine status of the fish (Schultz et al., 2005), odorants contained in the mucus may also be involved in chemical communication (Huertas et al., 2007; Saglio, 1982); the sole is known to produce large quantities of mucus. Thus bile, faeces and mucus are likely to be concentrated sources of odorants potentially involved in chemical communication in this species. The aim of the current study was to identify which crude solid-phase fractions of these conspecific body fluids (bile, intestinal fluid and mucus) contain olfactory potency—and thereby potential pheromones—and whether these fractions are detected differentially by the upper and lower epithelia.

2. Materials and methods

2.1. Conspecific body fluids

Body fluids were taken from adult *S. senegalensis* (of both sexes) that were being sampled as part of other studies (Aguilleiro et al., 2006). Bile fluid was taken directly from the gallbladder, intestinal fluid was extracted from the last 10 cm of intestine and mucus was scraped directly from the skin surface of freshly killed fish. Samples were pooled, diluted in distilled water (1:2), mixed thoroughly, centrifuged, aliquotted and frozen (−20 °C) until use.

2.2. Solid-phase extraction

Samples were first passed through reverse-phase C18 chromatography glass cartridges (IST International Sorbent Technology, Hengoed, UK). Extraction was carried out according to the manufacturer's instructions. Briefly, cartridges were previously conditioned with methanol (2 ml) and distilled water (2 ml), then samples were passed through the cartridges. Substances retained were eluted with 2 ml pure methanol (eluate), evaporated (under nitrogen) and the residue was dissolved in 12 ‰ sea water aliquot and stored at −20 °C until use. The filtrate (aqueous fraction) was divided into two halves. One-half was immediately aliquotted and frozen (−20 °C). The other half was passed through a C2/ENV+ cartridge, which retains a wider range of compounds than C18 cartridges. Extraction was as previously described for the C18 cartridges. The retained compounds were eluted with methanol (2 ml). This was then evaporated and the residue was re-dissolved in 12‰ seawater, aliquotted and stored at −20 °C until use. The filtrate was also aliquotted and frozen (−20 °C).

2.3. Bile acids

Bile acids (CHO, cholic acid; LCH, lithocholic acid; CDC, chenodeoxycholic acid; TCH, taurocholic acid; TLC, taurolithocholic acid and TCD, tauro-chenodeoxycholic acid) were all bought from Sigma Aldrich Chemical Co. (Portugal) except for 5β-scymnol (SCY), 5α-cyprinol (CYP), 5β-scymnol sulphate (SYS) and 5α-cyprinol sulphate (CYP-S), which were gifts from Prof. Alan F. Hofmann and Dr L.R. Hagey, University of California, USA. These were prepared as stock solutions of 10^{-2} M in ethanol kept at −20 °C and diluted in 12‰ seawater immediately prior to use.

2.4. Recording the electro-olfactogram (EOG)

The olfactory potency of the body fluids, their respective SPE fractions and bile acids was assessed by the electro-olfactogram (EOG) as previously described (Velez et al., 2005). Briefly, sole were adapted to dilute seawater (12‰) over four days. Prior to recording, the fish were anaesthetised by immersion in water containing 100 mg . l^{-1} MS222 (3-aminobenzoic acid ethyl ester, Sigma Aldrich) followed by intra-peritoneal injection of Saffan™ (300 µl . 100 g^{-1} body weight; Schering-Plough Animal Health, Welwyn Garden City, UK) and placed on a padded surface (with a slight backward tilt); aerated water (12‰) was pumped over the gills (approximately 100 ml . 100 g body $weight^{-1}$. min^{-1}) via a plastic tube inserted into the mouth. The body of the fish was covered by damp paper towel and the eyes covered with small pieces of black polythene. The upper olfactory rosette was exposed by cutting the overlying skin and musculature. The recording electrode was placed at a position that resulted in the largest response to the "standard" stimulus (10^{-3}M L-cysteine) and the reference electrode was placed lightly on the skin of the head nearby. The signal was digitised (DigiData 1322A, Axon Instruments, Molecular Devices Corporation, Union City, CA, USA) and stored on a P.C. running Axoscope™ software (version 9.0, Axon Instruments). All stimuli were dissolved directly in seawater of 12‰. At least one minute was allowed between successive stimuli. The order in which odorants were presented was varied between fish, but individual odorants were presented in order of increasing concentration. After all odorants had been tested on one olfactory epithelium (usually the lower, as the absolute amplitude of response is less; Velez et al., 2005) the fish was turned over and the same odorants were presented to the other epithelium. There was no apparent affect due to which epithelium was tested first. Between 10 and 12 fish were tested for sensitivity to the body fluids and their respective fractions, and four to five fish were tested for sensitivity to the bile acids. Although not all stimuli were tested on every fish, all odorants from a given body fluid were tested on both epithelia of each fish on which they were tested.

2.5. Data treatment and statistical analysis

The amplitude of the initial peak of the EOG was measured in millivolts, this was then blank-subtracted. Each EOG amplitude was normalised to the amplitude of response to a "standard" stimulus (10^{-3} M L-cysteine). Responses to the standard and blank were recorded

at regular intervals throughout the session. Differences in sensitivity to a given odorant between the upper and lower epithelia were then assessed by linear regression of log-transformed data (Hubbard et al., 2003b) and comparing both the slopes and elevations of the regressions (Zar, 1996). A P value of less than 0.05 was taken as statistically significant.

3. Results

Bile fluid, intestinal fluid and mucus all evoked significantly larger olfactory responses with lower thresholds of detection in the upper olfactory epithelium than the lower (Fig. 1). Bile fluid proved to be the most potent, followed by intestinal fluid and then mucus. However, given that skin mucus is continually in contact with the surrounding water and the rate of production is much higher than that of bile fluid or intestinal fluid, the relative importance of the odorants may follow a different order.

3.1. Bile fluid

After bile fluid was passed through C18 cartridges, significant olfactory activity was found in both the eluate and filtrate (Fig. 2a). However, the shape of the concentration–response curves was different; the eluate gave a more linear relationship when plotted semi-logarithmically, whilst the filtrate gave a more exponential response. This may be due to different classes of compounds being responsible for the activity in each fraction. After the filtrate was passed through C2/ENV+ cartridges the clear majority of olfactory activity remained in the eluate of C2/ENV+ (Fig. 2b). The eluate from the C18 cartridges evoked significantly larger amplitude EOGs in the upper epithelium than the lower (Fig. 2c), although the shape of the curve was similar. This was also true for the other fractions (data not shown).

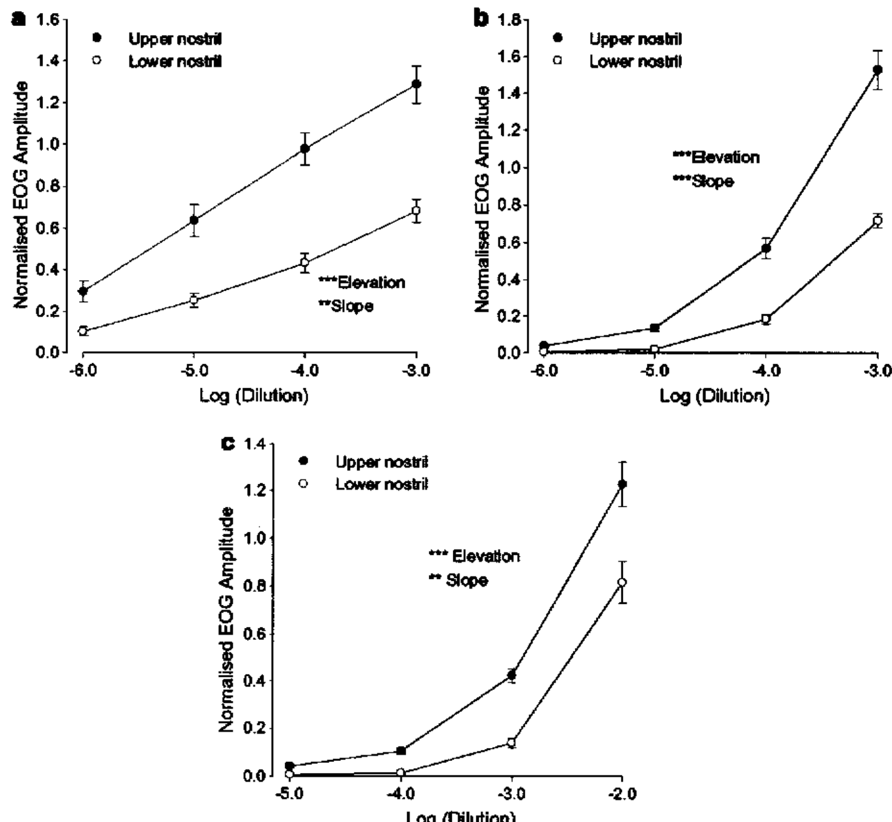

Fig. 1. Olfactory responses to conspecific body fluids recorded from both upper and lower olfactory epithelia of the Senegalese sole. Semi-logarithmic plots of pooled normalised EOG amplitude in response to dilutions of (a) bile fluid ($n = 12$), (b) intestinal fluid ($n = 12$) and (c) mucus ($n = 10$). Data are shown as means ± SEM; $^{**}P < 0.01$; $^{***}P < 0.001$ comparing responses from the upper epithelium with those from the lower.

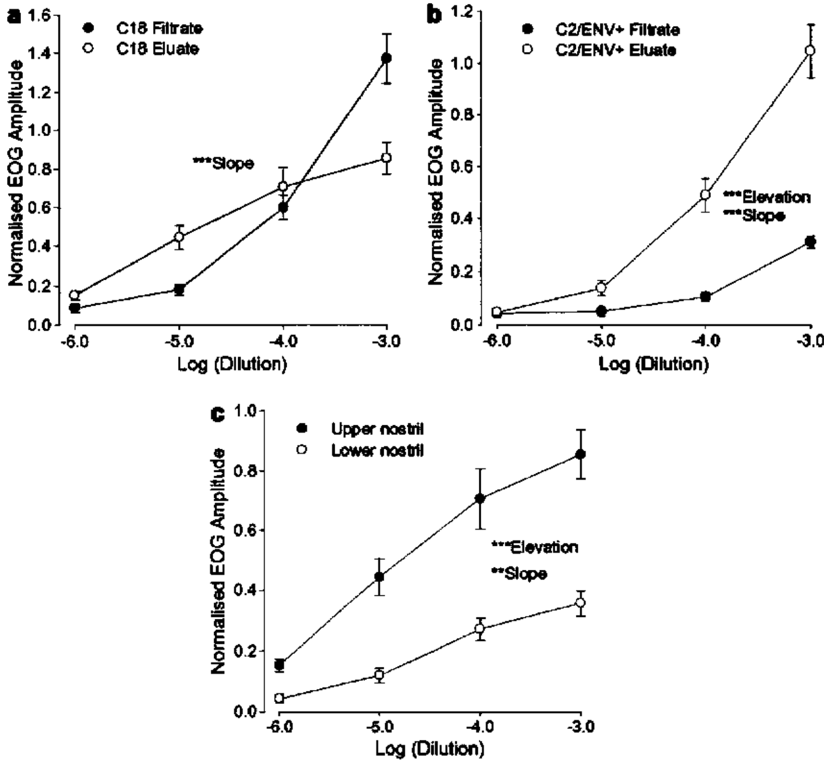

Fig. 2. Olfactory responses to solid-phase extracts of conspecific bile fluid recorded from the Senegalese sole. Semi-logarithmic plots of pooled normalised EOG amplitude in response to (a) C18 eluate (○) and filtrate (●) of bile fluid ($n = 12$) and (b) C2/ENV+ eluate (○) and filtrate (●) of the C18 filtrate of bile fluid ($n = 12$) recorded from the upper epithelium. (c) Semi-logarithmic plots of pooled normalised EOG amplitude in response to the C18 eluate of bile fluid recorded from the upper (●) and lower (○) olfactory epithelia ($n = 12$). Data are shown as means ± SEM; $^{**}P < 0.01$; $^{***}P < 0.001$ comparing responses to the eluate with those to the filtrate (a and b) or comparing responses from the upper epithelium with those from the lower (c).

3.2. *Intestinal fluid*

After intestinal fluid was passed through C18 cartridges, significant olfactory activity was found in both the filtrate and eluate, with the eluate being slightly more potent than the filtrate (Fig. 3a). In this case, the shape of the concentration–response curves was similar. However, when the C18 filtrate was passed through C2/ENV+ cartridges, the vast majority of the activity remained in the filtrate (i.e., was not retained by the cartridge). The C18 filtrate of intestinal fluid evoked significantly higher amplitude EOGs with a lower threshold of detection in the upper epithelium than the lower (Fig. 3c). Again, this was true for the other fractions (data not shown).

3.3. *Mucus*

After passing mucus through C18 cartridges, slightly more olfactory activity was found in the filtrate than the eluate (Fig. 4a), the shapes of the concentration–response curves being similar. The eluate and filtrate from the C2/ENV+ cartridges contained equal activity after the C18 filtrate had been passed through. Again, the C18 filtrate evoked significantly higher amplitude EOGs in the upper epithelium than the lower (Fig. 4c) and, again, this was true for the other fractions (data not shown).

3.4. *Bile acids*

Of the 10 bile acids tested, the olfactory response proved to be variable both among different bile acids and among individual fish. However, in all cases but one, the EOG responses were significantly higher in the upper epithelium (Fig. 5). The only exception was LCH (lithocholic acid) which was the least potent of the bile acids tested at either epithelium (data not shown). Of the C_{24} bile acids tested, the two most potent were CHO and CDC (cholic acid and chenodeoxycholic acid;

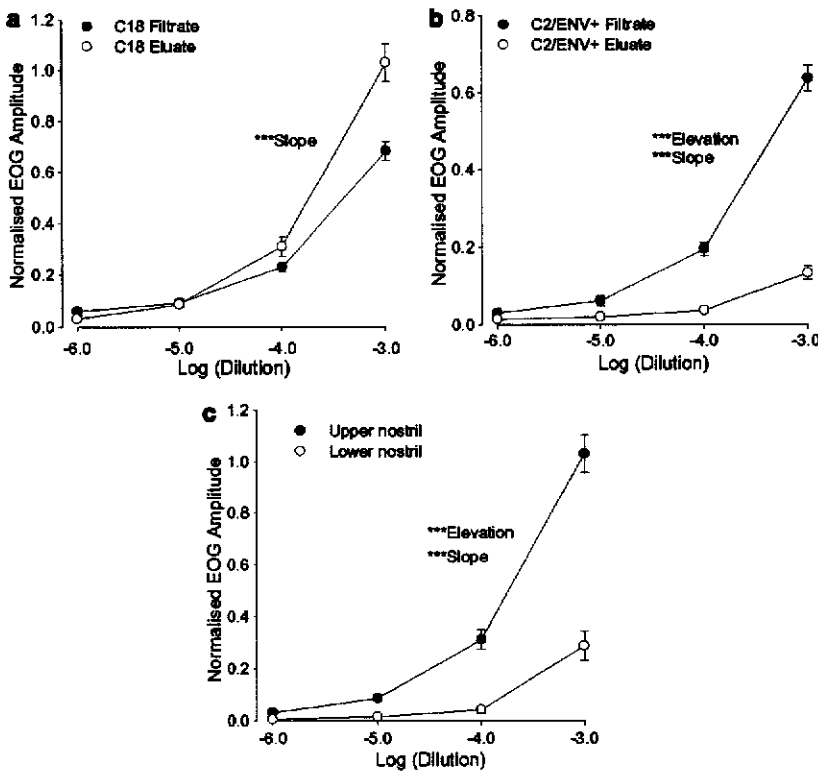

Fig. 3. Olfactory responses to solid-phase extracts of conspecific intestinal fluid recorded from the Senegalese sole. Semi-logarithmic plots of pooled normalised EOG amplitude in response to (a) C18 eluate (○) and filtrate (●) of intestinal fluid ($n = 12$) and (b) C2/ENV+ eluate (○) and filtrate (●) of the C18 filtrate of intestinal fluid ($n = 12$) recorded from the upper epithelium. (c) Semi-logarithmic plots of pooled normalised EOG amplitude in response to the C18 filtrate of intestinal fluid recorded from the upper (●) and lower (○) olfactory epithelia ($n = 12$). Data are shown as means ± SEM; *** $P < 0.001$ comparing responses to the eluate with those to the filtrate (a and b) or comparing responses from the upper epithelium with those from the lower (c).

Figs. 5a and b). The sulphated C_{27} bile acids, 5α-cyprinol sulphate and 5β-scymnol sulphate (Figs. 5c and d) were also potent, more so than their non-sulphated forms (data not shown).

4. Discussion

The current study demonstrates that a range of conspecific-derived odorants are more potent in the upper epithelium of the sole than the lower epithelium; this is consistent with structural (Rodríguez-Gómez et al., 2000) and functional asymmetry of the two olfactory epithelia (Velez et al., 2005). These results suggest that the upper olfactory epithelium may be more important in intra-specific chemical communication than the lower, i.e., the upper epithelium could be specialised for pheromone detection. Nevertheless, the olfactory potency of urine or substances that may be released across the gills was not assessed. In each of the body fluids tested, significant olfactory activity was found in more than one of the fractions. This suggests that each fluid contains more than one type, possibly more than one class, of odorant. The two more potent body fluids tested, bile and intestinal fluid, almost certainly contain bile acids; bile acids are known to be potent odorants in fish (Døving et al., 1980; Hara, 1994; Zhang et al., 2001) with thresholds of detection in the sub-nanomolar range. Bile acids are likely to be present in the C18 eluate fractions. However, the C2/ENV+ filtrate of the intestinal fluid contained markedly more olfactory activity than that of the bile fluid. If the olfactory activity of the intestinal fluid is due only to bile acids then this means that could be chemically modified during their transit down the gut, possibly by gut micro-fauna. Previous work has shown that, in fish, conjugated bile acids are released by the faeces (e.g., Frade et al., 2002; Vermeirssen and Scott, 1996). Alternatively other, possibly smaller molecular weight and/or hydrophilic, odorants may be present in the intestinal fluid.

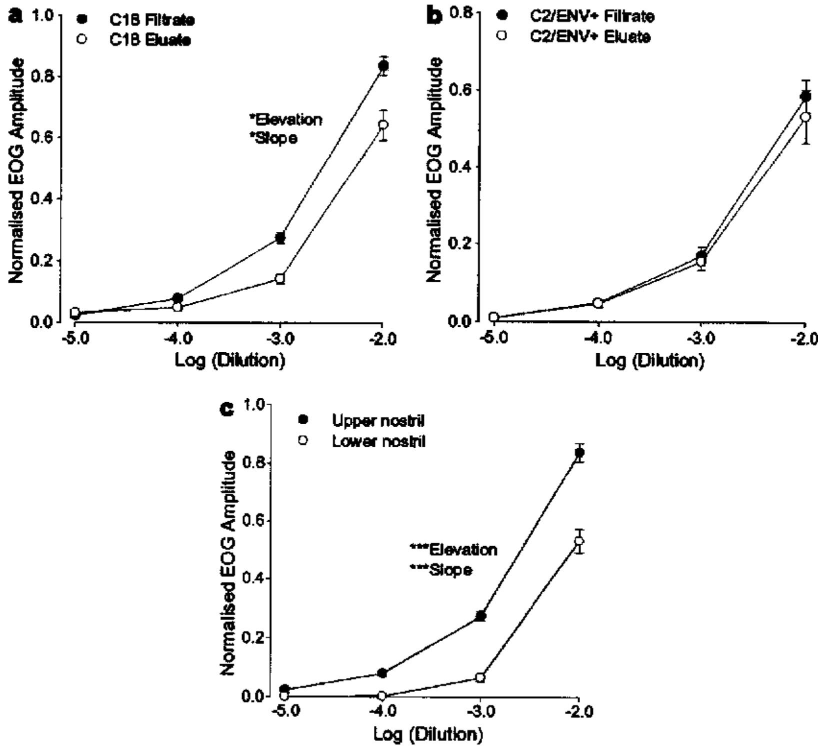

Fig. 4. Olfactory responses to solid-phase extracts of conspecific skin mucus recorded from the Senegalese sole. Semi-logarithmic plots of pooled normalised EOG amplitude in response to (a) C18 eluate (○) and filtrate (●) of mucus ($n = 10$) and (b) C2/ENV+ eluate (○) and filtrate (●) of the C18 filtrate of mucus ($n = 10$) recorded from the upper epithelium. (c) Semi-logarithmic plots of pooled normalised EOG amplitude in response to the C18 filtrate of mucus recorded from the upper (●) and lower (○) olfactory epithelia ($n = 10$). Data are shown as means ± SEM; $^{*}P < 0.05$; $^{***}P < 0.001$ comparing responses to the eluate with those to the filtrate (a and b) or comparing responses from the upper epithelium with those from the lower (c).

Although, on a volume for volume basis, the skin mucus proved to be less potent an odorant than bile or intestinal fluid, mucus still contains olfactory activity. As *S. senegalensis* produces large quantities of mucus, and mucus is in intimate contact with the surrounding seawater, the odorants may still play an important role in chemical communication. Perhaps surprisingly, mucus has received little attention as a possible vehicle for chemicals involved in intra-specific communication in fish (Huertas et al., 2007; Saglio, 1982; Stabell and Selset, 1980). Recent evidence has shown that plasma steroid hormone levels are well correlated with those in the mucus (Schultz et al., 2005), suggesting that this may be a reliable measure of the sex and/or physiological status of the donor. However, the role of mucus in chemical communication in any fish remains to be fully established.

The olfactory sensitivity of *S. senegalensis* to the ten bile acids tested was variable, but showed no clear correlation with either sex or size (data not shown). Both C_{24} and C_{27} bile acids were detected, and both taurine and sulphate conjugates. Which bile acids, if any, *S. senegalensis* releases to the water are not yet known; teleosts, in general, may possess both taurine and sulphate conjugates of C_{24} and C_{27} bile salts in the bile (Moschetta et al., 2005). Nevertheless, that bile acids are better detected by the upper olfactory epithelium is consistent with a role for bile acids as odorants within any, or all, of the body fluids tested.

Known fish pheromones have been identified as steroid hormones, prostaglandins (and some of their metabolites) plus the putative ostariophysan alarm pheromone hypoxanthine-3(*N*)-oxide (Brown et al., 2001; Chivers and Smith, 1998; Stacey and Sorensen, 2002). Recently, an amino acid metabolite, L-kynurenine, has been proposed to have a pheromonal role in the masu salmon (Yambe et al., 2006). As previously stated, teleosts also have highly developed olfactory sensitivity to bile acids, although a clearly defined role for this phenomenon is lacking. Whether the same, or

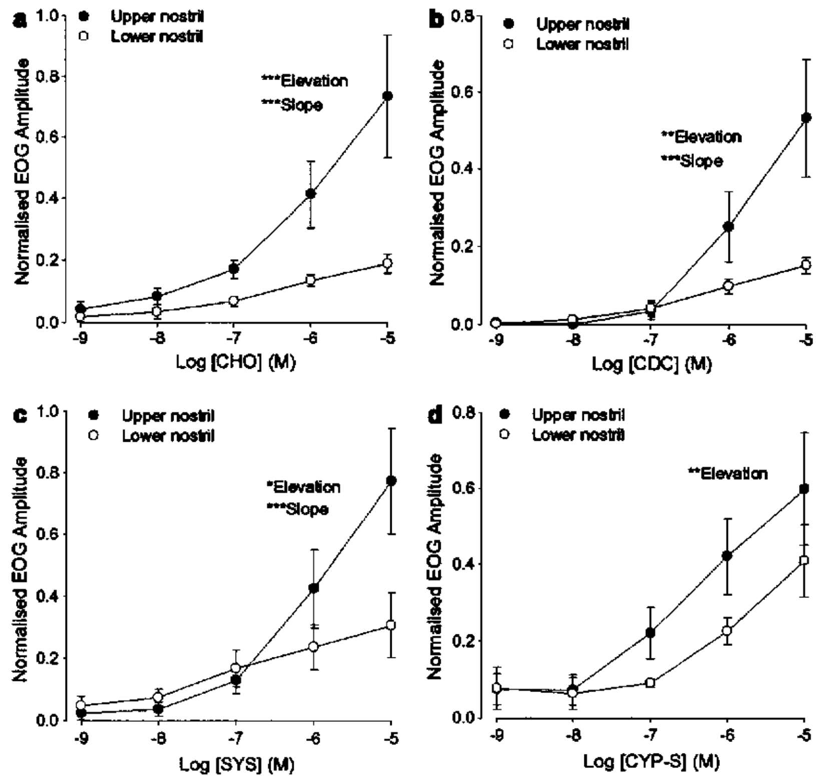

Fig. 5. Olfactory responses to bile acids recorded from the upper and lower olfactory epithelia of the Senegalese sole. Semi-logarithmic plots of pooled normalised EOG amplitude to the C_{24} bile acids (a) cholic acid (CHO; $n = 4$) and (b) chenodeoxycholic acid (CDC; $n = 5$) and the sulphated C_{27} bile acids (c) 5β-scymnol sulphate (SYS; $n = 4$) and (d) 5α-cyprinol sulphate (CYP-S; $n = 4$). Data are shown as means ± SEM; *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$ comparing responses from the upper (●) epithelium with those from the lower (○).

different, classes of compounds are involved in chemical communication in the sole is not yet known. Steroids, prostaglandins and bile acids are likely to be retained by the C18 cartridges so olfactory activity in the C18 eluates may be due to these compounds. Activity in the C18 filtrate may be due to amino acids or their derivatives. However, the current study cannot rule out other classes of compounds as being involved. Furthermore, in each body fluid, the overall odour is likely to be due to a mixture of different compounds. The chemical identity of some of the odorants released by sole is currently under investigation in order to clarify any putative pheromonal roles.

In summary, in a marine flatfish (*S. senegalensis*) bile, intestinal fluid and mucus all contain odorants that are better detected by the upper olfactory epithelium than the lower. Furthermore, all body fluids probably contain more than one class of odorants, one of which is likely to be bile acids. We suggest, therefore, that the upper olfactory epithelium is more involved in intra-specific chemical communication than the lower. However, the identities of the chemicals involved, and their biological roles, remain to be established.

Acknowledgments

This work was supported by grants from the Fundação para a Ciência e a Tecnologia, Portugal (POCTI/CVT/38831/2001 and SFRH/BD/16242/2004), funded by European Union FSE/FEDER and Ministério da Ciência e do Ensino Superior. The authors are grateful to Professor A.F. Hofmann and Dr L.R. Hagey of the Division of Gastroenterology, Department of Medicine, University of California, San Diego, USA, for their kind gift of 5α-cyprinol and 5β-scymnol and their respective sulphates. The authors are also grateful to Drs. Neil Duncan, Joan Cerdà and Mar Huertas (Center of Aquaculture—IRTA, Tarragona, Spain) for collecting the samples of bile, intestinal fluid and mucus.

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Identification, release and olfactory detection of bile salts in the intestinal fluid of the Senegalese sole (*Solea senegalensis*)

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Abstract

Although olfactory sensitivity to bile salts is a wide-spread phenomenon in teleosts, its precise biological function is poorly understood. This is due, in part, to lack of knowledge on the bile salts released to the water in sufficient quantities to be detected. The current study therefore identified bile salts in the intestinal and bile fluids of the Senegalese sole, *Solea senegalensis*, by mass spectrometry associated to liquid chromatography and assessed olfactory sensitivity to these bile acids using the electro-olfactogram (EOG). The main bile salts identified in the bile fluid were taurocholic acid (342 mM) and taurolithocholic acid (271 mM) plus a third, unidentified, bile salt of 532.3 Da. These three bile salts were also present in the intestinal fluid (taurocholic acid, 4.13 mM; taurolithochilic acid, 0.4 mM). In Senegalese sole-conditioned water, taurocholic acid (0.31 μM) was the only bile salt released in sufficient quantities to be measured. Senegalese sole had high olfactory sensitivity to taurocholic acid but not taurolithocholoc acid. Furthermore, the olfactory sensitivity was higher in the olfactory epithelium of the upper nostril (right) than in the lower (left). These two bile acids account for about 40% of the olfactory potency of intestinal fluid and are responsible for the difference in potency at the two epithelia. These results suggest that taurocholic acid can be used as a chemical signal in the Senegalese sole and that the olfactory epithelium of the upper nostril is specialised for this function.

Introduction

The main physiological function of bile acids and alcohols - steroidal compounds produced by the vertebrate biliary system - is to solubilise ingested lipids for easier digestion (e.g. Hofmann, 1999). Fish, however, have also evolved a high olfactory sensitivity to this class of compound. This phenomenon is generally believed to be involved in chemical communication and/or identification of potential predators and prey (Døving *et al.*, 1980; Hara, 1994a; Hara, 1994b; Sorensen and Caprio, 1998). Nevertheless, only in the sea lamprey have clearly defined roles for bile acids as pheromones been established (Li *et al.*, 2002; Sorensen *et al.*, 2005). In teleosts, the main bile acids are sulphated bile alcohols, mainly 5α-cyprinol, 5-chima-erol and C_{24} bile acids (cholic acid, chenodeoxycholic acid, deoxycholic acid and heamulcholic acid) and are conjugated mainly with taurine (Haslewood, 1967). However, some glycine-amidated bile acids and, in some marine fish, cysteinolic bile acids have been identified (Une *et al.*, 1991). To clarify the role of olfactory sensitivity to bile acids in fish, it is necessary not only to assess the sensitivity to different bile acids but also to identify those bile acids released in quantities sufficient to be detected by con-specifics and/or potential predators and prey.

The Senegalese sole (*S. senegalensis)*, hereafter 'sole', is a benthic flatfish of the family Pleuronectidae. This fish undergoes a metamorphosis which involves a 90° rotation of the body and the migration of the left eye to the right (upper) side. However, the left nostril containing the olfactory epithelium and associated structures do not migrate to the upper side but remain on the left (lower) side, facing the substratum (Rodríguez-Gómez *et al.*, 2001). Thus, the olfactory epithelia are exposed to different odour sources, which raises the possibility that the two olfactory epithelia have evolved

differential sensitivity to odorants and therefore functional asymmetry in the olfactory system (Velez *et al.*, 2005; Velez *et al.*, 2007). It is possible that the upper epithelium, facing the water column, has specialized mainly for intra-specific chemical communication and that bile acids are involved in this process; certainly, con-specific body fluids are better detected by the upper epithelium than the by the lower (Velez *et al.*, 2007). Although the majority of bile acids is reabsorbed during intestinal transit, it has been shown that fish release some bile acids *via* the faeces (Zhang *et al.*, 2001). The aim of the current study was, therefore, to identify the bile acids produced by the sole and assess their contribution to the olfactory potency of con-specific intestinal fluid and conditioned water.

Materials and Methods

Con-specific Body-Fluids and Sole-Conditioned Water

Body-fluids (intestinal and bile fluid) were taken from adult sole (both sexes) that were being sampled as part of another study (Agulleiro *et al.*, 2006). Bile fluid was taken directly from the gall bladder and intestinal fluid was extracted from the posterior 10 cm of intestine. Samples were pooled, diluted in distilled water (1:2), mixed thoroughly, centrifuged, aliquoted and frozen (-20°C) until use. Sole-conditioned water (1 l) was taken from a 400 l tank in which six sole (average weight, 150 g) were kept, unfed, in seawater (35 ‰) during four days. The water was filtered (1.2 µm; Whatman GF/C filters) and all samples were then passed through reverse-phase C18 chromatography cartridges (IST – International Sorbent Technology, Hengoed, UK). Extraction was carried out according to the manufacturer's instructions. Briefly, cartridges were conditioned with methanol (2 ml), washed with distilled water (2 ml) and then the samples were applied. Finally, retained substances were eluted with 2 ml methanol (eluate) and stored at –20 °C until use.

High-Performance Liquid Chromatography (HPLC)

The intestinal fluid eluate was fractionated using an HPLC system (Agilent 1100 series, Agilent Technologies, South Queensferry, West Lothian EH30 9TG, UK) consisting of a quaternary pump, a degassing device, an auto-sampling injector, an automatic sample collector, a column oven with a cooling device necessary to keep the column temperature at exactly 28 °C and a diode array detector scanning from 200 nm to 300 nm. The column was an Ascentis C18 column (25 cm x 4.6 mm, 5 µm). Samples were run with a linear gradient of water (pH 3.8) and acetonitrile (0-100%) over 30 min and a

flow rate of 0.7 ml min^{-1}. Fractions were collected every 3 min, evaporated under vacuum, dissolved in methanol, pooled and stored at -20ºC until use. Spectral data were collected and analysed with the software "Agilent ChemStation for LC and LC/MS system".

Recording the Electo-Olfactogram (EOG)

The olfactory potency of bile fluid, intestinal fluid and their respective HPLC fractions was assessed by the electro-olfactogram (EOG) as previously described (Velez *et al.*, 2005). To reduce the electrical shunting effect of seawater, sole were adapted to dilute seawater (12‰) over four days. The fish were anaesthetised by immersion in water containing 100 mg.l^{-1} MS222 (3-aminobenzoic acid ethyl ester, Sigma-Aldrich) followed by intra-peritoneal injection of Saffan™ (300 μl.$100g^{-1}$ body-weight; Schering-Plough Animal Health, Welwyn Garden City, U.K.) and placed on a padded surface (with a slight backward tilt); aerated water (12‰) was pumped over the gills (approximately 100 ml.100g body-$weight^{-1}$.min^{-1}) *via* a plastic tube inserted into the mouth. The body of the fish was covered by damp paper towel and the eyes covered with small pieces of black polythene. The upper olfactory rosette was exposed by cutting the overlying skin and musculature. The recording electrode was placed at a position that resulted in the largest response to the "standard" stimulus (10^{-3} M L-cysteine) and the reference electrode was placed lightly on the skin of the head nearby. The signal was digitised (DigiData 1322A, Molecular Devices Corporated, Sunny Vale, CA, USA) and stored on a computer running Axoscope™ software (version 9.2; Molecular Devices). All stimulants were dissolved directly in seawater of 12‰. At least one minute was allowed between successive stimuli. The order in which odorants were presented was varied between fish, but individual odorants were presented in order of

increasing concentration. After all odorants had been tested on one olfactory epithelium (usually the lower) the fish was turned over and the same odorants were presented to the other epithelium.

Data Treatment and Statistical Analysis

The amplitude of the initial peak of the EOG was measured in milivolts, this was then blank-subtracted. Each amplitude was normalised to the amplitude of response to the "standard" stimulus (10^{-3} M L-cysteine). Responses to the standard and blank were recorded at regular intervals throughout the session. Responses of the two olfactory epithelia to each HPLC fraction were compared using Student's *t*-test for paired samples. Differences in responsiveness to an artificial mixture of bile acids based on the concentrations measures in bile and intestinal fluid between the upper and lower epithelia were then assessed by linear regression of log-transformed data (Hubbard *et al.*, 2003) and comparing both the slopes and elevations of the regressions (Zar, 1996). Unless otherwise stated, data are presented as mean ± standard error of the mean (SEM)._A *P* value of less than 0.05 was taken to be statistically significant.

Quantification of Bile Salts

Bile salt concentration was measured using an enzymatic and fluorimetric approach described by Murphy *et al.* (1970). All chemicals (Tris-HCl buffer pH 7.2, EDTA, β-nicotinamide adenine dinucleotide (β-NAD+), 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* and hydrazine sulphate) were bought from Sigma-Aldrich (UK). All bile acids with a free 3α-hydroxy group can be detected with the enzyme 3α-hydroxysteroid dehydrogenase (Iwata and Yamasaki, 1964), however sulphated or glucuronidated bile acids require previous de-conjugation and solvolysis.

Enzymatic De-conjugation

The enzymatic de-conjugation was performed as described by (Murphy *et al.*, 1995). Briefly, the pH of each sample was adjusted to 5.6 (with 5 M HCl), and placed in a water bath at 37°C. After 10min, 10 μl of choloylglycine hydrolase solution (100 units/mg protein; Sigma-Aldrich, UK) was added, and incubation continued overnight. Bile salts were then extracted by SPE as described above. The eluate was evaporated to dryness under nitrogen.

Solvolysis

After the enzymatic de-conjugation 1.8 ml of methanol, 1.4 ml of dimethoxypropane and 10 μl of concentrated HCl were added to each sample. After mixing, the liquid was left in the dark overnight. Samples were then evaporated to dryness, re-dissolved in water and bile acids were extracted by SPE (as above) and quantified by liquid chromatography/mass spectrometry (see below).

Liquid Chromatography/Mass Spectrometry

The HPLC conditions were identical to those previously described for sample fractionation. LC/MS analysis was carried out using a Thermo-Finnigan LCQ Classic ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA) and a binary liquid chromatography (LC) pump (Series 200, Perkin Elmer, UK). The mass spectrometer was operated in negative ion electrospray mode with a spray voltage of 4 kV. Nitrogen gas flows of 60 arbitrary units (sheath flow) and 20 arbitrary units (auxiliary flow) and a capillary temperature of 270°C were employed to produce stable spray conditions. Data were collected in the full-scan mode, over the range m/z 150 to 1000. Xcalibur software

(Thermo Scientific, UK) was used to process the mass spectral data and produce total ion chromatograms for the separation. Bile acids were identified by comparing the retention times and the mass spectra with standards, whenever possible. For those bile acids identified (taurocholic acid and taurolithocholic acid), standards at various concentrations (5-500 μM) were subjected to the same procedure as the samples. Peak areas from the total ion chromatograms were plotted against the known concentrations from the standards to produce a calibration curve which was then used to estimate the concentrations in the samples.

Results

Intestinal Fluid

Of the ten HPLC fractions, only the first six contained appreciable olfactory activity (Fig. 1A). At the upper olfactory epithelium, fractions 1 (0-3 min) and 4 (9-12 min) were the most potent, each with about 40% of the total activity. At the lower epithelium, fraction 1 was the most potent. Both the unfractionated total and fraction 4 were more potent on the upper epithelium than the lower.

The largest amount of bile salts was detected in fraction 4 (Fig. 1B). Small amounts were also detected in fractions 3 and 6. The LC-MS chromatogram of fraction 4 had several peaks. The first peak, with a retention time of 9.38 min and an apparent molecular peak at m/z 515.3 (Fig. 2A), was identified as taurocholic acid (4.13 mM) by comparison with an authentic standard. The second peak, with a retention time of 9.97 min and an apparent molecular peak at m/z 499.3 (Fig. 2B), was identified as taurolithocholic acid (0.4 mM). The last well-defined peak, with a retention time of 10.56 min and an apparent molecular peak at m/z 532.4 (Fig. 2C), was not identified. The intestinal fluid eluate had a mass spectrum similar to that of fraction 4, except for one extra peak (retention time 5.15 min). The mass spectrum corresponding to this peak does not show the same peak profile as bile acids (Fig. 2D); for example, the peaks of double molecular mass present in Figs. 2A, 2B and 2C are absent in 2D. Also, a range of commercially available bile acids gave similar spectra to Figs. 2A - 2C (data not shown). Thus, this unknown compound is unlikely to be a bile acid.

Olfactory Sensitivity to Taurocholic Acid and Taurolithocholic Acid

The upper olfactory epithelium of sole had appreciable sensitivity to taurocholic acid, giving measurable responses down to 10^{-9} - 10^{-8} M (Fig. 3A). Due to the electrical shunting effect of the dilute seawater used, we suspect that these results may be an underestimation of the true olfactory sensitivity, so thresholds of detection were not calculated. Nevertheless, the lower epithelium gave significantly smaller EOG responses, suggesting that this epithelium is less sensitive to taurocholic acid than the upper. However, neither epithelium showed any appreciable sensitivity to taurolithocholic acid (Fig. 3B). The olfactory activity of fraction 4 and the artificial mixture of bile acids at the same concentration as measured in this fraction are statistically equal (Fig. 3C); bile acids are likely responsible for the olfactory potency of fraction 4.

Bile Fluid

In the bile fluid, two main peaks were detected (Fig. 4A). The first had a retention time of 9.58 min and an apparent molecular peak at m/z 515.3 and was identified as taurocholic acid, present at a concentration of 342 mM. The second peak had a retention time of 10.30 min and the mass spectrum showed the presence of taurolithocholic acid at 271 mM and another, unidentified, bile acid with an apparent molecular peak at m/z 532.3 (Fig. 4B). This peak may correspond to the same unidentified peak detected in intestinal fluid. Another minor peak was present with a retention time of about 12.42 min with an apparent molecular peak at m/z 516.3 and a mass spectrum that likely corresponds to a bile acid (Fig. 4C).

Sole-Conditioned Water

The mass spectrum of sole-conditioned water showed two main peaks (Fig. 5A). The first had a retention time of 9.81 min and was identified as taurocholic acid (0.31 μM). The second peak had a retention time of 10.95 min and a mass spectrum that suggests the existence of at least three different bile acids, one of which was identified as taurolithocholic acid. Although detectable, the amount of taurolithocholic acid was insufficient to quantify. The other two putative bile acids show molecular peaks at m/z 557.1 and m/z 584.2 (Fig. 5B).

Discussion

The current study shows that the main bile acids produced by the sole are taurocholic acid and taurolithocholic acid plus a minor third, unidentified, bile acid of 544.1 Da molecular mass. Although the concentrations of taurocholic acid and taurolithocholic acid are comparable in the bile fluid, their concentrations in the intestinal fluid are markedly different (10:1, taurocholic acid: taurolithocholic acid). This may reflect a higher reabsorption rate of taurolithocholic acid and/or transformation during intestinal transit. Furthermore, taurocholic acid was the only bile acid released into the water in sufficient quantities to be measured. This is matched by the olfactory sensitivity of sole to these bile acids; taurocholic acid is a significantly more potent odorant than taurolithocholic acid. The fraction containing most bile acids was also the fraction that evoked larger EOG responses from the upper olfactory epithelium than the lower and the artificial mixture of bile acids evoked similar amplitude EOGs to this fraction. Thus, it is likely that bile salts, taurocholic acid in particular, are responsible for the differential potency of intestinal fluid on the two epithelia. The third, unidentified, bile acid would not seem to make a significant contribution to the olfactory potency of intestinal or bile fluid. Nevertheless, other unidentified odorants in the intestinal fluid may also be important; bile acids only constitute about 43 % of the total olfactory activity of the intestinal fluid at the upper epithelium.

The role of olfactory sensitivity to bile acids in teleosts is not well understood. Bile acids are only produced by vertebrates (Haslewood, 1967) so the majority of bile acids in the marine environment are likely to come from fish with a minor, and variable, fraction coming from marine mammals, reptiles and birds. The fact that sole produce, release and smell taurocholic acid is consistent with a role for this bile acid in chemical

communication; the measured concentration of taurocholic acid in the sole-conditioned water is well above the calculated threshold of detection. However, other flatfish such as the Japanese flounder (*Paralichthys olivaceus*) also produce taurocholic acid (Kim *et al.*, 2007). Presumably, other components - whether derived from the faeces or other sources (such as the mucus; Velez et al., 2007) - would be required to produce species-specificity to the odour. Alternatively, the meaning of the chemical message may depend on context or timing. Nevertheless, taurocholic acid is not the only bile acid that sole can smell; they also have sensitivity to sulphated C_{27} bile salts 5β-scymnol sulphate and 5α-cyprinol sulphate (Velez *et al.*, 2007) produced by other fish species (Goto *et al.*, 2003; Haslewood, 1967). Thus, olfactory sensitivity to bile acids is not confined to those produced by conspecifics. Haslewood (1967) proposed an evolution from C_{27} 5α-alcohol sulphates to C_{24} 5β acids throughout the vertebrate lineage. Given that the role of bile salts as chemical signals occurred soon after, or even concurrently with, their role in lipid digestion (as seems likely given the use of bile salts as pheromones by the sea lamprey; Li *et al.*, 2002; Sorensen *et al.*, 2005), then it is possible that teleosts retained the olfactory receptor mechanisms for bile salts that they no longer produced in order to recognize other species - potential predators, prey or competitors. However, as the current study makes clear, in investigating this type of question it is important to verify which bile salts are actually *released* to the water (and are therefore available for olfactory detection) rather than simply which bile salts are present in the bile fluid (Zhang *et al.*, 2001).

In summary, the main bile salts produced by the sole are taurocholic acid and taurolithocholic acid plus a minor unidentified bile salt (532.3 Da). Taurocholic acid is released *via* the faeces, where it constitutes about 40% of the olfactory activity, to the water in sufficient quantities to be detected by the olfactory system of conspecifics,

especially by the upper (right) olfactory epithelium. These results suggest that taurocholic acid may be used as a chemical signal by the sole and that the upper epithelium is specialised for this function.

Acknowledgements

This study was financed by grants SFRH/BD/16242/2004 and POCTI/CVT/38831/2001 from the Portuguese National Science Foundation and the European Union Social Funds for Regional Development. The authors wish to thank Drs. Neil Duncan, Joan Cerdà and Mar Huertas (Center of Aquaculture-IRTA, Tarragona, Spain) for collecting bile and intestinal fluids from sole.

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Figure Legends

Figure 1. Bile acids as odorants in the intestinal fluid of sole. **A** Olfactory responses of the upper (black bars) and lower (white bars) olfactory epithelia of sole to HPLC fractions of conspecifics intestinal fluid. Note that differences in olfactory potency between the two epithelia are found in the total and fraction 4 only. Data are shown as mean ± SEM (n = 5). * $P<0.05$. **B** Histogram showing the total bile salt concentration measured in each HPLC fraction (mean + SEM of triplicate measures). Note that the majority of bile salts are found in fraction 4.

Figure 2. Identification of bile acids in the intestinal fluid of sole. **A** LC-MS mass spectrum of the first peak detected in HPLC fraction 4 of the intestinal fluid. This peak was identified as taurocholic acid. **B** LC-MS mass spectrum of the second peak detected in HPLC fraction 4. This peak was identified as taurolithocholic acid. **C** LC-MS mass spectrum of the minor peak detected in HPLC fraction 4 of intestinal fluid. This peak had a retention time of 10.6 min and an apparent molecular peak of (m/z) 532.4. The chemical identity is unknown. **D** Mass spectrum obtained by LC-MS of the first peak of the eluate of intestinal fluid sample. This peak had a retention time of 5.15 min and an apparent molecular peak of (m/z) 544.1. The chemical identity of this peak is unknown.

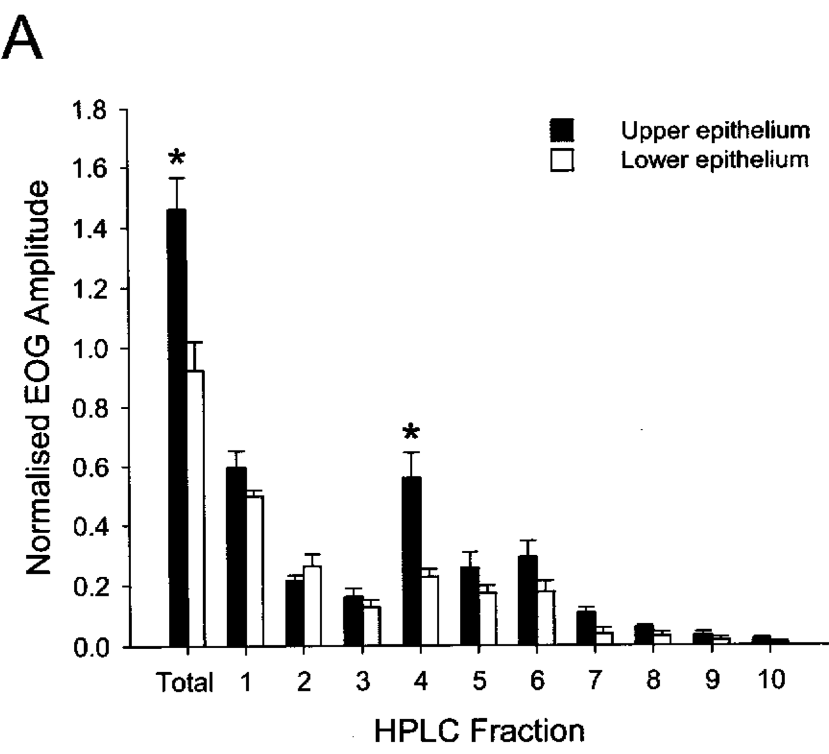
Figure 3. Olfactory sensitivity of sole to taurocholic acid (**A**) and taurolithocholic acid (**B**). Note that the upper epithelium gives significantly larger EOG responses to taurocholic acid than the lower acid but neither epithelium shows appreciable sensitivity to to taurolithocholic acid. **C**. Olfactory responses of the upper olfactory epithelium of

sole to an artificial mixture of bile acids based on those identified in fraction 4 and to fraction 4 itself. Data are shown as mean ± SEM (n=5), ** $P<0.01$.

Figure 4. LC-MS chromatograms of bile fluid. **A.** Two main peaks detected (R.T 9.58 and 10.3) and a minor thirst peak with a R.T. of 12.42 min. **B.** LC-MS mass spectrum of the second peak (R.T. 10.3 min) detected on the Bile fluid sample. On this peak there are at least two different bile acids, the larger was identified as taurolithocholic acid and the smaller, unidentified, had an apparent molecular peak of (m/z) 532.4. **C** LC-MS mass spectrum of a peak with R.T. 12.4 min detected on the bile fluid sample. This peak had an apparent molecular peak of (m/z) 516.3. The chemical identity of this peak is unknown.

Figure 5. LC-MS chromatograms of sole conditioned water. **A.** Two main peaks detected (R.T. 9.81 and 10.95 min). **B.** LC-MS mass spectrum of the second peak detected in sole conditioned water. This peak had a retention time of 10.95 min and corresponded to three different bile acids, of which one was identified as taurolithocholic acid. The other two unidentified peaks had an apparent molecular peak at m/z 557.1 and m/z 584.2.

Figure 1



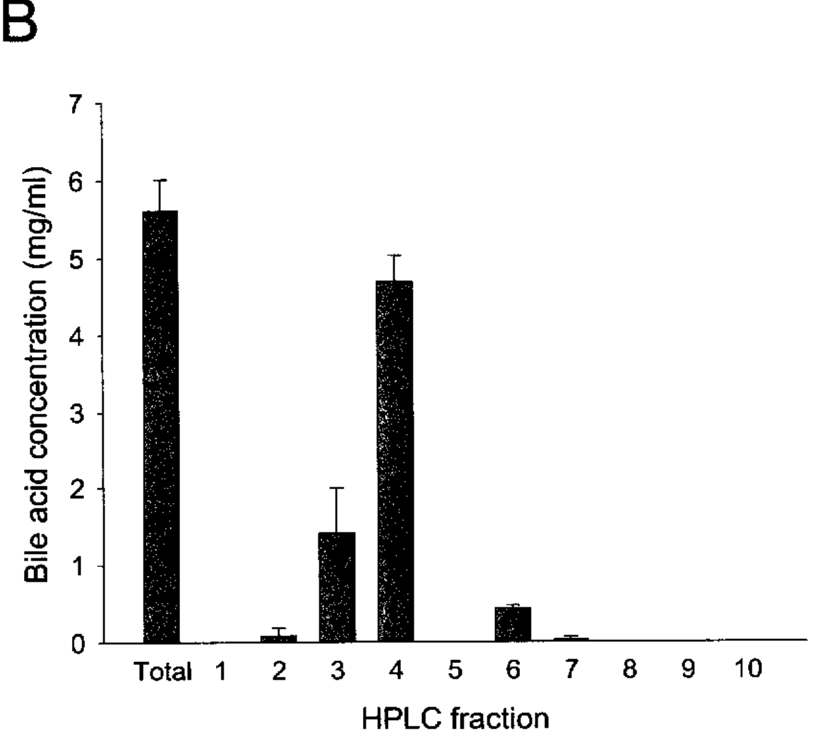
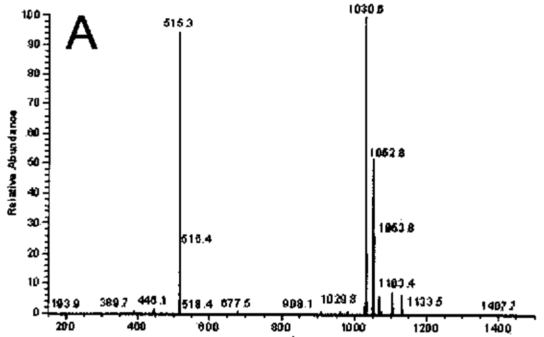
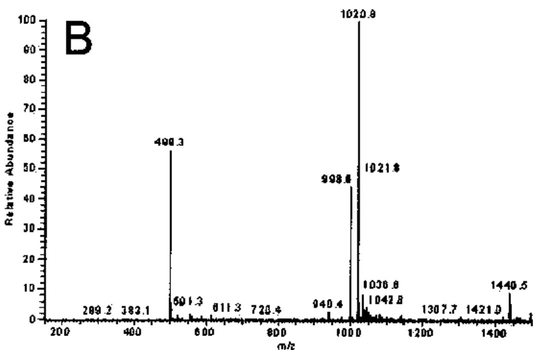
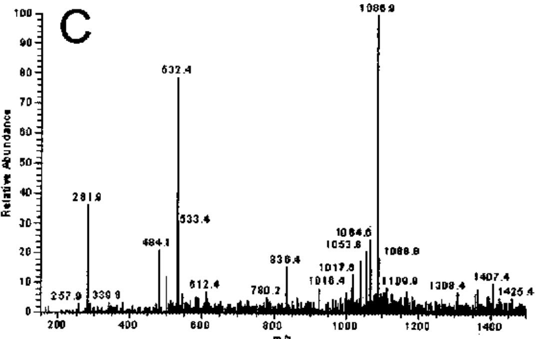


Figure 2







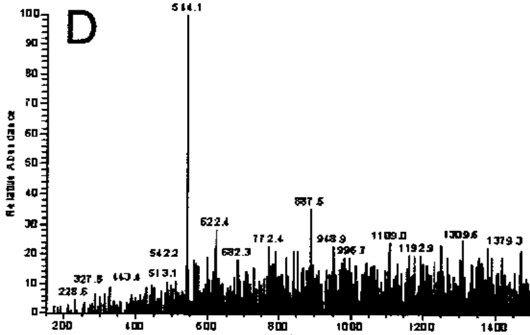


Figure 3

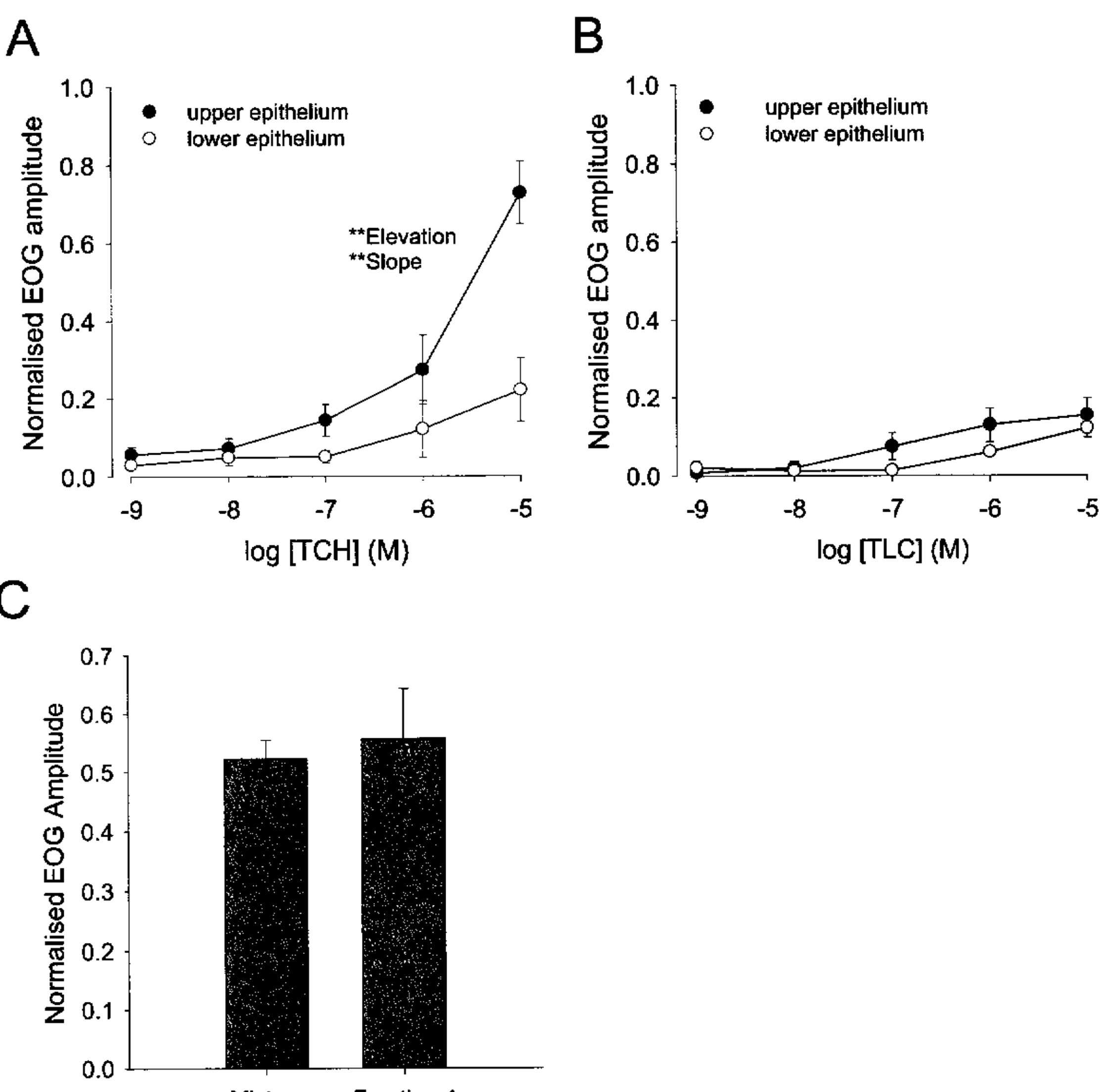
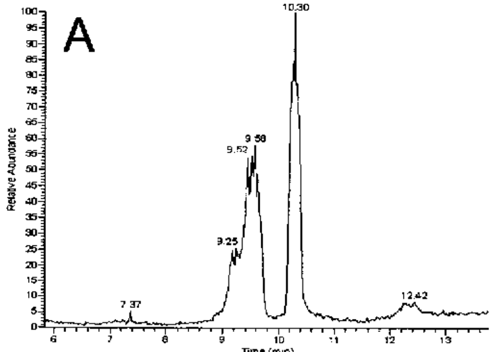
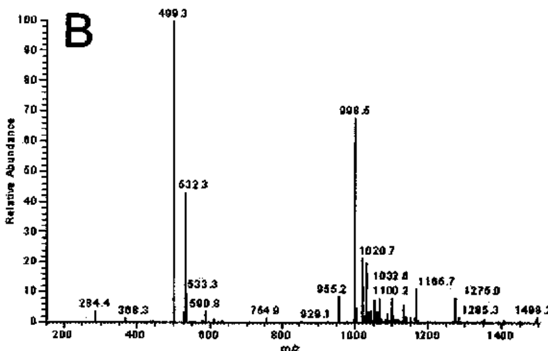


Figure 4





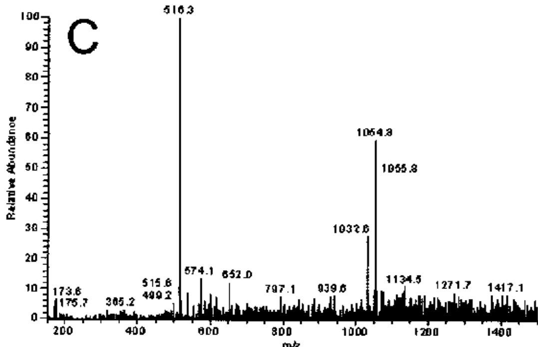
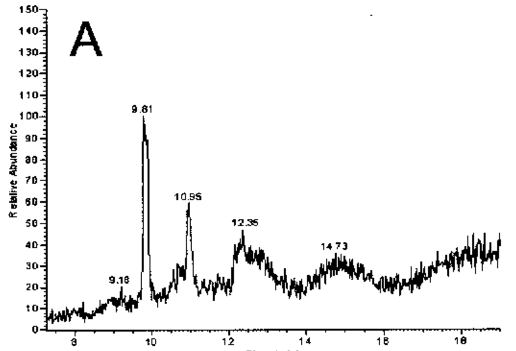
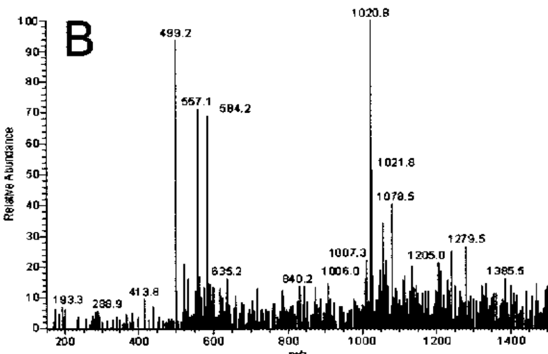


Figure 5





2.4 Transduction pathways in olfactory receptor neurons

Olfactory transduction pathways in the Senegalese sole *Solea senegalensis*

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Abstract

Previous work has shown that there is functional asymmetry in the olfactory system of the Senegalese sole; different odorants are detected better by the upper or lower olfactory epithelium: The current study was designed to test whether these differences are associated with different odorant receptor types and/or transduction pathways. Differences in receptor mechanisms were assessed by cross-adaptation with amino acid odorants (L-cysteine, L.phenylalanine and 1-methyl-L-tryptophan) and bile acid odorants (taurocholic acid and cholic acid). Transduction pathways were assessed by using a phospholipase C (PLC) inhibitor (U73122) and an adenylate cyclase (AC) inhibitor (SQ-22536). Olfactory responses were measured using the electro-olfactogram (EOG) in both cases. Cross-adaptation suggested that there are relatively specific receptors for 1-methyl-L-tryptophan and L-phenylalanine (food-related odorants) in the lower epithelium and for taurocholic acid (conspecific-derived odorant) in the upper epithelium. Inhibition by U73122 (IC_{50}; 15-55 nM) suggested that olfactory responses to amino acids were mediated mostly, but not entirely, by the PLC/IP_3 transduction pathway whereas bile acid responses were mediated by both PLC/IP_3 and AC/cAMP pathways. Simultaneous application of both drugs rarely inhibited olfactory responses completely, suggesting the involvement of non-PLC/non-AC mediated transduction pathways; this was more evident for the bile acids than amino acids. With the aromatic amino acids and bile acids, but not L-cysteine, there were clear differences in the contribution of each transduction pathway (PLC, AC and non-PLC/non-AC) between the upper and lower epithelia. Taken together, these results suggest that the differences in sensitivity between the upper and lower olfactory epithelia are associated with differences in odorant receptors and transduction mechanisms.

Introduction

In vertebrates, an odorant is initially detected by binding to one or more different olfactory receptor proteins (Touhara, 2002). There are two main types of olfactory receptor proteins, the vomeronasal receptor type (VR-type) and the olfactory receptor type (OR-type). In mammals, it is commonly assumed that OR-type (found in the main olfactory epithelium) deal with common (*i.e.* non-pheromonal) odours whereas VR-type (found in the vomeronasal organ) are specialized for detecting pheromones (Alekseyenko *et al.*, 2006; Korsching, 2004). Fish, however, do not have a vomeronasal organ; both OR-type and VR-type receptors are distributed throughout the main olfactory epithelium (Asano-Miyoshi *et al.*, 2000; Hansen *et al.*, 2004). It is generally accepted that olfactory signal transduction occurs through hetero-trimeric G proteins, but it remains unclear which transduction pathways are involved. In mammals, the adenylate cyclase (AC)/cyclic AMP (cAMP) acting *via* cyclic-nucleotide-gated ion-channels is the predominant pathway involved in olfaction (Bakalyar and Reed, 1990; Boekhoff *et al.*, 1990; Breer *et al.*, 1990; Jones and Reed, 1989; Schild and Restrepo, 1998; Sklar *et al.*, 1986). Genetically altered mice, in which various components of the AC/cAMP pathway were deleted, showed that this is the common pathway for all receptor neurons (Wong *et al.*, 2000). However, recent evidence also suggests a role for the phospholipase C (PLC)/inositol tri-phoshate (IP_3) pathway in olfactory transduction in mammals (Elsaesser *et al.*, 2005; Lin *et al.*, 2004), crustaceans (Boekhoff *et al.*, 1994; Hatt and Ache, 1994; Munger *et al.*, 2000; Xu and McClintock, 1999) and fish (Gdovskii and Ruzhinskaya, 2001; Hansen *et al.*, 2003; Kalinoski *et al.*, 1992; Lo *et al.*, 1994; Lo *et al.*, 1993; Ma and Michel, 1998; Sorensen and Sato, 2005). In lobsters, the PLC pathway mediates neuronal depolarization whereas AC mediates hyperpolarization

(Boekhoff *et al.*, 1994; Fadool *et al.*, 1995; Hatt and Ache, 1994; McClintock and Ache, 1989; Michel and Ache, 1992; Schmeidel-Jakob *et al.*, 1990). In fish, knowledge of the transduction mechanisms involved in olfaction is limited. As in crustaceans, there is evidence for the existence of inhibitory responses (Kang and Caprio, 1995); however, the neuronal and/or transduction pathways involved are unknown. In goldfish, *Carassius auratus*, the olfactory sensitivity to prostaglandins and sex steroids (pheromones) is mediated by the AC pathway, whereas responses to amino acids are mediated by both AC and PLC pathways (Sorensen and Sato, 2005). In catfish, *Ictalurus puntactus*, amino acids seem to act *via* an increase in PLC activity and consequent IP_3 production (Restrepo *et al.*, 1993; Restrepo *et al.*, 1990). In the Atlantic salmon, *Salmo salar*, both amino acids and bile acids odorants use the PLC pathway (Lo *et al.*, 1994; Lo *et al.*, 1993) but, in zebrafish, *Danio rerio*, PLC pathway blockers inhibit the olfactory response to amino acids but not to bile acids (Ma and Michel, 1998).

The Senegalese sole (*Solea senegalensis*, hereafter 'sole') is a marine, benthonic flatfish. We have previously shown that the upper (right) and lower (left) olfactory epithelia of sole have different sensitivities to some odorants; the upper epithelium is more sensitive to con-specific body-fluids and bile acids (Velez *et al.*, 2005; Velez *et al.*, 2007a) whereas the lower nostril is more sensitive to aromatic amino acids (Velez *et al.*, 2005). In addition, there are receptors in the lower nostril that respond to 1-methyl-L-tryptophan, an odorant released by one of their main prey species (Velez *et al.*, 2008a). This suggests that both olfactory epithelia are specialised for detecting different functional odorants; the upper epithelium is specialised for chemical communication whereas the lower is specialised in food detection (Velez *et al.*, 2007a; Velez *et al.*,

2008a; Velez *et al.*, 2008b). The objective of the current study was twofold; to evaluate the involvement of adenylate cyclase and phospholipase C mediated pathways involved in the olfactory detection of food-related stimuli and conspecific-derived odorants (by selective inhibition) and, secondly, to test whether differences exist in the receptors and/or predominance of these two transduction pathways between the upper and lower epithelia.

Materials and Methods

Stimuli preparation and delivery

Odorants used were the amino acids 1-methyl-L-tryptophan, L-phenylalanine and L-cysteine and the bile acids taurocholic acid and cholic acid. L-methyl-L-tryptophan and phenylalanine are released by one of the sole's main prey species, *Hediste diversicolor*, and are better detected by the lower olfactory epithelium (Velez *et al.*, 2005; Velez *et al.*, 2007b; Velez *et al.*, 2008a) whereas taurocholic acid was identified as the main bile acid in the bile and intestinal fluid and is better detected by the upper epithelium (Velez *et al.*, 2008b). Cholic acid was used to assess the specificity of olfactory receptors for taurocholic acid; both are structurally similar and are better detected by the upper epithelium (Velez *et al.*, 2007a). L-cysteine is equally well detected by the two epithelia (Velez *et al.*, 2005). Solutions of L-cysteine, L-phenylalanine, 1-methyl-L-tryptophan (10^{-3}M), taurocholic acid and cholic acid (10^{-5} M) (Sigma-Aldrich) were prepared daily in dilute seawater (12‰). The inhibitors of signal transduction used were U73122 (PLC inhibitor; Tocris, Park Ellisville, MO, USA; Hansen *et al.*, 2003) and SQ-22536 (AC inhibitor, Sigma-Aldrich; Chen *et al.*, 2000). For the pharmacological studies, each stimulus was tested in the absence and presence of each drug. The responses in the presence of the drug were normalised to the mean of the response before application and after 15 to 30 minutes wash-out.

Recording the Electo-Olfactogram (EOG)

The electro-olfactogram (EOG) was recorded in fish adapted to 12‰ seawater as previously described (Velez *et al.*, 2005) except that the fish were anesthetised by

immersion in water containing 200 $mg.l^{-1}$ MS222 (3-aminobenzoic acid ethyl ester; Sigma-Aldrich) followed by intramuscular injection of gallamine triethiodide (Sigma-Aldrich; 0.6 mg.100g body $weight^{-1}$). At least one minute was allowed between successive stimuli. Different stimuli were given in a randomised order, but individual odorants were presented in order of increasing concentration.

Cross Adaptation

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 minutes), and test stimulus (B) prepared with the adapting solution is tested. Percent adaptation was calculated by pre-and active adaptation responses (Hara, 2005; Laberge and Hara, 2004). The cross-adapted responses were calculated as a percentage of the control (un-adapted) response (*i.e.* stimulus B alone). The concentrations of the adapting and test stimuli were chosen, on the basis of concentration/response curves, to give similar sized EOG responses. After one minute wash-out, all responses returned to pre-adapted levels.

Pharmacological Studies

To investigate the signalling cascade used by olfactory receptor neurons in odorants signal transduction the olfactory sensitivity to the selected stimuli was tested in the absence and presence of drugs that selectively inhibit PLC (U73122), or AC (SQ-22536). Stimuli were tested first in the absence and then in the presence of drugs. Before testing stimuli in the presence of the AC or PLC inhibitors, the olfactory epithelium was superfused for 10 minutes with seawater (12‰) containing the drug. All

odorants tested during this stage were prepared in the presence of the pharmacological agent at the same concentration as the water superfusing the olfactory epithelium. After testing, the olfactory epithelium was washed out for 15 to 30 minutes by perfusion with seawater (12‰) alone. In all cases, response amplitudes returned to pre-drug levels. The percentage of response was calculated by dividing amplitude of the olfactory response pre-and during the application of the pharmacological agent. In each experiment, the effect of one drug was tested on one epithelium (upper or lower) only.

Data Treatment and Statistical Analysis

The amplitude of the initial 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). For comparison between nostrils in cross-adaptation and pharmacological studies, data were normalised to the amplitude of the control response (*i.e.* in the absence of adapting stimulus or drug respectively) similarly blank subtracted. Differences in responses between the two epithelia were analysed using Student's *t*-test. Differences in the effects of the two drugs were tested by ANOVA followed by the Tukey test. A *P* value of less than 0.05 was taken to be statistically significant. The effect of increasing concentrations of either drug on olfactory responses to different odorants were analysed by fitting the normalised responses to a conventional three-parameter Hill equation. In the case of the bile acids, the U73122-insensitive fraction of the response was subtracted before the Hill plot was fitted. As SQ-22536 only inhibited olfactory responses at concentrations of 10-6 and 10-5 M, no attempt was made to fit Hill curves to these data.

Results

Cross-adaptation

Cross-adaptation with taurocholic acid (10^{-5}M) as the adapting odorant reduced the olfactory responses to L-cysteine and cholic acid equally in both olfactory epithelia. However, the response to 1-methyl-L-tryptophan was reduced more in the upper epithelium than the lower epithelium (Fig. 1A). The presence of taurocholic acid reduced the olfactory responses to L-cysteine by about 30% and those to cholic acid by 94%. Responses to 1-methyl-L-tryptophan were reduced by 54% at the upper epithelium but only 12% at the lower. Conversely, olfactory responses to taurocholic acid during adaptation with L-cysteine (10^{-3} M) were reduced by 37% at the upper epithelium but by 75% at the lower (Fig. 1B). Adaptation with 1-methyl-L-tryptophan (10^{-3} M) decreased the olfactory responses to cholic acid by only 38% at the upper epithelium but by 74% at the lower. Finally, adaptation with cholic acid (10^{-5} M) reduced olfactory responses to taurocholic acid by 60% at the upper olfactory epithelium but by 85% at the lower.

Effect of Adenylate Cyclase and Phospholipase C Inhibition

L-cysteine

The effect of 10^{-6} M U73122 (phospholipase C inhibitor) on the olfactory responses to 10^{-3} M L-cysteine was similar between the two epithelia. Responses to L-cysteine were reduced by 83% in the upper epithelium and 89% in the lower (Fig. 2A). The AC inhibitor SQ-22536 (10^{-5} M) had little effect on the responses to 10^{-3} M L-cysteine in

either epithelium reducing them by only 14%. In the presence of both drugs simultaneously (SQ-22536 and U73122 at 10^{-5} M and 10^{-6} M respectively), the responses to L-cysteine were reduced to 4% (96% inhibition) of control responses in both epithelia. In the upper epithelium, the two drugs acting together were more effective than U73122 alone. The inhibitory effect of U73122 was concentration-dependent and could be fitted to conventional Hill equation (Figs. 2A and 2B). The IC_{50} in the upper epithelium was $5.5 \pm 1.8 \times 10^{-8}$ M and the Hill coefficient was 0.61 ± 0.11 (Fig. 2B). In the lower epithelium, the IC_{50} was $3.2 \pm 0.9 \times 10^{-8}$ M and the Hill coefficient was 0.80 ± 0.17 (Fig. 2C).

1-methyl-L-tryptophan/L-phenylalanine

The inhibition of U73122 on the olfactory responses to 1-methyl-L-tryptophan was, again, statistically equal in both epithelia; the amplitude was reduced by 70% in the upper epithelium and 85% in the lower (Fig. 3A). The effect of SQ-22536, however, was different between the two epithelia; responses were inhibited by 32% but only 2% at the upper and lower epithelia, respectively. The effect of U73122 and SQ-22538 acting together also differed between the two epithelia. In the upper epithelium, the olfactory responses to 1-methyl-L-tryptophan were nearly abolished completely. At the lower epithelium, however, the inhibition by U73122 and SQ-22538 together was statistically equal to that induced by U73122 alone. The concentration-dependent inhibition of U73122 on the olfactory responses to 1-methyl-L-tryptophan on the lower epithelium gave an IC_{50} of $2.7 \pm 1.1 \times 10^{-8}$ M and a Hill coefficient of 0.4 ± 0.1 (Fig. 3B).

The effects of the drugs on the olfactory responses to L-phenylalanine were, overall, similar to that of 1-methyl-L-tryptophan. Inhibition by U73122 (10^{-6} M) reduced EOG amplitudes in response to 10^{-3} M L-phenylalanine by 79% in the upper epithelium and 86% in the lower (Fig. 4A). Again, inhibition by SQ-22536 on the olfactory responses to L-phenylalanine was markedly different between the two epithelia; 44% in the upper epithelium but only 2% in the lower. The presence of U73122 and SQ-22536 together completely abolished the olfactory sensitivity to L-phenylalanine in the upper epithelium; however, in the lower epithelium, some response remained (albeit low; 6%). The concentration-dependent inhibition of U73122 on olfactory responses of the lower epithelium to L-phenylalanine gave an IC_{50} of $2.4 \pm 1.0 \times 10^{-8}$ M and a Hill coefficient of 0.5 ± 0.1 (Fig. 4B).

Taurocholic Acid and Cholic Acid

The inhibitory effect of 10^{-6} M U73122 on the olfactory responses to taurocholic acid was greater on the lower epithelium than the upper (Fig. 5A); EOG responses to 10^{-5} M taurocholic acid were inhibited by 38% and 67% at the upper and lower epithelium respectively. Conversely, SQ-22536 inhibited responses to 10^{-5} M taurocholic acid by 50% at the upper olfactory epithelium but only 23% at the lower. However, simultaneous exposure to both U73122 and SQ-22536 failed to further reduce the response amplitudes than U73122 or SQ-22536 alone at the lower and upper epithelia respectively; 61% inhibition at the upper epithelium and 75% inhibition at the lower. The concentration-dependent inhibition of U73122 on the olfactory responses to taurocholic acid from the upper epithelium gave an IC_{50} of $2.0 \pm 0.9 \times 10^{-8}$ M and a Hill coefficient of 0.4 ± 0.1 (Fig. 5B).

The effects of the two drugs on the olfactory responses to cholic acid were similar to that of taurocholic acid. U73122 was more effective in inhibiting the olfactory responses to cholic acid in the lower epithelium than the upper; 70% in the lower epithelium and 41% in the upper (Fig. 6A). Conversely, the inhibitory effect of SQ22536 was greater on the upper epithelium than the lower; 49% in the upper and 29% in the lower (Fig. 6A). The inhibition induced by both U73122 and SQ-22536 simultaneously in the upper epithelium (68%) was greater than that induced by either of them individually. In the lower olfactory epithelium, however, the inhibitory effect of both drugs simultaneously (79%) was equal to that of U73122 alone but greater than that induced by SQ-22536 alone. The concentration-dependent inhibition of U73122 on olfactory responses to cholic acid on the upper epithelium gave an IC_{50} of $1.5 \pm 0.8 \times 10^{-8}$ M and a Hill coefficient of 0.4 ± 0.2 (Fig. 6B).

Discussion

The current study suggests that there are differences in both the olfactory receptors and the signal transduction pathways between the upper and lower olfactory epithelia of the sole. This is consistent with previous studies which have shown that sensitivities to different odorants differ between the two epithelia; certain food-related odorants (*e.g.* L-phenylalanine and 1-methyl-L-tryptophan) are detected better by the lower epithelium whilst conspecifics-derived odorants (*e.g.* taurocholic acid) are detected better by the upper epithelium (Velez *et al.*, 2005; Velez *et al.*, 2007a; Velez *et al.*, 2007b; Velez *et al.*, 2008a).

Cross-adaptation

Taurocholic acid was only able to partially block olfactory responses to L-cysteine in both epithelia. This is not surprising since the two odorants are structurally very different and are likely to be detected by different receptors. However, a proportion of the response (approximately 30%) is due to (a) common mechanism(s) and suggests the existence of relatively non-specific odorant receptors. Conversely, taurocholic acid was able to block the response to cholic acid nearly completely (approximately 95%) in both epithelia, consistent with the similarity in structure between the two odorants. Nevertheless, these results suggest that a small percentage of receptors can differentiate between these closely related odorants; sole release taurocholic acid, but not cholic acid, to the water (Velez *et al.*, 2008b). The response to 1-methyl-L-tryptophan, on the other hand, was hardly affected by the presence of taurocholic acid on the lower epithelium, whereas the response was more than halved in the upper epithelium. This suggests that the selectivities and/or sub-types of odorant receptors expressed in the two epithelia are

different. This is consistent with our previous finding that 1-methyl-L-tryptophan is released by ragworms (*Hediste diversicolor*), one of the sole's main prey (Cabral, 2000), which live buried in the substrate, and may reflect a specialisation of the lower epithelium in specifically detecting this odorant.

With L-cysteine, 1-methyl-L-tryptophan or cholic acid as the adapting odorant, however, the olfactory responses to taurocholic acid were consistently reduced more in the lower epithelium than the upper. Similarly, this may reflect a greater proportion of odorant receptors in the upper epithelium that are more selective for taurocholic acid than the lower epithelium. Again, this is consistent with our previous finding that sole release taurocholic acid into the water and the upper olfactory epithelium is more sensitive to it than the lower (Velez *et al.*, 2008b). This suggests that the upper epithelium may be more important in the detection of con-specifics - *e.g.* pheromones - and/or other fish species than the lower.

Transduction pathways

The olfactory responses to L-cysteine were markedly inhibited by the PLC inhibitor U73122 (approximately 80-90%); the AC inhibitor SQ-22536 was only able to reduce the response by around 10%. These results suggest that the PLC/IP_3 pathway is the predominant transduction mechanism for the olfactory sensitivity to this amino acid, with only a minor role for the AC/cAMP pathway. Furthermore, some response, albeit small, remained in the presence of both drugs simultaneously; this suggests that part of the response to L-cysteine is mediated by non-PLC/non-AC pathways and/or that either or both of the drugs are not 100% effective at the concentrations used (see below). Significantly, however, the inhibitory effects of these drugs were equal in both nostrils.

This is consistent with our previous observation that olfactory sensitivity to L-cysteine is equal in the two epithelia (Velez *et al.*, 2005). Conversely, the effects of the drugs on olfactory responses to L-phenylalanine and 1-methyl-L-tryptophan - both putative food-related odorants (Velez *et al.*, 2007b; Velez *et al.*, 2008a) - differed between the two epithelia. This is again consistent with our previous findings that the lower epithelium is more sensitive to these amino acids than the upper; possibly different receptors, acting *via* different transduction pathways, are responding to the same odorant in the two epithelia. In both epithelia, however, the effect of U73122 was greater than that of SQ-22536, suggesting that the PLC/IP_3 transduction pathway is, again, more important than the AC/cAMP pathway for these amino acids. In the lower epithelium especially, the two drugs applied simultaneously were unable to inhibit olfactory responses to either odorant completely. Again, this suggests the presence of non-PLC/non-AC dependent olfactory transduction pathways in the sole.

The inhibitory effects of both drugs were similar for the two bile acid odorants used (taurocholic acid and cholic acid); this supports the cross-adaptation results that suggest that, for the most part, they are acting *via* the same receptors. In the upper epithelium - and in contrast to the amino acid odorants - the effects of either drug were approximately equal; if anything SQ-22536 was more effective than U73122. Given that the upper epithelium of the sole is more sensitive to bile acids (Velez *et al.*, 2005; Velez *et al.*, 2007a), this suggests that, in contrast to the amino acid odorants, the AC/cAMP transduction pathway is of equal importance to the PLC/IP_3 pathway in bile acid detection. However, application of both drugs simultaneously did not inhibit responses to either bile acid completely; 20-40% of control responses remained. This may indicative that non-PLC/non-AC mediated pathways may be more important for

bile acid than amino acid detection, at least in the sole. What these mechanisms may be was not addressed by the present study. Nevertheless, some studies have suggested, in rats at least, the involvement of phosphodiesterase and gaseous second messengers, such as nitric oxide and carbon monoxide, in olfactory transduction (Breer *et al.*, 1992; Juilfs *et al.*, 1997; Verma *et al.*, 1993). Whether similar mechanisms exist in fish remains to be tested.

Efficacy of U73122 and SQ-22536

In no case in the present study was the olfactory response to a given odorant completely inhibited by either drug alone. This may be because, as previously suggested, more than one transduction pathway is involved for these odorants. However, it may also be because the efficacy of the drugs is not 100% in the sole; these two explanations are not mutually exclusive. Nevertheless, the IC_{50} values estimated for U73122 (15-55 nM) are sufficiently low to suggest that this drug is effective in the sole as it is in other non-mammalian vertebrates (Brann and Fadool, 2006; Jung *et al.*, 2005; Wellerdieck *et al.*, 1997) and there were no clear differences in the IC_{50} values between odorants or epithelia. The AC inhibitor SQ-22536 only induced inhibitory responses at concentrations of 10^{-6} and 10^{-5} M so IC_{50} values could not be calculated. Nevertheless, in the case of L-phenylalanine stimulating the upper olfactory epithelium, both drugs acting together were able to completely inhibit any response. This supports the idea that odorants act *via* more than one transduction pathway, rather than the drugs are less than 100% effective. Whether one odorant receptor - or one olfactory receptor neuron - may act *via* more than one transduction pathway (as in crustaceans; Ache and Zhainazarov, 1995; Hatt and Ache, 1994) is an open question. Current work in our laboratory is

aimed at identifying the isoforms of adenylate cyclase and phospholipase C in the sole, and to assess whether these may also differ between the two epithelia.

Conclusions

In summary, the current study provides evidence that, in the olfactory system of the sole, the PLC/IP_3 transduction pathway is more important than the AC/cAMP pathway for amino acid odorants whereas both pathways are approximately equally involved in bile acid detection. In both cases, however, a proportion of the olfactory response may be due to non-PLC/non-AC mediated mechanisms. Furthermore, there are apparent differences in the receptors and transduction mechanisms for the same odorant between the upper and lower olfactory epithelia. Where present, these differences correlate with differential olfactory sensitivity to food-related and conspecific-related odorants

Acknowledgements

This study was supported by grants SFRH/BD/16242/2004 and POCI/BIA-BMC/55467/2004 from the Portuguese National Science Foundation and European Union Social Funds for Regional Development.

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Figure Legends

Figure 1. A Effect of cross-adaptation to 10^{-5} M taurocholic acid on the olfactory responses (EOG) evoked by L-cysteine (10^{-3} M), 1-methyl-L-tryptophan (10^{-3} M) and cholic acid (10^{-5} M) recorded from the upper (black bars) and lower epithelia (white bars) of sole. Data are shown as mean + S.E.M. (n=6). *** $P<0.001$ between the two epithelia. **B** Effect of cross-adaptation to 10^{-3} M L-cysteine, 10^{-3} M 1-methyl-L-tryptophan and 10^{-5} M cholic acid (CHO) on the olfactory responses to 10^{-5} M taurocholic acid recorded from the upper (black bars) or lower epithelium (white bars) of sole. Data are shown as mean + S.E.M. (n=6). *** $P<0.001$ between the two epithelia.

Figure 2. A. Olfactory responses to 10^{-3} M L-cysteine recorded from the upper (black bars) and lower (white bars) olfactory epithelia of sole during exposure to U73122 (10^{-6} M) and/or SQ-22536 (10^{-5} M). Bars with different letters (a, b or c and x or y) are significantly different ($P<0.05$) between treatments (drugs) but within each epithelium. No differences were found between the two epithelia. Data are shown as mean + S.E.M. (n=6). **B** and **C**. Normalised olfactory responses to 10^{-3} M L-cysteine as a function of increasing [U73122] recorded from the upper (**B**) and lower (**C**) olfactory epithelia. Data are shown as mean ± S.E.M. (n=6). A Hill plot has been fitted to the mean values.

Figure 3. A. Olfactory responses to 10^{-3} M 1-methyl-L-tryptophan recorded from the upper (black bars) and lower (white bars) olfactory epithelia of sole during exposure to U73122 (10^{-6} M) and/or SQ-22536 (10^{-5} M). Data are shown as mean + S.E.M. (n=6).

Bars with different letters (a, b or c and x or y) are significantly different ($P<0.05$) between treatments (drugs) but within each epithelium. ** $P<0.01$ between the two epithelia with the same treatment (drug). **B.** Normalised olfactory responses to 10^{-3} M 1-methyl-L-tryptophan as a function of increasing [U73122] recorded from the lower olfactory epithelium. Data are shown as mean ± S.E.M. (n=6); only the results from the lower epithelium are shown as it is significantly more sensitive to 1-methy-L-tryptophan than the upper. A Hill plot has been fitted to the mean values.

Figure 4. A. Olfactory responses to 10^{-3} M L-phenylalanine recorded from the upper (black bars) and lower (white bars) olfactory epithelia of sole during exposure to U73122 (10^{-6} M) and/or SQ-22536 (10^{-5} M). Data are shown as mean + S.E.M. (n=6). Bars with different letters (a, b or c and x or y) are significantly different ($P<0.05$) between treatments (drugs) but within each epithelium. ** $P<0.01$, *** $P<0.001$ between the two epithelia with the same treatment (drug). **B.** Normalised olfactory responses to 10^{-3} M L-phenylalanine as a function of increasing [U73122] recorded from the lower olfactory epithelium. Data are shown as mean ± S.E.M. (n=6); only the results from the lower epithelium are shown as it is significantly more sensitive to L-phenylalanine than the upper. A Hill plot has been fitted to the mean values.

Figure 5. A. Olfactory responses to 10^{-5} M taurocholic acid recorded from the upper (black bars) and lower (white bars) olfactory epithelia of sole during exposure to U73122 (10^{-6} M) and/or SQ-22536 (10^{-5} M). Data are shown as mean + S.E.M. (n=6). Bars with different letters (a or b and x or y) are significantly different ($P<0.05$)

between treatments (drugs) but within each epithelium. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ between the two epithelia with the same treatment (drug). **B.** Normalised olfactory responses to 10^{-5} M taurocholic acid as a function of increasing [U73122] recorded from the upper olfactory epithelium. Data are shown as mean ± S.E.M. (n=6); only the results from the upper epithelium are shown as it is significantly more sensitive to taurocholic acid than the lower. A Hill plot has been fitted to the mean values (N.B. the U73122-insensitive fraction of the response has been subtracted).

Figure 6. A. Olfactory responses to 10^{-5} M cholic acid recorded from the upper (black bars) and lower (white bars) olfactory epithelia of sole during exposure to U73122 (10^{-6} M) and/or SQ-22536 (10^{-5} M). Data are shown as mean + S.E.M. (n=6). Bars with different letters (a or b and x or y) are significantly different ($P<0.05$) between treatments (drugs) but within each epithelium. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ between the two epithelia with the same treatment (drug). **B.** Normalised olfactory responses to 10^{-5} M cholic acid as a function of increasing [U73122] recorded from the upper olfactory epithelium. Data are shown as mean ± S.E.M. (n=6); only the results from the upper epithelium are shown as it is significantly more sensitive to cholic acid than the lower. A Hill plot has been fitted to the mean values (N.B. the U73122-insensitive fraction of the response has been subtracted).

Figure 1A

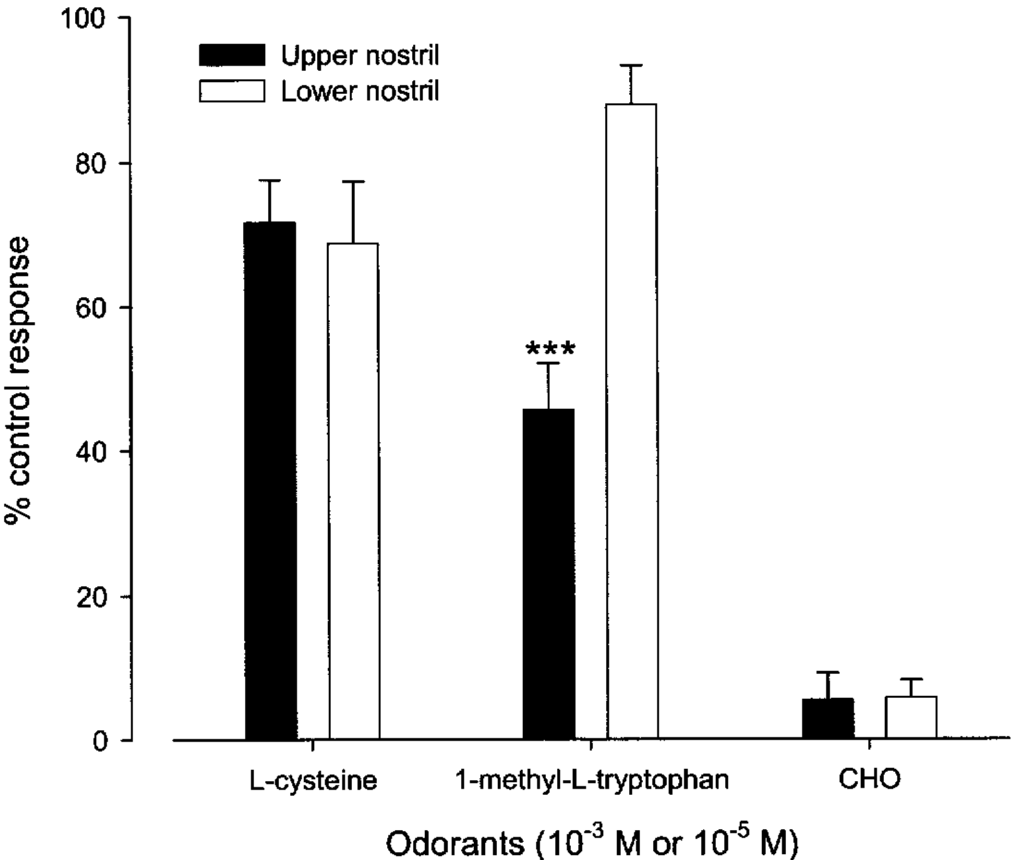


Figure 1B

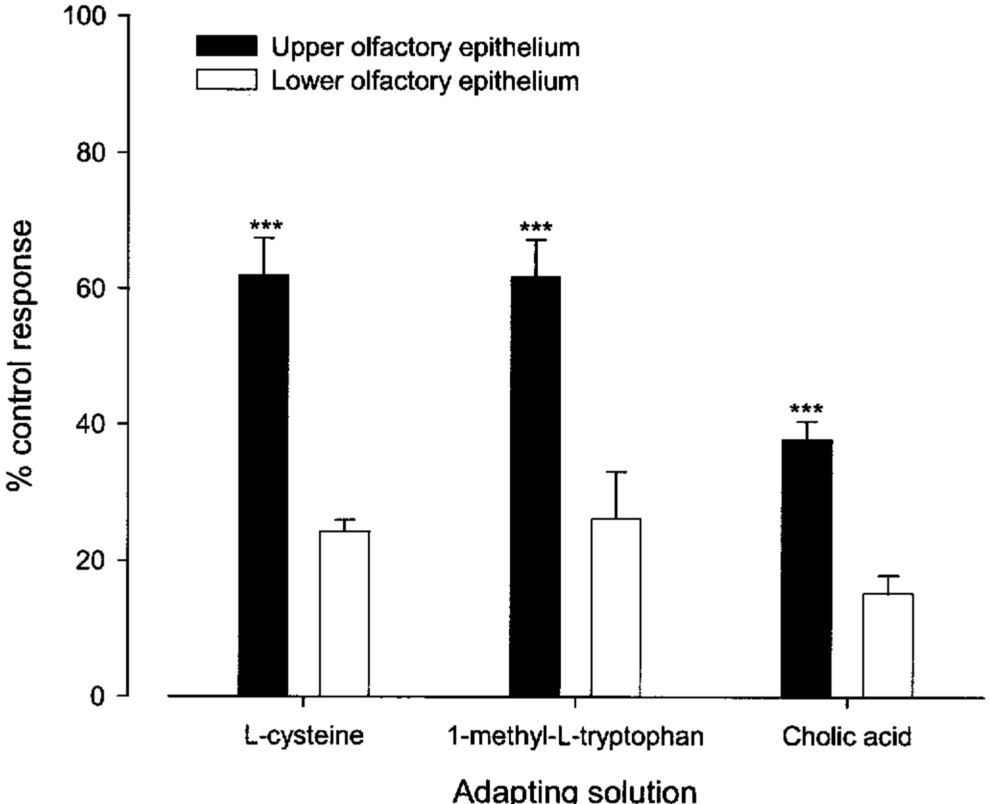


Figure 2A

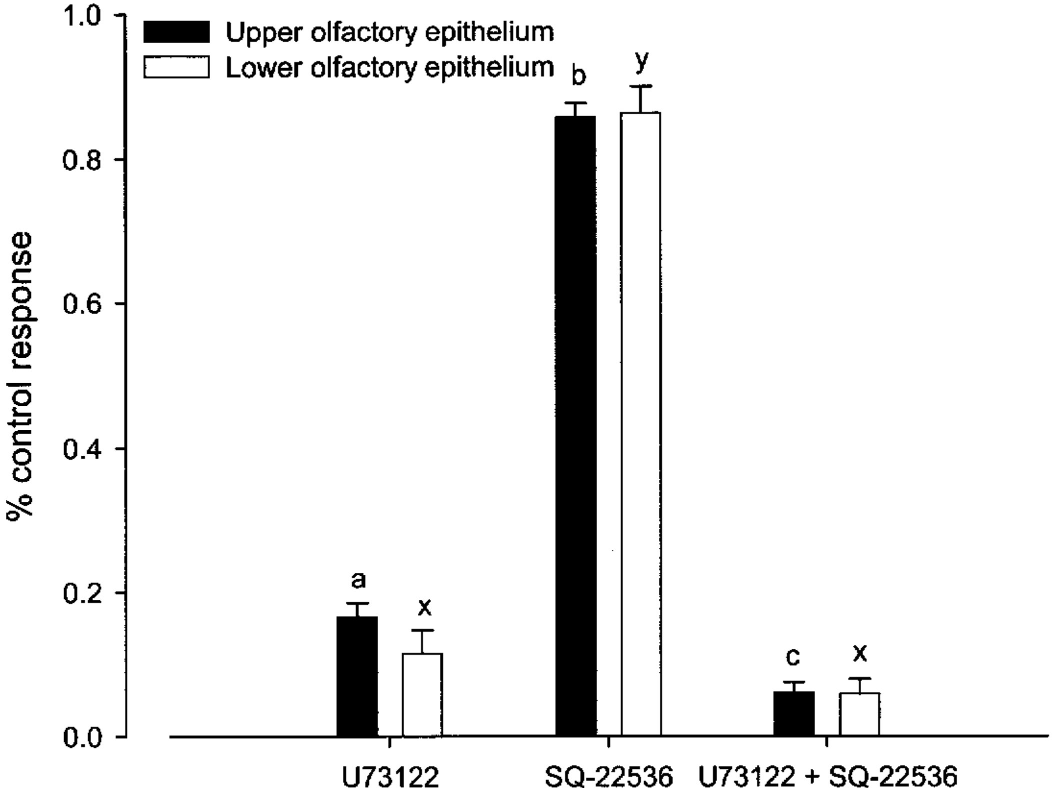
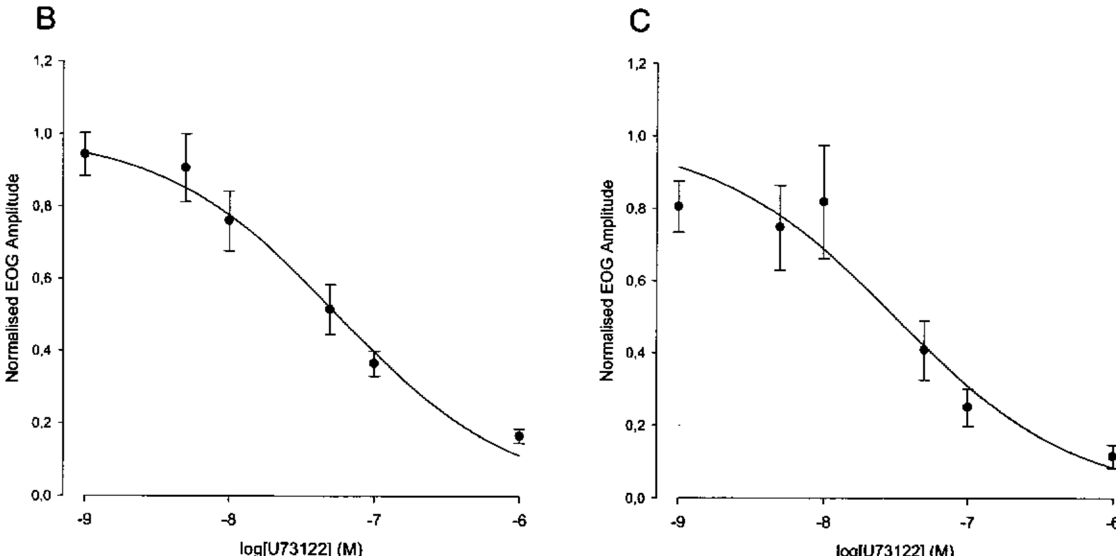


Figure 2B and 2C



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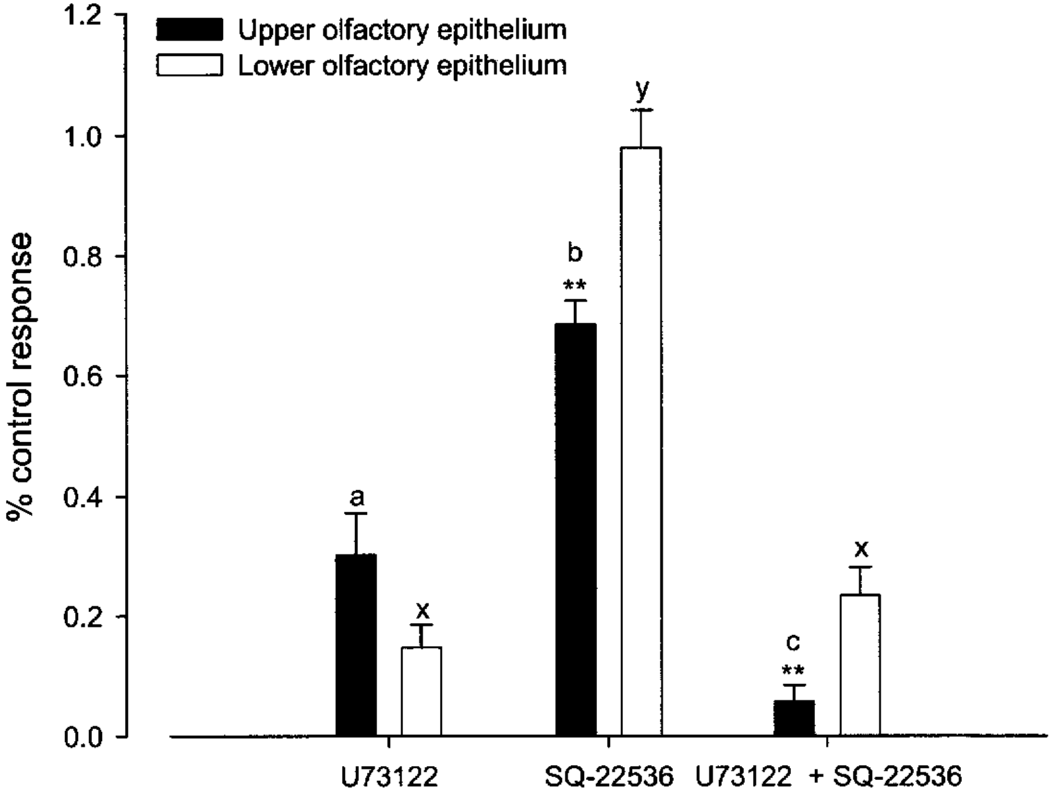

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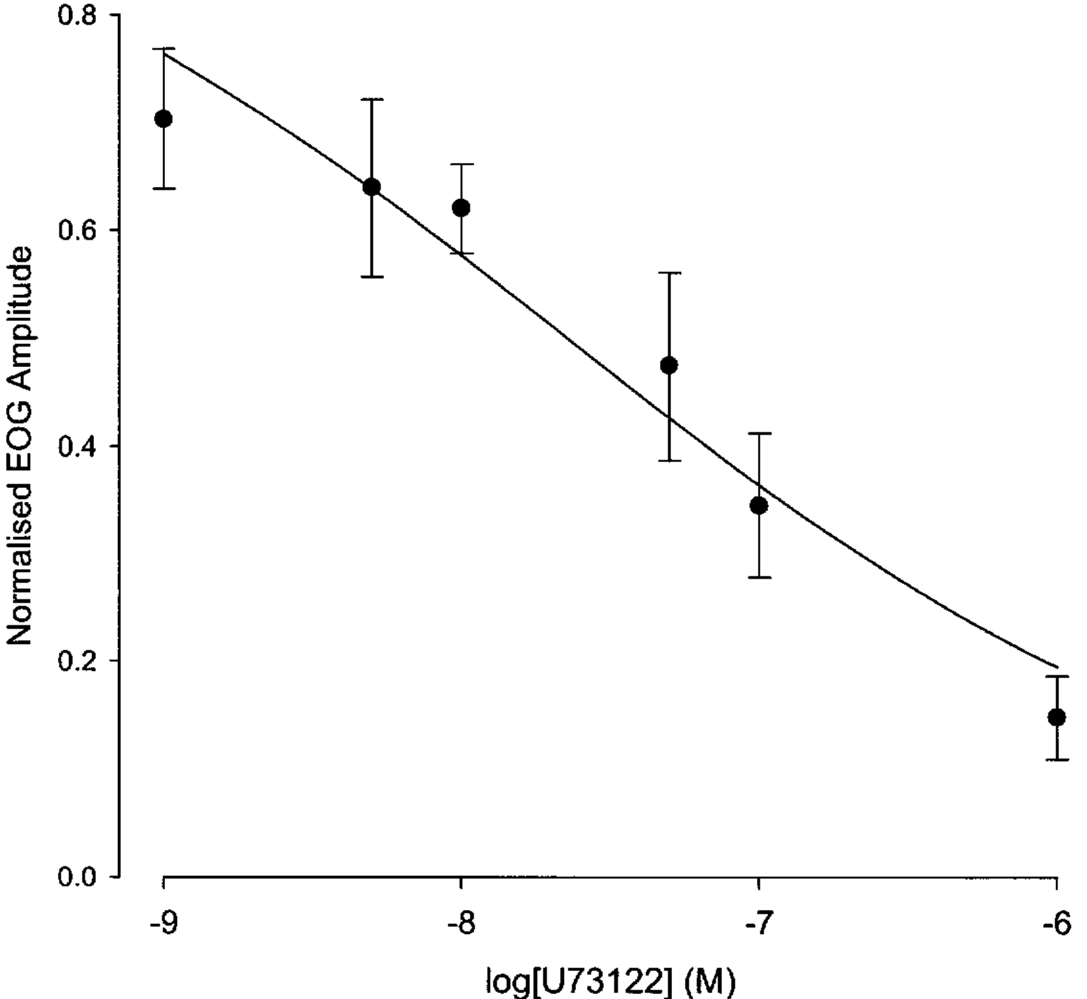

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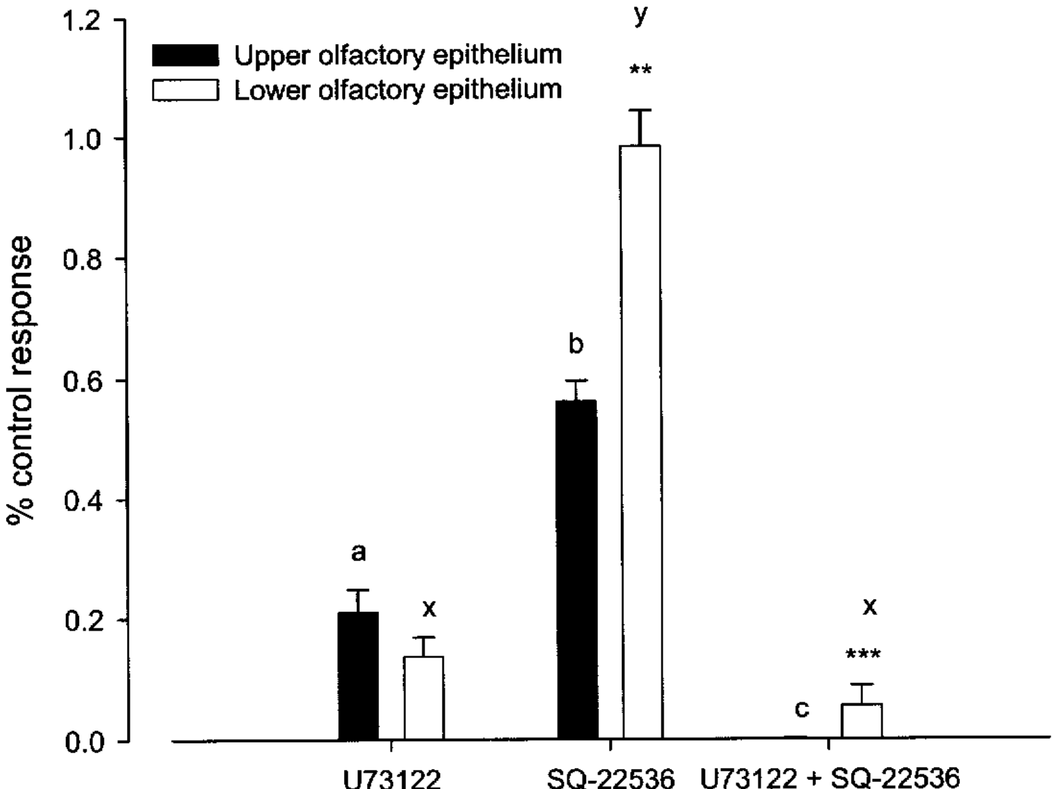

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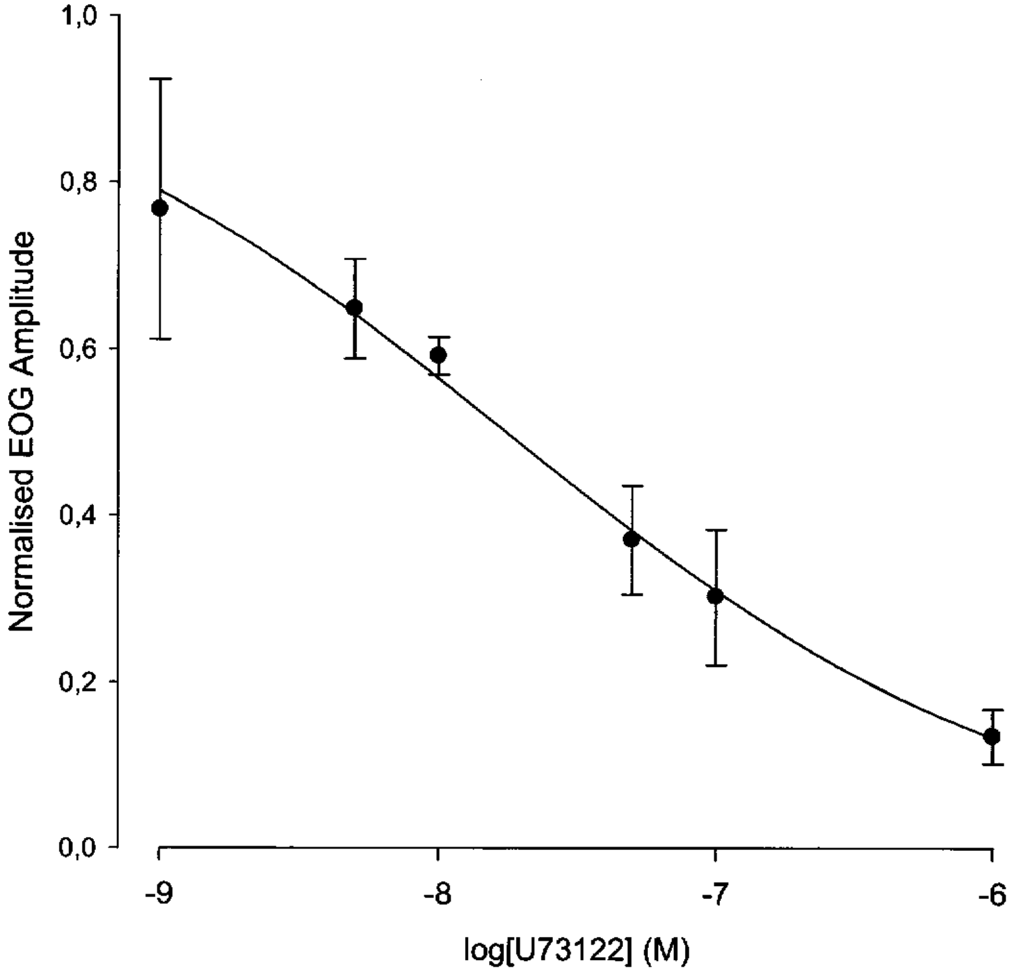

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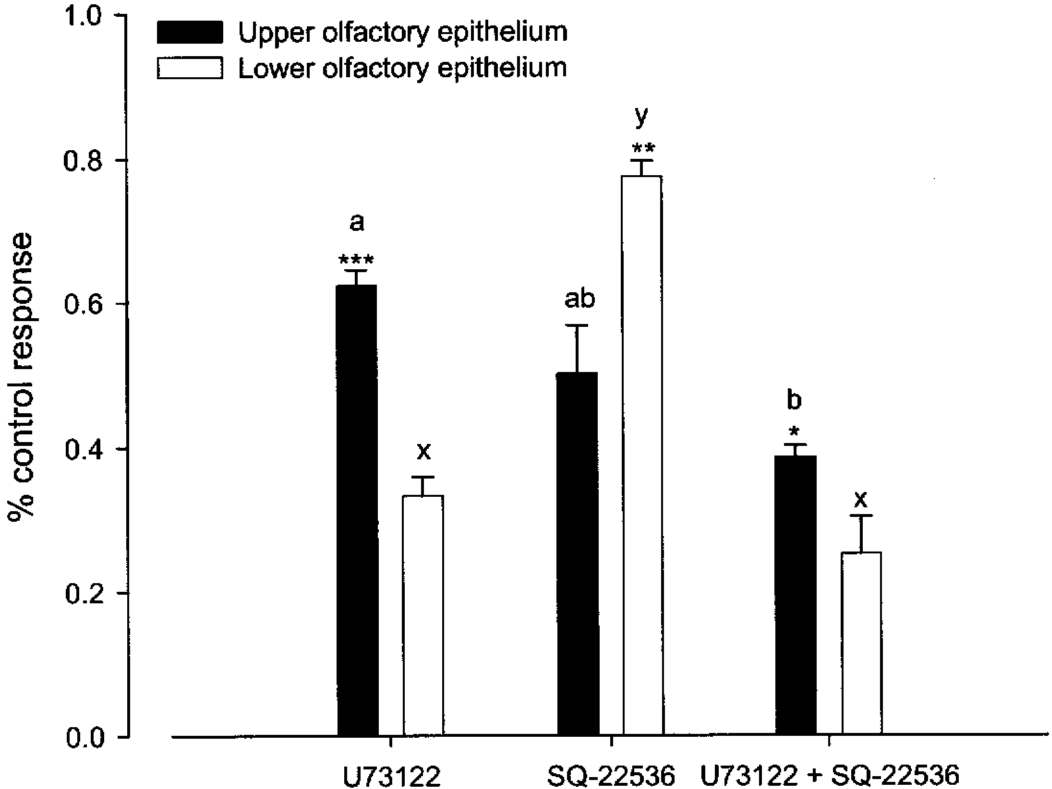

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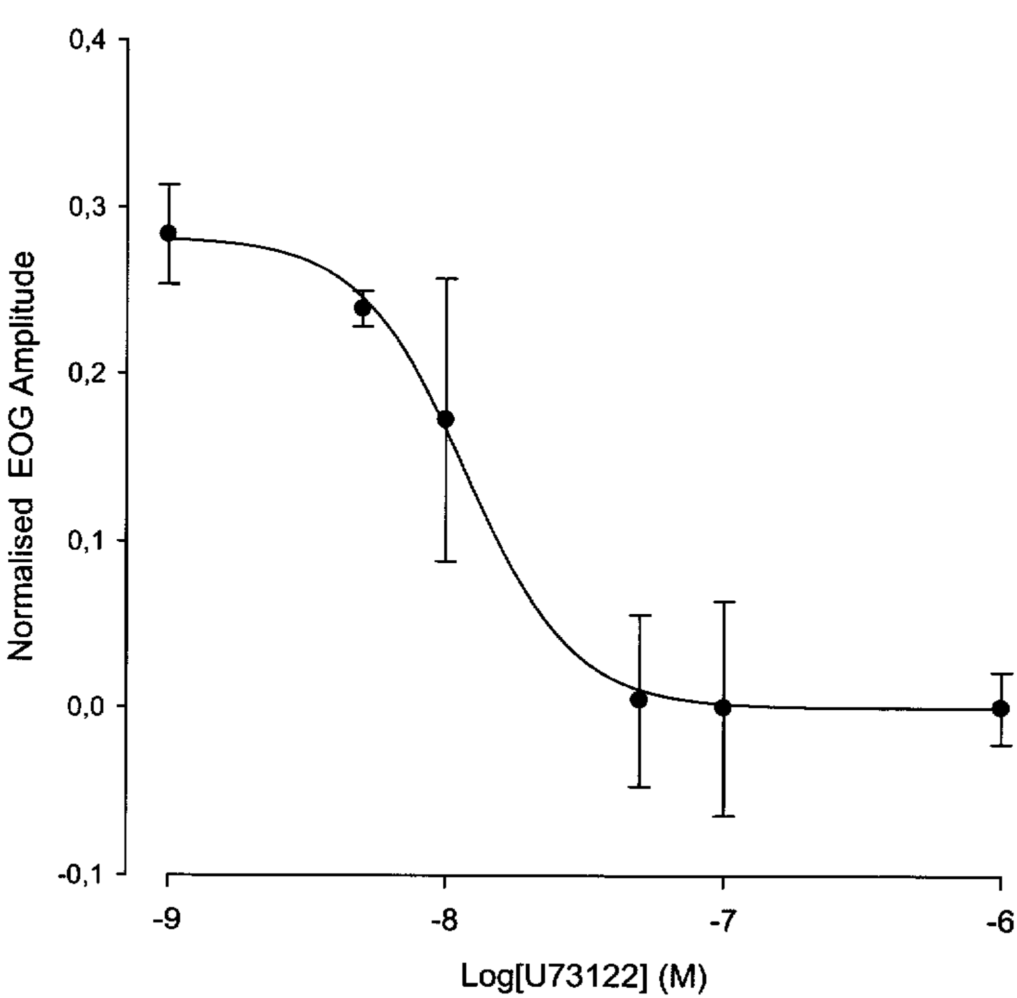


Figure 6A

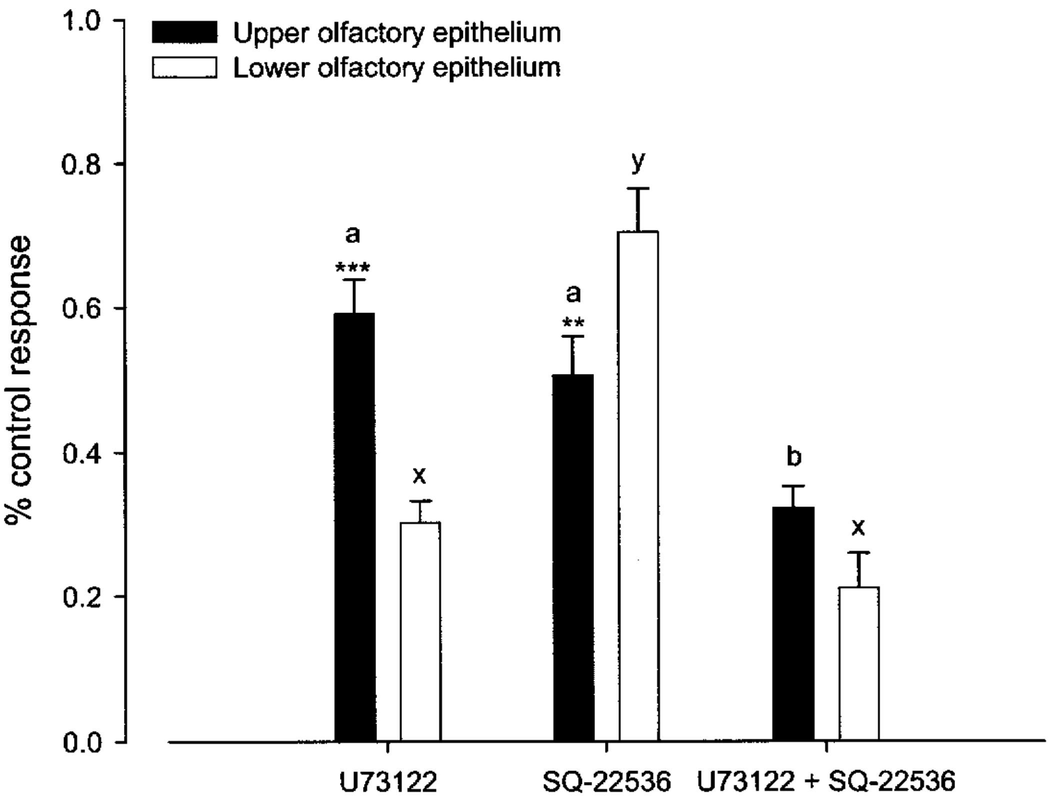
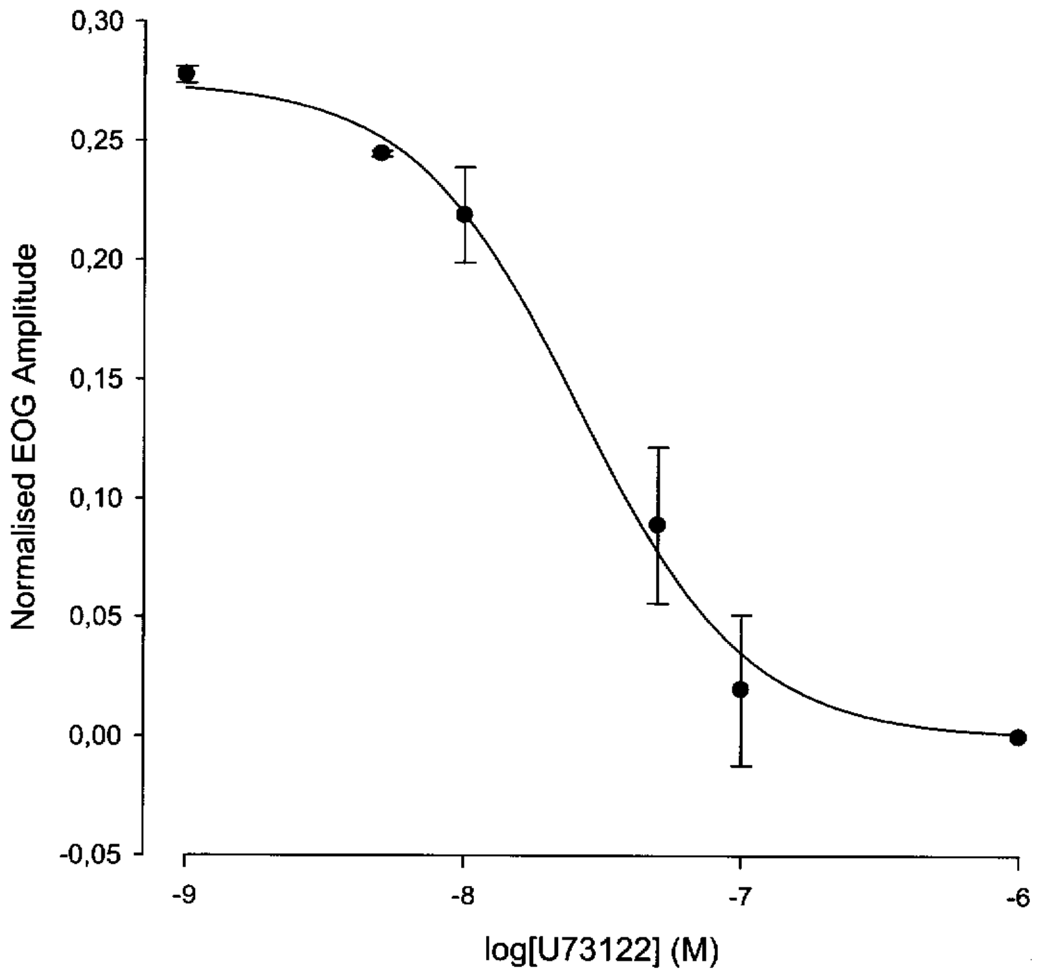


Figure 6B



2.5 Olfactory sensitivity to environmental inorganic cations

Adaptation to reduced salinity affects the olfactory sensitivity of Senegalese sole (*Solea senegalensis*) to Ca^{2+} and Na^{+} but not amino acids.

This manuscript will be submitted after revision to *Journal of Experimental Biology*, with the following co-authors: Zélia Velez[1,2], Peter C. Hubbard[1], Eduardo N. Barata[1,2] and Adelino V.M. Canário[1]

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Abstract

The Senegalese sole is a marine flatfish which often penetrates into brackish water to feed. It cannot, however, survive in full freshwater. The current study investigated the effect of adaptation to low salinity (10‰) on olfactory sensitivity to changes in environmental [Ca^{2+}] and [Na^{+}] and amino acids by the electro-encephalogram recorded from the olfactory bulb. The sole showed olfactory sensitivity to increases in environmental [Na^{+}] and decreases in environmental [Ca^{2+}]; sensitivity to Na^{+} higher was greater at 10‰ whereas that to Ca^{2+} was greater at 35‰. Decreased environmental [Na^{+}] increased sensitivity to changes in [Ca^{2+}], whereas increased environmental [Ca^{2+}] decreased bulbar responses to changes in [Na^{+}]. Sensitivity to amino acids was unaffected by external salinity. However, the absence of external Na^{+} strongly decreased bulbar responses to amino acids in fish adapted to 35‰ seawater, but not in those at 10‰. The absence of external Ca^{2+} had no such effect at either salinity. This suggests that odorant-receptor binding and/or olfactory transduction is reliant on external Na^{+} (but not Ca^{2+}) at higher salinities but the olfactory system is able to adapt to lower environmental [Na^{+}]. Taken together, these results suggest that reductions of external salinity modulate olfactory sensitivity to environmental Ca^{2+} and Na^{+} but not amino acids. However, at low salinities, olfactory sensitivity to amino acids is maintained by decreasing reliance on external Na^{+}.

Introduction

Maintenance of constant plasma [Ca^{2+}] and [Na^{+}] is crucial to vertebrates; both ions are involved in a multitude of physiological processes (*e.g.* muscular contraction, action potential generation and cellular signalling). In the aquatic environment, levels of these ions can vary from vastly in excess of physiological needs (*e.g.* seawater) to nearly zero (soft freshwater). As yet, however, the mechanisms by which fish perceive these environmental levels are poorly understood. Accumulating evidence suggests that teleosts have olfactory sensitivity to changes in environmental [Ca^{2+}] and [Na^{+}] (Bodznick, 1978; Hubbard *et al.*, 2000; Hubbard *et al.*, 2002; Nearing *et al.*, 2002). Olfactory sensitivity to changes in environmental [Ca^{2+}] has been described in both freshwater and marine fish; goldfish (*Carassius auratus*) and freshwater-reared sockeye salmon (*Oncorhynchus nerka*) have olfactory sensitivity to increases in environmental [Ca^{2+}] (Bodznick, 1978; Hubbard *et al.*, 2002) whereas the seabream (*Sparus aurata*) is sensitive to reductions in [Ca^{2+}] (Hubbard *et al.*, 2000). Bodznick (1978) proposed that the olfactory sensitivity of freshwater-reared sockeye salmon to changes in environmental [Ca^{2+}] contributed to their ability to identify natal rivers. However, goldfish and seabream are non-migratory, which suggests that there may be other reasons for fish to be able to monitor environmental [Ca^{2+}] (Hubbard *et al.*, 2000; Hubbard *et al.*, 2002). Seabream, although predominantly marine, can survive in brackish water but not freshwater; it has been suggested that olfactory sensitivity to changes in environmental [Ca^{2+}] is used to warn the fish that it is reaching the limit of its salinity tolerance (Hubbard *et al.*, 2000; Nearing *et al.*, 2002). It also may affect the physiological mechanisms regulating internal calcium homeostasis.

Goldfish and salmon also have olfactory sensitivity to increases in environmental [Na^+] (Bodznick, 1978; Hubbard and Canário, 2007; Hubbard *et al.*, 2002). The physiological significance of this phenomenon is not yet known; however, the relatively high detection threshold for Na^+ suggests that this sensitivity is not involved in routine monitoring but may serve as a warning that fish is approaching the limits of its osmoregulatory capacity and/or activate osmoregulatory mechanisms (Hubbard *et al.*, 2002). Nevertheless, in the goldfish at least, Ca^{2+} and Na^+ are detected by distinct olfactory mechanisms (Hubbard and Canário, 2007).

As well as acting as putative odorant in their own right, both Ca^{2+} and Na^+ are intimately involved in olfactory transduction of 'conventional' odorants (Menini, 1999; Nakamura, 2000; Schild and Restrepo, 1998). How the large changes in external availability of these ions that occur on movement between sea- and freshwater (and *vice versa*) may affect olfactory sensitivity has been little studied. In euryhaline fish, such as salmonids, olfactory sensitivity to amino acids seems to be largely independent of external salinity (Shoji *et al.*, 1994; Shoji *et al.*, 1996). However, in the seabream, short-term exposure to Ca^{2+}-free seawater caused a temporary reduction in the olfactory response to L-serine (Hubbard *et al.*, 2000). Thus, olfactory transduction in fish may be independent of external Ca^{2+} and Na^+ or they may be able to adapt to changing levels. If and how olfactory sensitivity to these ions changes with external salinity has not yet been investigated.

The Senegalese sole (*Solea senegalensis*), hereafter 'sole', is a marine flatfish of the Mediterranean Sea and north-west Atlantic Ocean. It can, and often does, penetrate estuarine waters to feed (Cabral, 2000), although it cannot survive in full freshwater. As it is nocturnal and its main prey live buried in the substrate (Bayarri *et al.*, 2004; Cabral, 2000), it is likely that olfaction plays an important role in food-search. Previous work

has shown that the amino acids L-phenylalanine and glycine are released by one of the sole's main prey and that the sole has olfactory sensitivity to both (Velez *et al.*, 2007). The current study, therefore, investigated how adaptation to reduced salinity may affect olfactory sensitivity to Ca^{2+}, Na^{+} and these amino acids and the reliance on external Ca^{2+} and Na^{+} in olfactory transduction.

Materials and Methods

Experimental animals

Sole were obtained from local aquaculture facilities (IPIMAR, Olhão, Portugal). Fish were grown according to procedure described by Dinis et al. (1999) and were fed daily on commercial pellets (AQUASOJA 2-3.5 mm, Sorgal SA, Portugal). Experiments were carried out on fish kept in seawater (35‰) and fish adapted to low-salinity seawater (10‰). Adaptation to low salinity was carried gradually over one week; sole were then kept at 10‰ for one week prior to use. At the time of experiments animals were between 100-300g.

Recording the electro-encephalogram (EEG) from the olfactory bulb

Prior to recording, fish were anaesthetised by immersion in water containing 200 $mg.l^{-1}$ MS222 (3-aminobenzoic acid ethyl ester; Sigma-Aldrich) followed by intramuscular injection of gallamine triethiodide (Sigma-Aldrich; 0.6 $mg.100g^{-1}$ body weight) and placed on a padded surface with a slight forward tilt (to prevent water from entering the wound). The gills were irrigated with a constant flow (approximately 1 $ml.g^{-1}$ body $weight.min^{-1}$) of aerated water (of the appropriate salinity) containing MS-222 (100 $mg.l^{-1}$). The body of the fish was covered by damp paper towel and the eyes covered with small pieces of black polythene. The olfactory rosette was exposed by cutting the skin and connective tissue covering the epithelium. The nostril was constantly irrigated with charcoal-filtered seawater (without anaesthetic) under gravity (flow-rate: 6–8 $ml.min^{-1}$) *via* a glass tube. Test solutions were delivered to the tube irrigating the nasal cavity *via* a computer-operated three-way solenoid valve for a period of 5 s. The olfactory bulb was exposed by removal of the skin, connective tissue and the overlying

bone. The electro-encephalogram (EEG) was recorded with a purpose-built 'suction' electrode (Brierley *et al.*, 2001; Hubbard and Canário, 2007) connected to Neurolog NL104 AC pre-amplifier (Digitimer Ltd., Welwyn Garden City, UK). The signal was filtered (low-pass 300 Hz, high pass 3 Hz; Neurolog NL125, Digitimer Ltd) and integrated (time constant 1 s, Neurolog NL703, Digitimer Ltd). Both the direct and integrated signals were digitized (Digidata 1300A, Molecular Devices Corporation, Sunny Vale, CA, USA) and displayed on a computer running Axoscope 9.2 software (Molecular Devices Corporation).

Stimulus Solutions

Artificial seawaters (ASW) at 35‰ and 10‰, with and without calcium, with and without sodium, and with and without choline were prepared as shown in Table 1. The appropriate ranges of [Ca^{2+}] or [Na^{+}] were made up by appropriate mixing of these solutions. Choline chloride was used to maintain osmolality and [Cl^{-}] in Ca^{2+}- and Na^{+}-free solutions. Thus, the only difference in ionic composition between ASW and Na^{+} and/or Ca^{2+}-free ASW is that Ca^{2+} and Na^{+} were replaced by choline. Control experiments using Ca^{2+} free and Na^{+} free ASW without choline and ASW plus 20 $mmol.l^{-1}$ choline chloride as a stimulus were also carried out. Amino acids were prepared the same water perfusing the olfactory epithelium.

To test the effects of absence of external Ca^{2+} and Na^{+} on olfactory sensitivity, stimuli were prepared in Ca^{2+}- or Na^{+}-free ASW depending of the ion being tested. When testing the olfactory sensitivity in the absence of external Ca^{2+}, the background water perfusing the olfactory epithelium was Ca^{2+}-free ASW (10‰ or 35‰ as appropriate). When assessing the olfactory sensitivity in the absence of external Na^{+} the

background water superfusing the olfactory epithelium was Na^+ free ASW (10‰ or 35‰ as appropriate).

Data Treatment and Statistical Analysis

The amplitude of each integrated EEG response was blank subtracted (using the appropriate blank solution). The bulbar response to 1.0 $mmol.l^{-1}$ L-cysteine (in 35‰-ASW or 10‰-ASW, as appropriate) was checked at regular intervals throughout the recording period. To obtain concentration/response curves, integrated response amplitudes were normalised to that of 10^{-3} M L-cysteine. To evaluate the effect of Na^+ and Ca^{2+} on olfactory sensitivity, the integrated response in the absence of these ions was normalised to control responses recorded in the presence of that ion. Responses to changes in external $[Ca^{2+}]$ were fitted to a three-parameter Hill equation as previously described (Hubbard *et al.*, 2000) to obtain the apparent IC_{50} (concentration giving a 50% maximal response), Hill co-efficient and I_{max} (maximum response) values. Apparent IC_{50} and I_{max} values were compared using Student's t-test. Differences in responsiveness to amino acids and sodium between 35‰ and 10‰ were assessed by linear regression of log-transformed data (Hubbard *et al.*, 2003; Velez *et al.*, 2005) and comparing both the slopes and elevations of the regressions (Zar, 1996). For the effect of absence of external Ca^{2+} and Na^+ on bulbar responses to amino acids, the data were analysed by repeated-measures ANOVA followed by Dunnett's test (SigmaStat 2000, SPSS Science). In all cases, $P<0.05$ was taken to represent statistical significance.

Results

Olfactory responses to changes in environmental [Ca^{2+}]

Sole responded to a reduction of [Ca^{2+}] in ASW with an increase of EEG wave amplitude in the olfactory bulb (Figs. 1A and 1B); this increase was higher in fish at 35‰ seawater than those adapted to 10‰ (Figs. 1C - 1D). At 10‰, the apparent IC_{50} was 2.01 ± 0.44 mmol.l^{-1} Ca^{2+} (mean ± S.E.M., $N = 6$) with a Hill co-efficient of -2.08 ± 0.59 and an I_{max} of 0.5 1± 0.07. At 35‰, the olfactory sensitivity to reductions in environmental [Ca^{2+}] was higher, as reflected by a lower IC_{50} of 0.72 ± 0.17 mmol.l^{-1} (mean ± S.E.M.; N=6), a Hill coefficient of -1.48 ± 0.27 and a higher I_{max} of 1.29 ± 0.19. Both IC_{50} and I_{max} were statistically different between 10‰ and 35‰. During stimulation with reductions in external [Ca^{2+}], EEG wave frequency in the olfactory bulb was similar to that from brain activity and there were no differences between 10‰ and 35‰ adapted fish (data not shown). Substitution of $CaCl_2$ with choline chloride did not affect the responses; neither changes in osmolality nor [Cl^-] are responsible for the observed responses.

Olfactory sensitivity to changes in [Na^+]

As with Ca^{2+}, during stimulation with Na^+, EEG wave frequency in the olfactory bulb was similar to basal brain activity, neither were there differences between 10‰ and 35‰ sea water. However, sole responded to increases of external [Na^+] with an increase of wave amplitude in the olfactory bulb (Figs. 2A and 2B). This increase in wave amplitude was higher in fish adapted to 10‰ sea water than in those kept at 35‰ (Fig. 2C). In contrast to the responses to changes in [Ca^{2+}], bulbar responses were only seen to increases in external [Na^+]; reduction of external [Na^+] failed to evoke any response,

independent of salinity (data not shown). As the bulbar responses to increases of external $[Na^+]$ showed no sign of reaching a maximum, even at 414 mmol.l^{-1}, concentration/response curves could not be fitted to a conventional three-parameter Hill equation. The concentration/response curves to changes in $[Na^+]$ at 35‰ and 10‰ ASW were, nevertheless, statistically different in both the slopes and elevations of the regressions (Fig. 2C). Substitution of NaCl with choline chloride did not affect the responses; neither changes in osmolality nor $[Cl^-]$ were responsible for the observed responses.

Olfactory sensitivity to amino acids

Stimulation of the olfactory epithelium with L-cysteine evoked an increase of EEG wave amplitude in the olfactory bulb in a concentration-dependent manner (Fig. 3). However, there was no statistically significant effect of salinity on the concentration/response curve to L-cysteine (Fig. 3C). Bulbar EEG wave frequency in response to stimulation with L-cysteine was similar to basal activity and there were, again, no differences between 10‰ and 35‰ adapted fish.

There were no effects of salinity on the olfactory sensitivity to amino acids glycine and L-phenylalanine (Figs. 4 and 5). Bulbar responses to amino acids in both salinities showed no sign of reaching a maximum, even at 1.0 mmol.l^{-1}, thus concentration/response curves were not fitted to a Hill equation.

Effect of absence of external Ca^{2+} and Na^+ on olfactory sensitivity to amino acids

The effects of absence of external Ca^{2+} and Na^+ on olfactory sensitivity to amino acids were similar for all three amino acids tested (Fig. 6). The bulbar responses to amino acids at both salinities were not altered by the absence of external Ca^{2+}, whereas the

absence of Na^+ strongly decreased the amplitude of bulbar responses to all three amino acids but only at 35‰, not at 10‰.

At 10‰, the absence of Ca^{2+} did not alter bulbar responses to increases in external [Na^+]. However, at 35‰, bulbar responses to increases in external [Na^+] increase more than three times compared to control (Fig. 7). Conversely, the absence of external Na^+ completely attenuated the bulbar response to decreases in external [Ca^{2+}] at both salinities (Fig. 7). The bulbar responses to reductions (10 $mmol.l^{-1}$ to 0) of external [Ca^{2+}] at different background [Na^+] levels were severely bluntened when the external [Na^+] was below 46 $mmol.l^{-1}$. Above this [Na^+], the amplitude of bulbar responses to decreases in external [Ca^{2+}] increased exponentially with background [Na^+] (Fig. 8A). Conversely, the dependence of bulbar responses to increases in external [Na^+] (from 0 to 460 $mmol.l^{-1}$) showed the opposite trend. At low background [Ca^{2+}] levels (0.1 - 0.2 $mmol.l^{-1}$), responses to Na^+ were similar to those in the absence of external Ca^{2+} (Fig. 8B). However, at a background [Ca^{2+}] of 0.5 $mmol.l^{-1}$ or more, the bulbar responses to increases in external [Na^+] became progressively smaller. Nevertheless, bulbar responses to Na^+ were never completely attenuated; even at a background [Ca^{2+}] of 9 $mmol.l^{-1}$, appreciable responses to increases in external [Na^+] were recorded.

Discussion

Olfactory Sensitivity to Ca^{2+} and Na^{+}

The current study shows that the olfactory system of the sole responds to decreases in environmental [Ca^{2+}] and increases in environmental [Na^{+}]. Both ions evoked large-amplitude, low-frequency (~10 Hz) wave activity in the olfactory bulb, very similar to that previously described in goldfish (Hubbard and Canário, 2007) and similar to that evoked by 'conventional' odorants, such as amino acids, in the sole. The response to decreases in [Ca^{2+}] was similar to, but slightly more sensitive than, that of the seabream in terms of the apparent IC_{50} (seabream; 1.7 mmol.l^{-1}; sole; 0.7 mmol.l^{-1}) and amplitude (Hubbard *et al.*, 2000); this may be due to the different recording methods used (*i.e.* bulbar EEG and multi-unit recording from the olfactory nerve respectively). However, continual exposure of the olfactory epithelium of the seabream to Ca^{2+}-free ASW caused a decrease in the apparent IC_{50} to 0.5 mmol.l^{-1} (*i.e.* increased sensitivity); in the sole, adaptation to 10‰ seawater reduced the sensitivity (apparent IC_{50}; 2.0 mmol.l^{-1}) and amplitude of the response. This discrepancy may be explained by the effect of Na^{+} on the olfactory response to Ca^{2+}; the current study shows that the olfactory response to changes in environmental [Ca^{2+}] is increased by high external [Na^{+}], whereas the response to Na^{+} is decreased by high external [Ca^{2+}]. The olfactory response to reductions of external [Ca^{2+}] was recorded against a background [Na^{+}] of 460 mmol.l^{-1} in the seabream, whilst the olfactory response of sole adapted to 10‰ seawater was recorded against a background [Na^{+}] of 131 mmol.l^{-1}. Further work will clarify whether this change in sensitivity reflects an active adaptation on the part of the olfactory system to lower environmental [Ca^{2+}] (*i.e.* the sensitivity to a given stimulus is 'tuned' to its

prevailing intensity) or if it can be explained simply as a direct result of the lower [Na^+] of 10‰ seawater (see below).

The olfactory system of sole responded to increases in environmental [Na^+] in a similar way to freshwater-reared salmon and goldfish (Bodznick, 1978; Hubbard *et al.*, 2002). As far as the authors are aware, this is the first time that an olfactory response to changes in environmental [Na^+] has been documented in a marine fish. In contrast to the olfactory response to changes in external [Ca^{2+}], the olfactory system did not respond to decreases in external [Na^+] but only to increases. The functional significance of this observation remains unclear. Nevertheless, the sensitivity to changes in [Na^+] was increased by adaptation to low salinity (10‰). This is opposite to the effect of increasing [Ca^{2+}] on the olfactory sensitivity to Na^+ in the goldfish (Hubbard and Canário, 2007), although the absolute environmental levels of either ion are different; this may be expected of a marine in comparison with a freshwater teleost. Whether this reflects an active adaptation of the olfactory system to lower prevailing [Na^+] or is due to the lower environmental [Ca^{2+}] and subsequent lower inhibition by calcium awaits further research.

Detection Mechanisms for Ca^{2+} and Na^+

It has been suggested that the olfactory sensitivity of fish to Ca^{2+} is mediated by the Ca^{2+}-sensing receptor (Ca^{2+}-SR), similar to that cloned from bovine parathyroid gland (Brown *et al.*, 1993); this receptor is present in the olfactory epithelia of several fish (Hubbard *et al.*, 2002; Nearing *et al.*, 2002). Nevertheless, calcium is not the only ligand of the Ca^{2+}-SR; it also binds other divalent and trivalent cations, including Mg^{2+} (present in seawater at ~50 $mmol.l^{-1}$), although its affinity for Mg^{2+} is much lower that for Ca^{2+} (Chang and Shoback, 2004). Consistent with this, the olfactory sensitivity of

goldfish to changes in external [Mg^{2+}] is correspondingly less (Hubbard *et al.*, 2002) and the sensitivity to reductions in [Ca^{2+}] in the seabream remains acute despite the continued presence of 50 $mmol.l^{-1}$ Mg^{2+} (Hubbard *et al.*, 2000). Olfactory sensitivity to Mg^{2+} and the effect of Mg^{2+} on the sensitivities to Ca^{2+} and Na^{+} were not investigated in the present study.

The affinity for Ca^{2+} of the mammalian Ca^{2+}-SR is reduced by elevated extracellular [Na^{+}] (Quinn *et al.*, 1998); this may be due to 'shielding' of the Ca^{2+}-binding site by Na^{+} ions (Loretz, 2008). Similarly, the olfactory sensitivity of the goldfish to changes in [Ca^{2+}] is reduced by increasing environmental [Na^{+}] (Hubbard and Canário, 2007). However, in the sole, increased environmental [Na^{+}] increased the olfactory sensitivity to changes in [Ca^{2+}]. This may be due to a reliance on external Na^{+} ions of the transduction pathway for the sensitivity to external Ca^{2+}. This discrepancy may be explained by further studies into the olfactory transduction pathways in fish.

In mammals, activation of the Ca^{2+}-SR elicits a variety of G protein mediated intracellular, signals including activation of PLC, PLA_2, PI_4K, MAP kinases (ERKs and JNK) and the inhibition of AC (Breitwieser, 2008; Hofer and Brown, 2003). Assuming that responses to Ca^{2+} are mediated by a Ca^{2+}-SR, a possible explanation for the olfactory sensitivity of sole to decreases of [Ca^{2+}] is that, in sea water, Ca^{2+} is constantly bound to the receptor and, consequently, AC activity is inhibited. As the external [Ca^{2+}] decreases, Ca^{2+} is released from the receptor and AC becomes activated, leading to olfactory neuron depolarization. In contrast to the goldfish (Hubbard and Canário, 2007), olfactory responses to changes in [Ca^{2+}] in the sole are increased by increasing external [Na^{+}]; in the absence of external Na^{+}, olfactory sensitivity to Ca^{2+} is completely abolished. Thus, in the sole, olfactory sensitivity to Ca^{2+} depends on the presence of external Na^{+}.

Na^+ ions may compete with Ca^{2+} ions for the Ca^{2+}-binding site; however, Na^+ has a negative effect on the activation of Ca^{2+}-SR (Quinn *et al.*, 1998). Thus, decreasing $[Na^+]$ leads to a higher activation of the Ca^{2+}-SR by a given increase in $[Ca^{2+}]$ and a subsequent increase in sensitivity. This may explain why increasing $[Na^+]$ attenuates olfactory sensitivity to changes in $[Ca^{2+}]$ in the goldfish (Hubbard and Canário, 2007) but it cannot explain the higher sensitivity to $[Ca^{2+}]$ in 35‰ compared to 10‰ seawater in the sole. Activation of non-selective cation channels by Ca^{2+}-SR has been described in hippocampal pyramidal cells (Ye *et al.*, 1996a; Ye *et al.*, 1996b). This could explain the abolishment of olfactory response to Ca^{2+} in the absence of Na^+ and the higher olfactory sensitivity to Ca^{2+} recorded in fish adapted to 35‰ compared to those adapted to 10‰ seawater in the sole.

Sole has olfactory sensitivity to increases in external $[Na^+]$ but to decreases in external $[Ca^{2+}]$; however, the olfactory responses to changes in $[Na^+]$ seem to be mediated by a different mechanism from those to changes in $[Ca^{2+}]$. Furthermore, the concentration/response curve to changes in external $[Ca^{2+}]$ is sigmoidal suggesting a receptor-mediated mechanism, whereas the concentration/response curve to $[Na^+]$ does not reach a maximum (within the range of concentrations tested), suggesting that responses to Na^+ are channel-mediated (Hubbard and Canário, 2007). In addition, if both Ca^{2+} and Na^+ responses were due to activation of the same cells, the absence of Ca^{2+} would depolarise these neurons - thus inhibiting responses to Na^+ - whereas decreases of background $[Ca^{2+}]$ have the opposite effect on olfactory sensitivity to Na^+. Furthermore, high external $[Na^+]$ would inhibit sensitivity to Ca^{2+}. In sole, however, the absence of external Ca^{2+} increases olfactory bulbar responses to Na^+ whereas responses to Ca^{2+} are attenuated by low external $[Na^+]$. We suggest that olfactory responses to Na^+ are mediated by a channel that allows the influx of Na^+ ions and, possibly, other cations.

Ca^{2+} ions may also enter this channel but, instead of entering the cell, remain in the pore and block it. The existence of Na^+-activated non-selective cation channels has been shown in the olfactory receptor neurons of lobsters (Zhainazarov and Ache, 1998; Zhainazarov *et al.*, 1998); these Na^+ activated channels are (as are other Na^+-activated channels from different systems) blocked by Ca^{2+} (Armstrong and Cota, 1999).

Olfactory Sensitivity to Amino Acids

Fish, in general, have high olfactory sensitivity to amino acids (Hara, 1994; Michel, 2006) and the sole is no exception (Velez *et al.*, 2005; Velez *et al.*, 2007). The slightly higher sensitivity of sole to amino acids reported in the current study may simply be a reflection of the different method used. Previous studies used the electro-olfactogram (EOG) which is likely to underestimate the true sensitivity due to the shunting effect of seawater (Velez *et al.*, 2005). Recording from the olfactory bulb (EEG) eliminates this problem and allows a direct comparison of sensitivity at different salinities. Sole are able to maintain olfactory sensitivity to amino acids independently of external salinity; no differences were seen between the bulbar responses to L-cysteine, L-phenylalanine and glycine at 35‰ and 10‰ with thresholds of detection around 10^{-7} to 10^{-8} M in all cases. This is consistent with previous studies with euryhaline salmonids (Shoji *et al.*, 1994; Shoji *et al.*, 1996) and would be expected for a fish, such as sole, that often penetrates estuaries in order to feed (Cabral, 2000); L-phenylalanine and glycine are two of the main amino acids released by one of the sole's chief prey species, the ragworm *Hediste diversicolor* in such an environment (Velez *et al.*, 2007). However, the olfactory transduction mechanism(s) must somehow adapt to the lower availability of Na^+ in estuarine water; at 35‰, the olfactory response to amino acids is dependent on external Na^+ but is independent of Na^+ at 10‰. Whether this is achieved by regulating

the ionic component of the mucus layer overlying the olfactory epithelium (as in mammals; Schild and Restrepo, 1998) or by shifting the transduction pathway to use extra-cellular (rather than external) Na^+ is not yet known. In the seabream, exposure of the olfactory epithelium to Ca^{2+}-free ASW caused a temporary diminution of the response to L-serine, suggesting some reliance of the transduction mechanism on external Ca^{2+} which, again, could be overcome (Hubbard *et al.*, 2000). Olfactory responses of the sole to amino acids seem to be independent of external Ca^{2+}. This may reflect different ion channels (Na^+-selective or Ca^{2+}-selective) being involved in the transduction mechanisms of the different species. The olfactory transduction pathways in sole are currently under investigation (Velez *et al.*, 2008).

Summary

The olfactory system of sole responds to decreases in environmental [Ca^{2+}] but increases in [Na^+]. Sensitivity to Ca^{2+} is higher at 35‰ but that to Na^+ is higher in 10‰ seawater. Although the sensitivities to the two ions are mediated by apparently different mechanisms, changes in the background levels of one affect the sensitivity to the other. Olfactory sensitivity to amino acids remains constant in 35‰ and 10‰ seawater. At 10‰ the response to amino acids depends on neither external Ca^{2+} nor Na^+. However, at 35‰, absence of external Na^+ (but not Ca^{2+}) markedly reduces the response to amino acids. This suggests that the olfactory system must adapt to lower salinities by reducing the dependence on environmental Na^+.

Acknowledgements

This work was supported by grants from the Portuguese National Science Foundation and the European Union Social Funds for Regional Development (grant numbers: POCI/BIA-BMC/55467/2004 and SFRH/BD/16242/2004) funded by European Union FSE/FEDER and Ministério da Ciência e do Ensino Superior.

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Figure Legends

Figure 1. Typical EEG recordings from the olfactory bulb of the sole in response to stimulation of the olfactory epithelium with Ca^{2+}-free ASW against a background of (**A**) 10‰ ASW and (**B**) 35‰ ASW in fish adapted to 10‰ and 35‰ seawater respectively. Upper trace; integrated activity of raw signal (middle trace), lower trace; expansion of the portion in the dashed rectangle to show wave-form. (**C**) Semi-logarithmic plot of pooled data showing the olfactory responses to changes in external [Ca^{2+}] of sole adapted to 10‰ (open circles) or 35‰ (filled circles). Data are shown as mean ± S.E.M. (N = 6). Apparent I_{max} (**D**) and IC_{50} (**E**) values calculated from Hill plots fitted to the data shown in **C** (N = 6). * P<0.05, ** P<0.01.

Figure 2. Typical EEG recordings from the olfactory bulb of sole in response to an increase in [Na^+] of 460mM ASW against a background of (**A**) 10‰ ASW (Na^+-free) and (**B**) 35‰ ASW (Na^+-free) in fish adapted to 10‰ and 35‰ seawater respectively. Upper trace; integrated activity of raw signal (middle trace), lower trace; expansion of the portion in the dashed rectangle to show wave-form. (**C**) Semi-logarithmic plot of pooled data showing the olfactory responses to increases in external [Na^+] of sole adapted to 10‰ (open circles) and 35‰ (filled circles) seawater. Note that the responses were recorded against a background of in Na^+-free ASW (10‰ or 35‰ as appropriate). Data are shown as mean ± S.E.M. (N = 6). ***P<0.001.

Figure 3. Typical EEG recordings from the olfactory bulb in response to 10^{-3} M L-cysteine from sole adapted to (**A**) 10‰ and (**B**) 35‰ seawater. Upper trace; integrated

activity of raw signal (middle trace), lower trace; expansion of the portion in the dashed rectangle to show wave-form. **(C)** Semi-logarithmic plot of pooled data showing the olfactory responses of sole to 10^{-3} M L-cysteine in ASW-10‰ (open circles) and ASW-35‰ (filled circles) in fish adapted to 10‰ and 35‰ seawater respectively. Data are shown as mean ± S.E.M. ($N = 6$). No statistically significant differences were found between slope or elevation of the the regression curves fitted to the data at the two different salinities.

Figure 4. Semi-logarithmic plot of pooled data (N=6) showing the olfactory responses of sole to 10^{-3} M glycine in ASW-10‰ (open circles) and ASW-35‰ (filled circles) in fish adapted to 10‰ and 35‰ seawater respectively. Data are shown as mean ± S.E.M. ($N = 6$). No statistically significant differences were found between the slope or elevation of the regression curves fitted to the data at the two different salinities.

Figure 5. Semi-logarithmic plot of pooled data (N=6) showing the olfactory responses of sole to 10^{-3} M L-phenylalanine in ASW-10‰ (open circles) and ASW-35‰ (filled circles) in fish adapted to 10‰ and 35‰ seawater respectively. Data are shown as mean ± S.E.M. ($N = 6$). No statistically significant differences were found between slope or elevation of the regression curves fitted to the data at the two different salinities.

Figure 6. Effect of the absence of external Ca^{2+} (black bars) or Na^+ (white bars) on the olfactory bulbar responses to **(A)** L-cysteine, **(B)** glycine and **(C)** L-phenylalanine (all at 10^{-3} M) stimulating the olfactory epithelium in fish adapted to 35‰ or 10‰ seawater. Data are shown as mean ± S.E.M. ($N = 6$). ** $P<0.01$ compared to control.

Note that the amplitude of responses is reduced only by the absence of Na^+ in fish adapted to 35‰ seawater only.

Figure 7. Effect of the absence of external Ca^{2+} on the olfactory bulbar response to Na^+ and the absence of external Na^+ on the olfactory bulbar response to Ca^{2+} recorded from sole adapted to 35‰ (black bars) or 10‰ (white bars) seawater. Data are shown as mean ± S.E.M. (N = 6). ** P<0.01 compared to the appropriate control. Note that absence of external Na^+ attenuated the bulbar response to changes in [Ca^{2+}] independent of salinity, whereas the bulbar responses to changes in [Na^+] are increases in the absence of Ca^{2+} in water of 35‰ but not 10‰.

Figure 8. **A** Semi-logarithmic plot of pooled data showing the effect of increasing background [Na^+] on the olfactory bulbar response to changes in external [Ca^{2+}] (10 mM to 0) in the sole. B Semi-logarithmic plot of pooled data showing the effect of increasing background [Ca^{2+}] on the olfactory bulbar response to changes in external [Na^+] (0 to 460 mM). Data are shown as mean (N = 2). Both experiments were recorded from sole adapted to 35‰ seawater. Note that increasing background [Na^+] increases the olfactory response to Ca^{2+} whereas increasing background [Ca^{2+}] decreases the response to Na^+.

Table 1. Composition of artificial seawaters (ASW) used in the current study. Ranges of concentrations were made by appropriate mixing of the solutions.

	[NaCl] (mM)	[KCl] (mM)	[$CaCl_2$] (mM)	[$MgSO_4$] (mM)	[$MgCl_2$] (mM)	[Choline chloride] (mM)
ASW	460	10	10	25	25	—
Ca^{2+} free ASW	460	10	—	25	25	20
Na^{+} free ASW	—	10	10	25	25	460
Na^{+} and Ca^{2+}-free ASW	—	10	—	25	25	480
10‰ ASW	131.4	2.9	2.9	7.1	7.1	—
Ca^{2+} free ASW 10‰	131.4	2.9	—	7.1	7.1	5.8
[Ca^{2+}] 10mM ASW 10‰	131.4	2.9	10	7.1	7.1	—
Na^{+} free 10 ‰ ASW	—	2.9	2.9	7.1	7.1	131.4
[Na^{+}] 460mM ASW 10‰	460	2.9	2.9	7.1	7.1	—

Figure 1

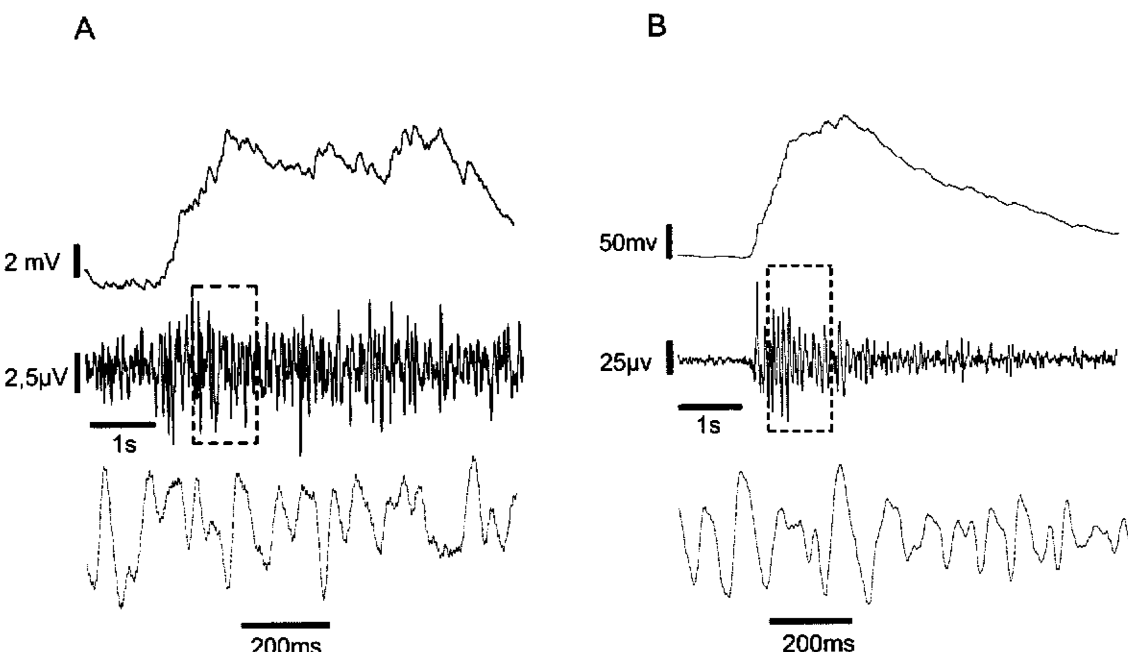
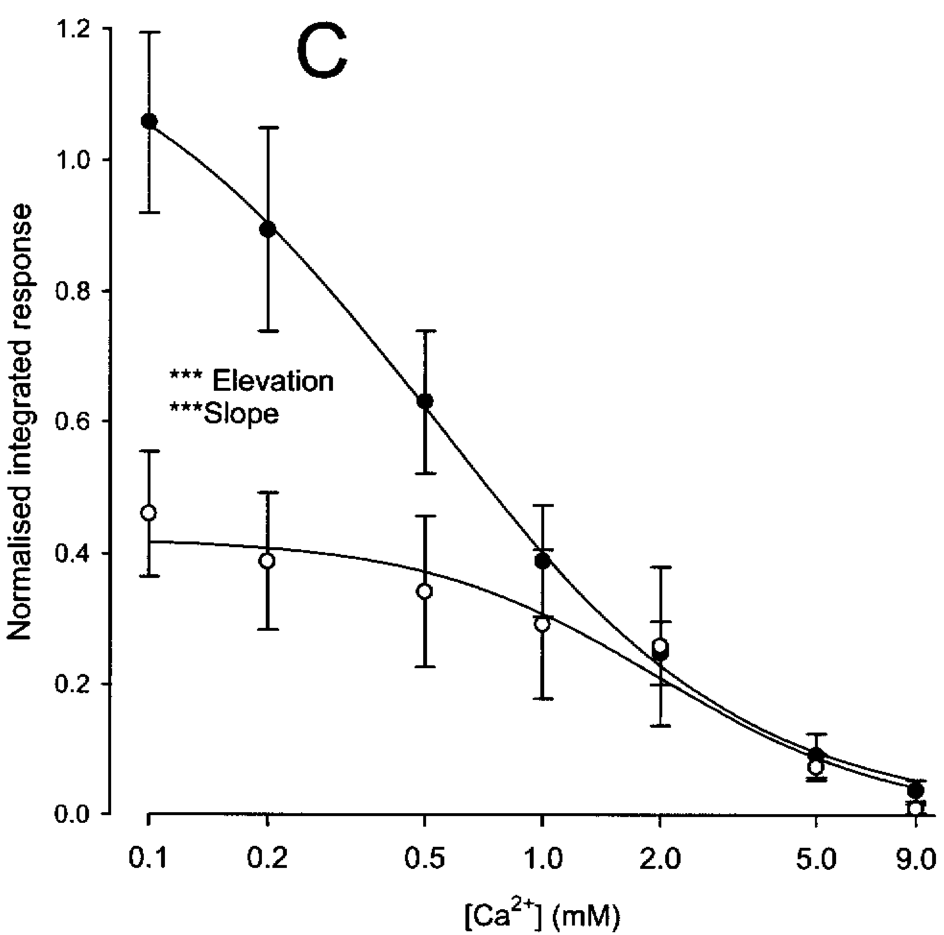
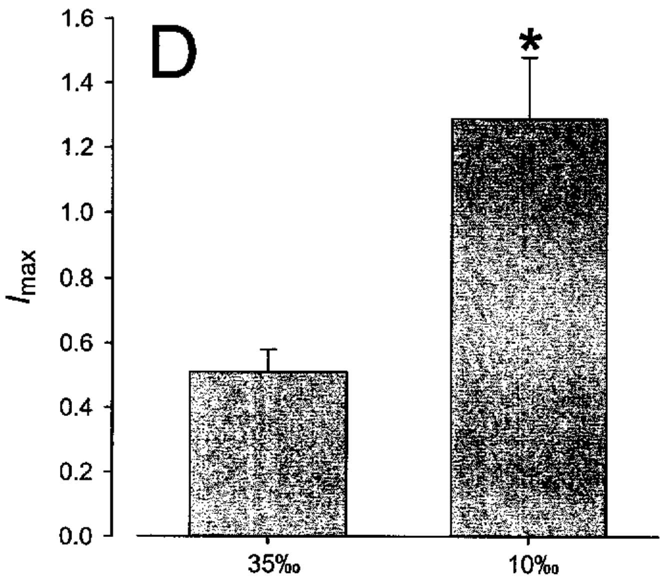
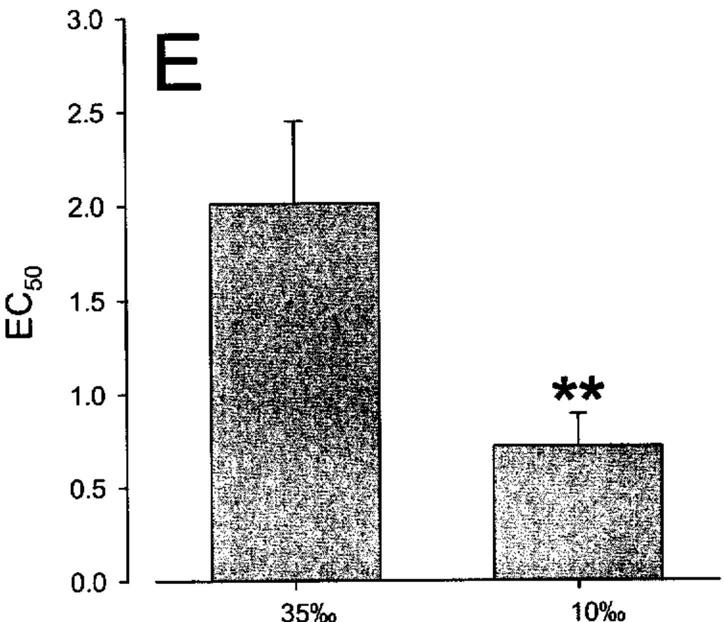


Figure 1C






E
3.0
2.5
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EC_{50}
**
35‰
10‰

Figure 2

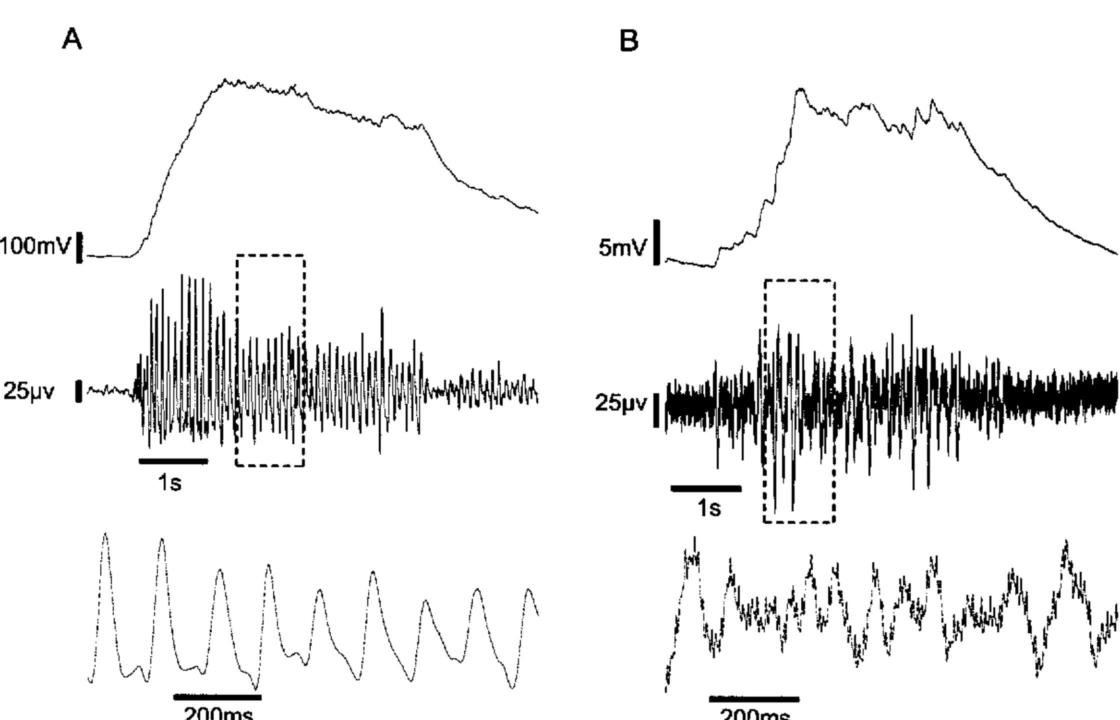


Figure 2C

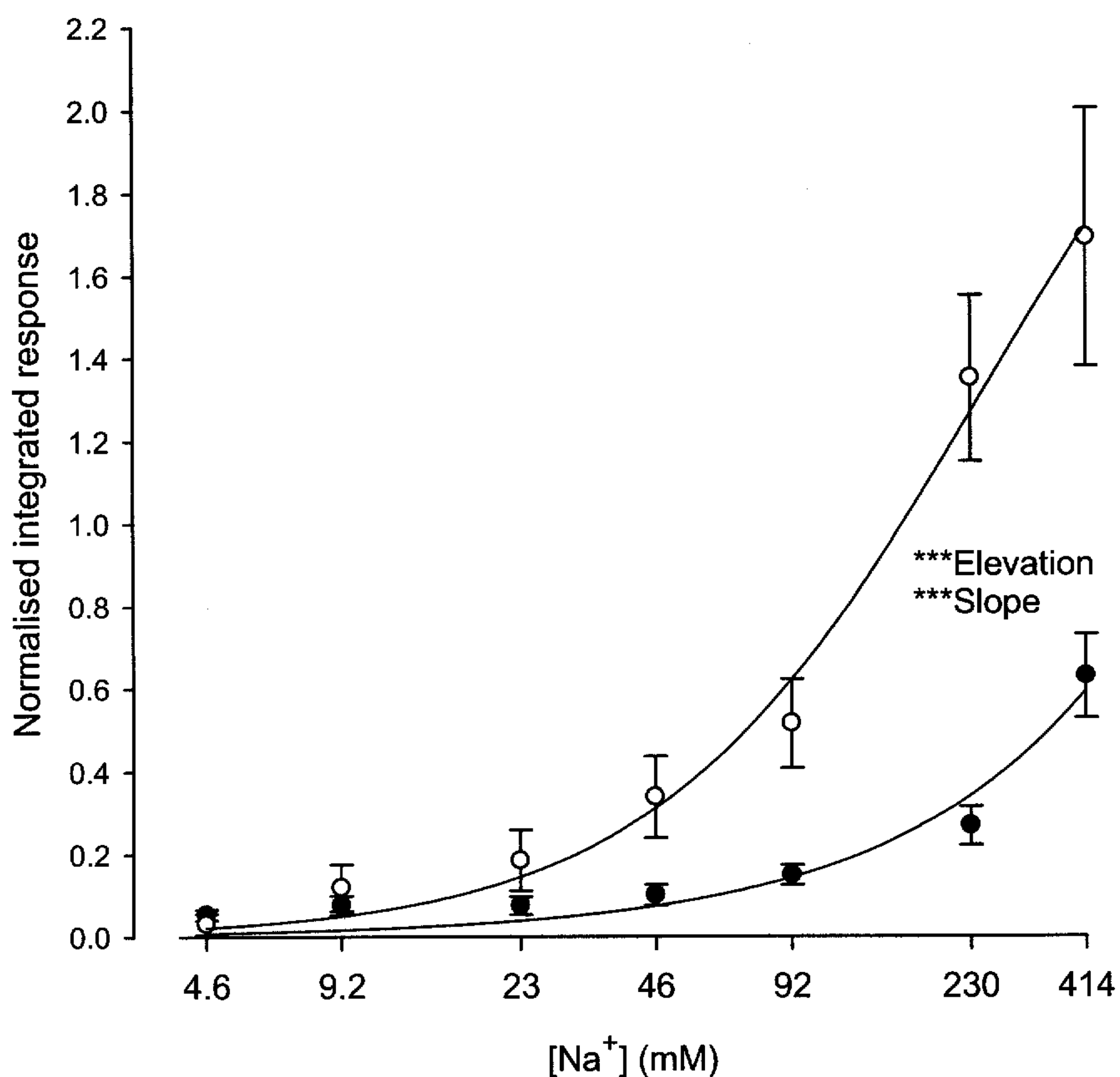


Figure 3

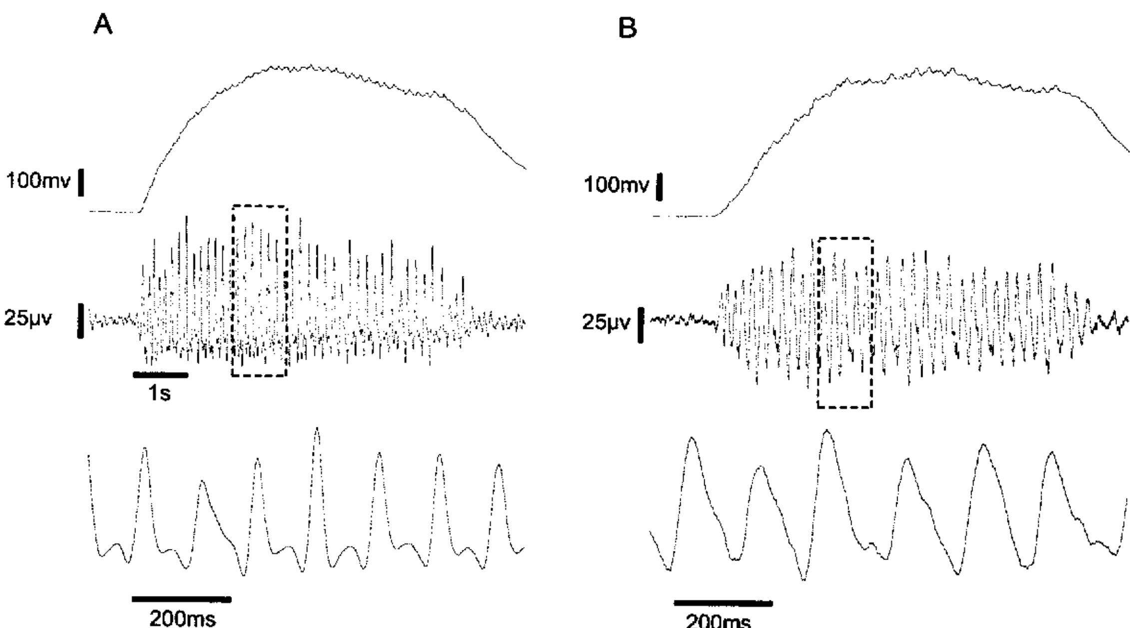


Figure 3C

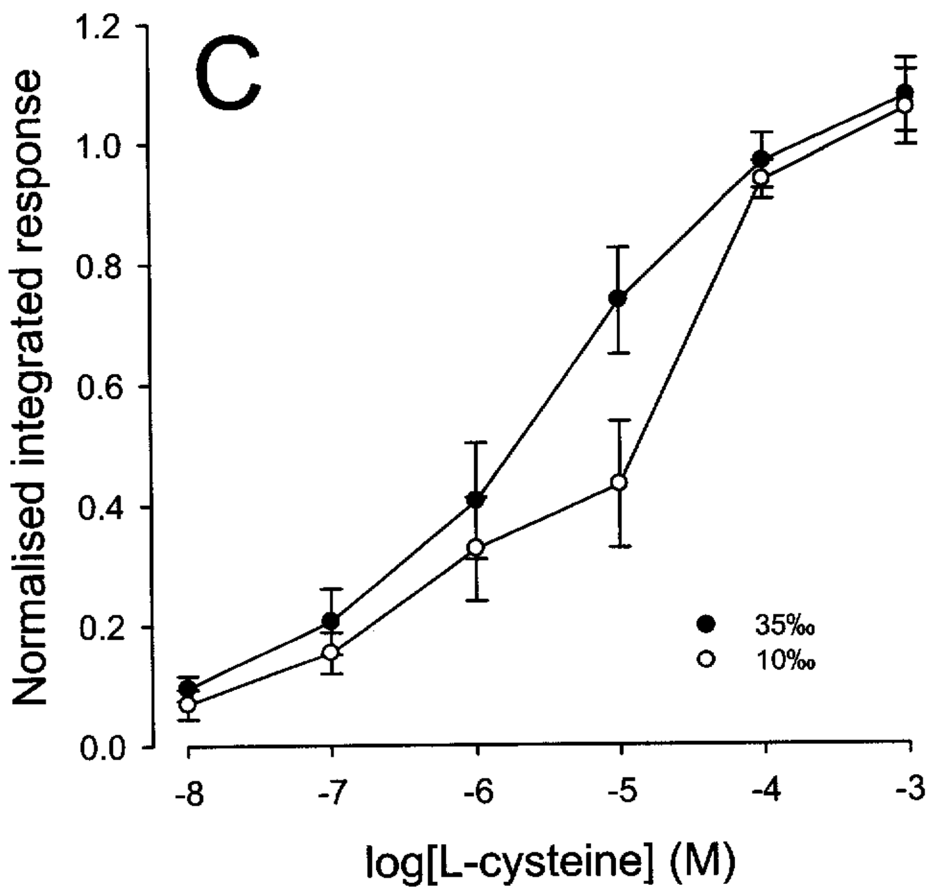


Figure 4

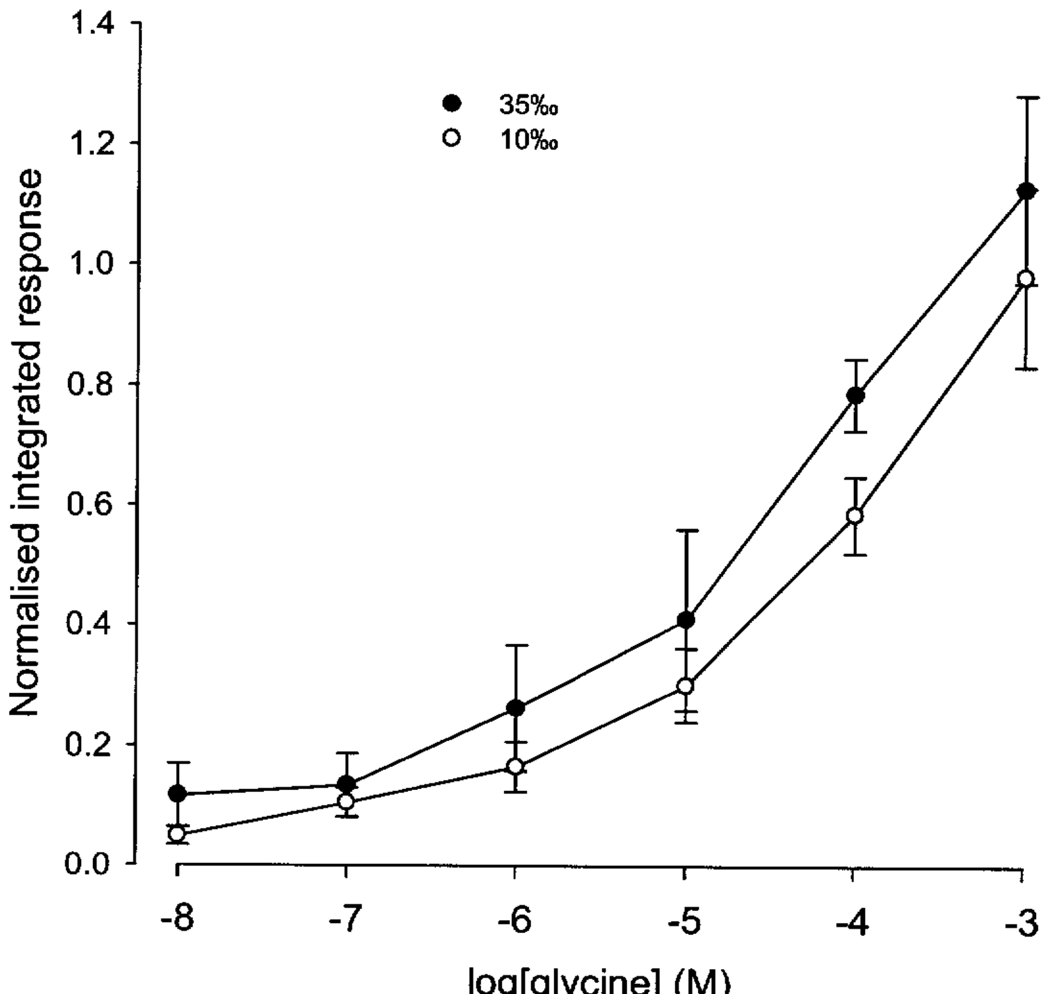


Figure 5

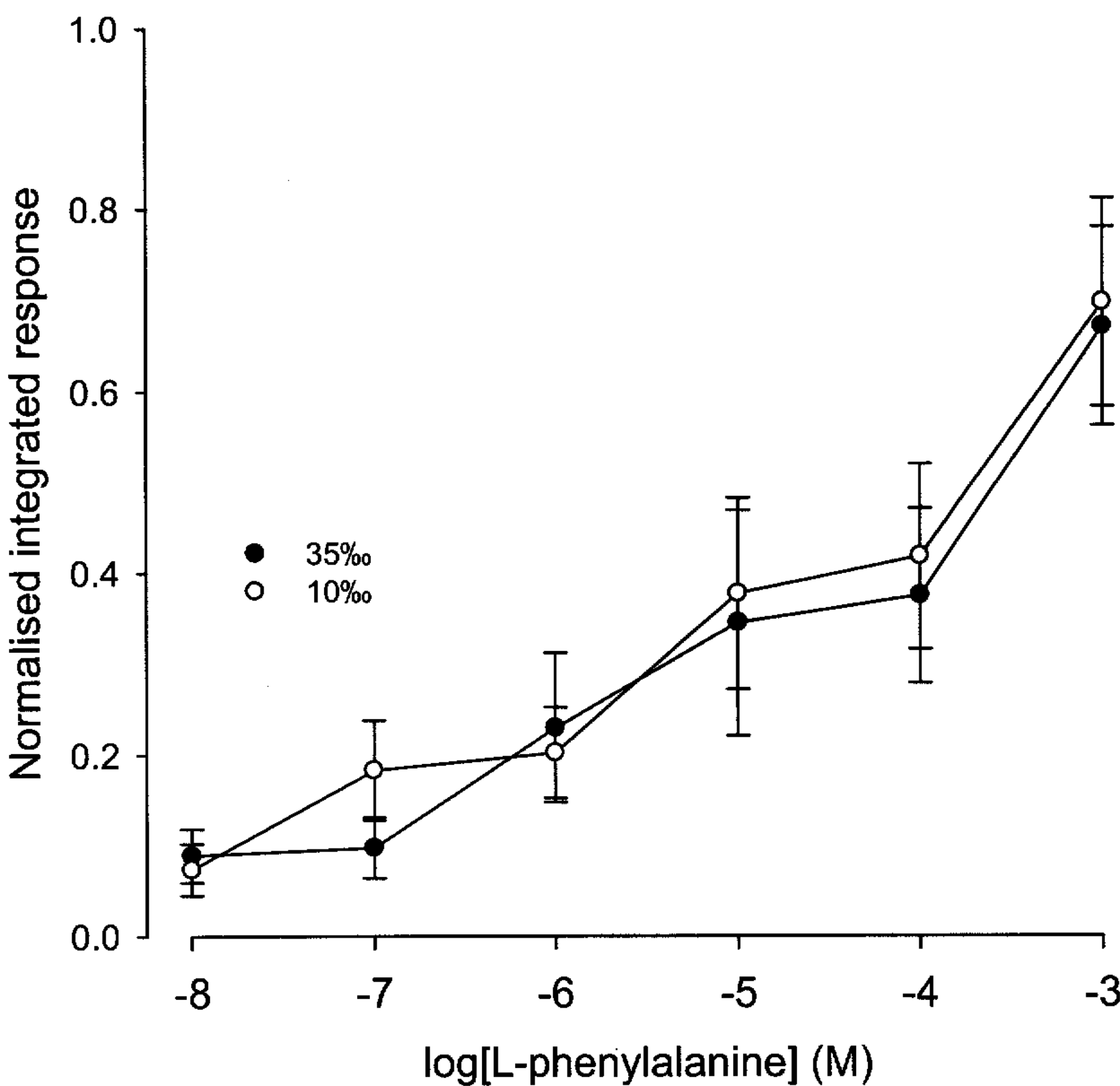


Figure 6

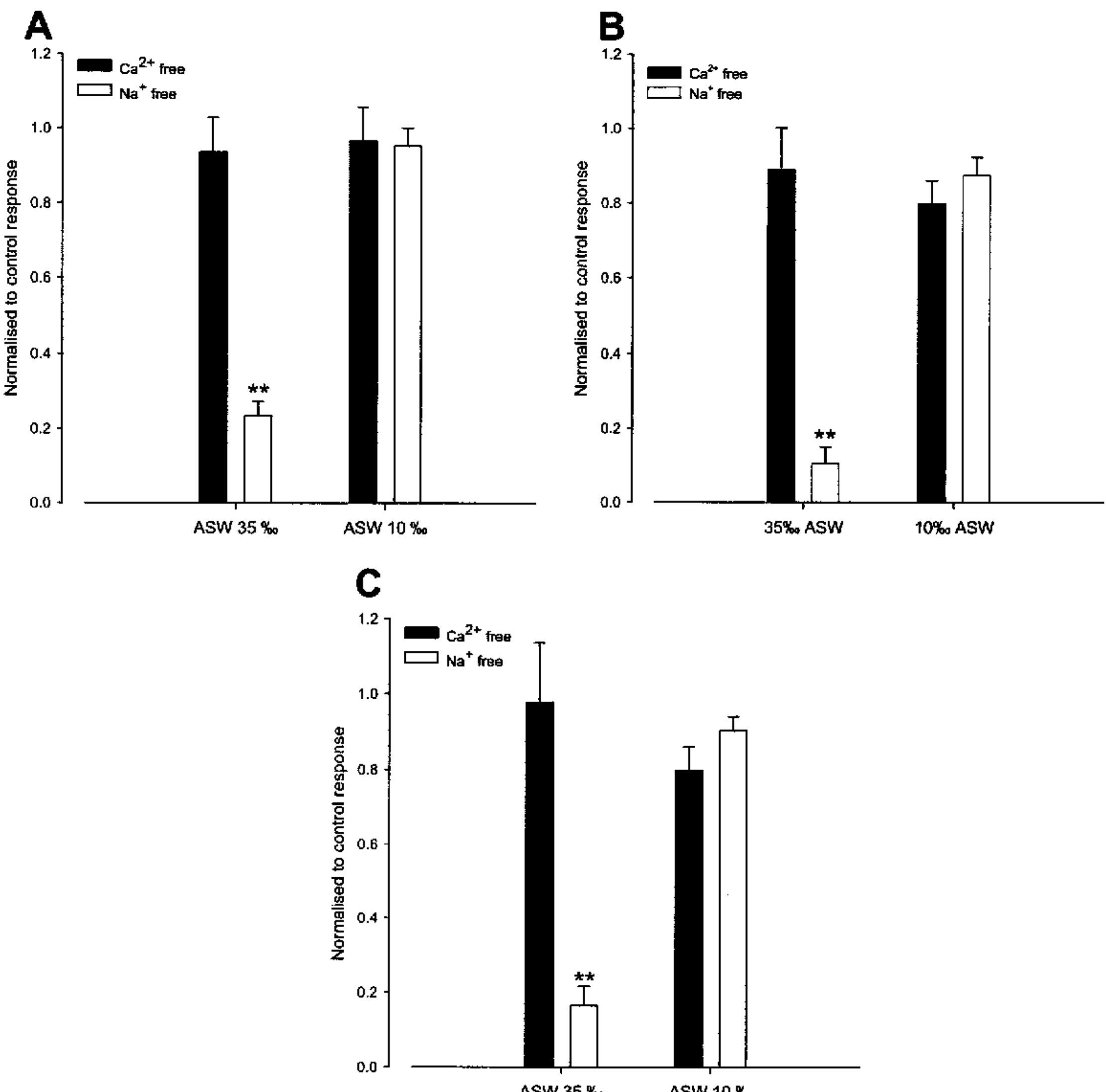


Figure 7

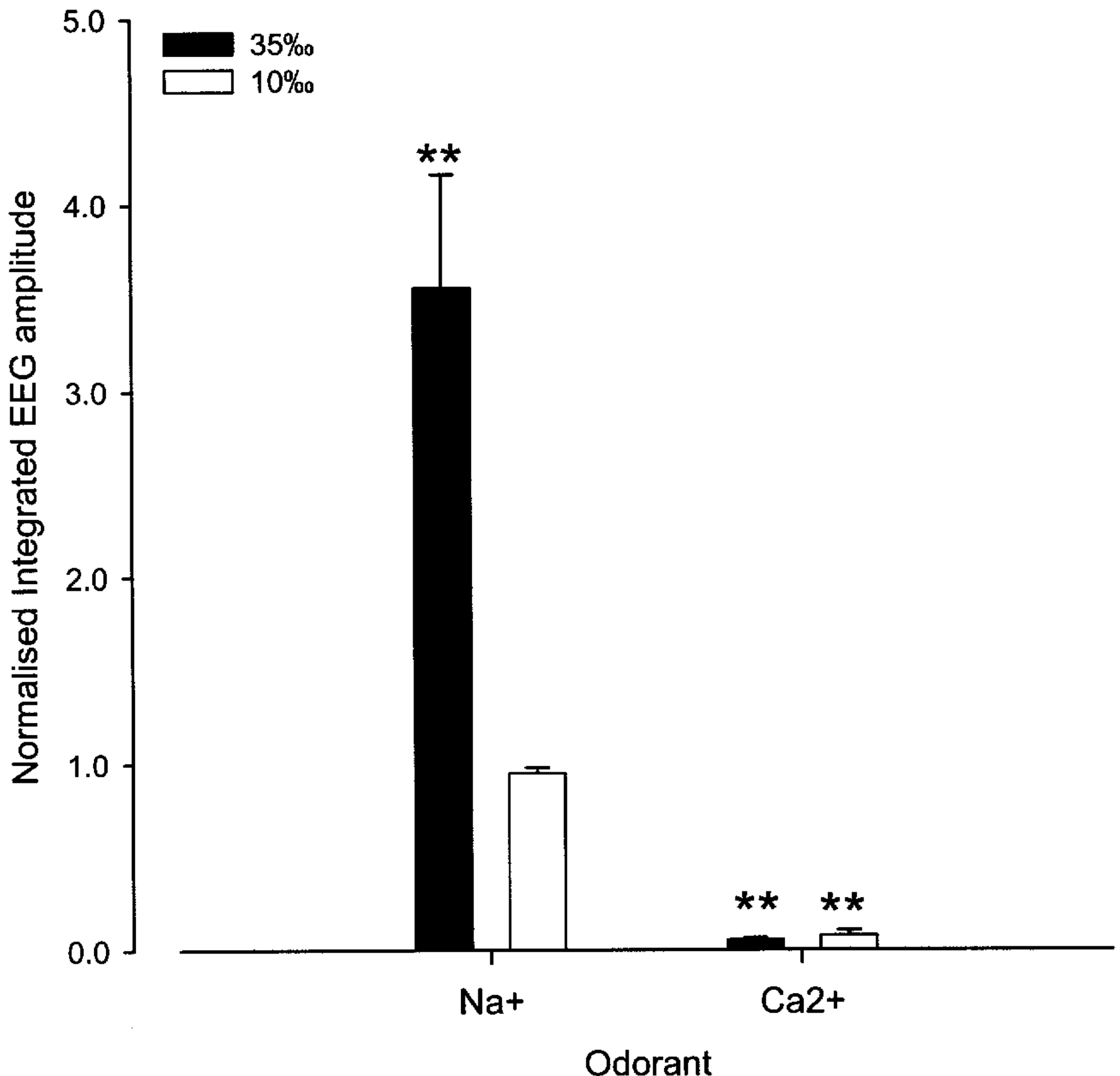
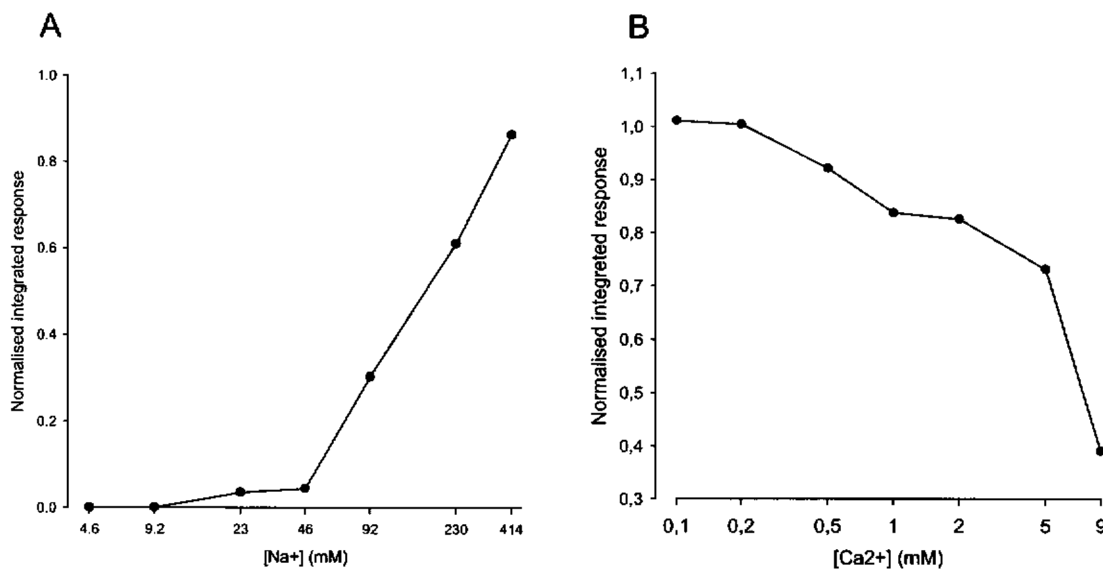


Figure 8



Chapter III

General Discussion

III General Discussion

The working hypothesis of the current study was that the two olfactory epithelia of sole are specialised for detecting different odorants with distinct ecological roles. The studies included in this thesis have shown that some compounds released by ragworms have olfactory potency and that this is greater at the lower olfactory epithelium than the upper (paper I and II). In addition, it was also shown that some odorants responsible for the higher sensitivity of the upper olfactory epithelium to con-specific body fluids are released into water (paper IV). This supports the hypothesis that the lower olfactory epithelium is specialised for prey detection and the upper for the detection of odorants released by con-specifics (possible pheromones).

The first part of general discussion focuses on the ecological importance of differential olfactory detection in sole. The second part discusses the olfactory transduction pathways involved in the detection of both food-related stimuli and con-specific body-fluids that are differently detected by the two epithelia. The last part of this discussion focuses on the ecological importance of the olfactory detection of changes in environmental cation concentrations and the possible mechanisms involved.

3.1 Differential detection of odorants by the two olfactory epithelia of sole

3.1.1 Food related odorants

Our studies showed that the main odorants present in both *H. diversicolor* macerate and worm-conditioned water are amino acids and their derivatives (Paper I and II). In the macerate, most of the olfactory potency is likely to be due to the presence of amino acids (Paper I), whereas in worm-conditioned water, amino acids constitute only a small fraction of the total olfactory activity (paper I). Chemical identification studies suggested that although amino acids and, possibly, small peptides also contribute to the total olfactory activity of worm-conditioned water, the majority of the olfactory potency of this sample is due to one amino acid derivative, 1-methyl-L-tryptophan (paper II). Furthermore, our results showed that 1-methyl-L-tryptophan is likely to be the main odorant responsible for the olfactory sensitivity of the lower olfactory epithelium to worm-conditioned water (paper II).

In aquaculture, the use of feeding stimulants can enhance the acceptance of artificial food leading to an increase in growth. In the last few years, many studies have focused on the identification of feeding stimulants in fish (*e.g.* Burrells, *et al.*, 2001; Carr, *et al.*, 1996; Kubitza, *et al.*, 1997; Mackie, *et al.*, 1980; Papatryphon, Soares Jr., 2000; Reig, *et al.*, 2003). Most of these studies were preformed using macerates of natural prey organisms; however, it is not known whether any of these compounds are released by the prey under natural conditions. Our results shows that the compounds released into the environment

by ragworms may be substantially different from those present in their body tissues (paper I and II). Given that the compounds released are more probably used by fish to detect their prey, our results suggest that future studies in chemical identification of food related stimuli should be done with prey-conditioned water rather than macerates of prey organisms.

In addition to the higher olfactory sensitivity of the lower epithelium to food related stimuli and 1-methyl-L-triptophan, the results from cross-adaptation studies also support the hypothesis of specialization of the lower olfactory epithelium in the detection of food related odorants. It was previously suggested by different authors that olfactory receptor neurons are not highly specific, but rather have a relatively broad response spectrum to the olfactory stimuli (Kang, Caprio, 1995; Restrepo, *et al.*, 1990). Our results from cross-adaptation studies showed that all the tested stimuli interfere with the olfactory potency of the others, suggesting that responses to certain odorants always involve some degree of non-specificity (Paper II). Response of the upper epithelium to 1-methyl-L-tryptophan is probably largely non-specific; all the tested stimuli cross-adapted with 1-methyl-L-tryptophan were able to markedly reduce the responses of the upper epithelium. In contrast, receptors of the lower epithelium are apparently more specific, and have a higher affinity, for 1-methyl-L-tryptophan; L-cysteine, glycine or even the structurally similar L-phenylalanine were unable to reduce the response by more than 50 % (Paper II). This suggests that in sole the olfactory sensitivity to a given odorant is due to activation of individual sensory neurons that respond to many odorants and functionally distinct olfactory sensory neurons which are more specific to a given odorant. Furthermore, the existence of olfactory receptor mechanisms responding specifically to 1-methyl-L-tryptophan

suggests that the detection and discrimination of this compound is important to sole.

As far we are aware, 1-methyl-L-tryptophan has never been described as a feeding stimulant. We have shown that one of the sole's main prey organisms releases this compound into the environment and that sole can smell; the higher olfactory sensitivity of the lower olfactory epithelium to water conditioned by ragworm is due to 1-methyl-L-tryptophan. Together these results suggest that 1-methyl-L-tryptophan is an important odorant for feeding. However, before any definitive conclusions are drawn about the ecological importance of this compound, behavioural studies need to be carried out.

The acquisition of food by flatfish is a process that usually comprises four basic elements: searching, encountering, capturing and ingesting a prey item (reviewed in Gibson, 2005). In order to evoke feeding behaviour, from initial detection to ingestion, a number of different stimuli need to be present. These stimuli required depend on the relative importance of the sense organs and the environmental conditions in which fish lives. Individuals from the same species inhabiting different environments may require different stimuli. Some flatfish rely mainly on sight to find food, others use both vision and olfaction, whereas non-visual feeders mainly use olfaction for detecting prey (reviewed in De Groot, 1971; Gibson, 2005). Previous studies have shown that the behavioural effect of a single compound can be different from that of the same compound within a mixture (Tabor, *et al.*, 2004; Valentincic, Koce, 2000; Valentincic, *et al.*, 2000a). A specific behavioural response (*e.g.* feeding behaviour) is more likely to be evoked by a mixture of odorants than by a single odorant (Mackie, *et al.*, 1980; Valentincic, *et al.*, 2000a; Valentincic, *et al.*, 2000b). Our studies showed that

although 1-methyl-L-tryptophan is the main odorant responsible for the preferential detection of worm-conditioned water in the lower olfactory epithelium (paper II), other compounds - namely amino acids - also contribute to its total olfactory potency (paper I). Previous studies have shown that, in general, the sole has highly olfactory sensitivity to amino acids (Velez, *et al.*, 2005). Cross-adaptation studies showed that in both the upper and lower olfactory epithelium, responses to amino acids are due at least in part to non-specific receptors, thus the "cocktail" of odorants released by ragworms can be detected by both olfactory epithelia. We hypothesised that the detection of amino acids alerts fish to the possible presence of prey. Given that both epithelia are sensitive to amino acids; this would allow fish to detect a putative prey even at distance. In addition the activation of receptors that specifically detect a compound released by a specific prey (*e.g.* 1-methyl-L-tryptophan) would allow the fish to better identify its prey. Also, due to the specificity of this receptor to a given compound, it is possible that it could be used to detect differences in the concentration of that odorant; this would be helpful in finding the source of the odour.

Evidence obtained from the wild suggests flatfish can be generally divided in two groups according to their prey types: Polychaete and crustacean eaters, and piscivores (reviewed in Link, *et al.*, 2005). According to Cabral (2000), the main organisms identified in the stomach contents of *S. senegalensis* caught from the River Tagus estuary were *Corophium spp* (copepod)., *H. diversicolor* (polychaete) and *Scrobicularia plana* (bilalve mollusc); the importance of larger prey items in sole diet, namely *H. diversicolor*, increases with fish size. In an environment such as the Tagus estuary where polychaete species are highly diverse (*e.g. Nephthys hombergi, Glycera convolute, Diopatra neapolitana, Stresblospio shrubsolii*) and

some species are more abundant than *H. diversicolor*, the clear abundance of ragworms in the stomach contents of sole suggests a marked preference for this prey (Cabral, 2000). Behavioural studies are needed to evaluate whether 1-methyl-L-tryptophan is required for prey recognition and/or selection. It is not known whether other marine organisms also release this compound or whether other fish species (including flatfish) can smell it. The ability to detect a compound that other fish can not detect could represent an advantage in avoiding feeding competition between similar species.

The upper olfactory epithelium is not completely insensitive to 1-methyl-L-tryptophan. However, large olfactory responses occur only when this stimulus is present at high concentrations. Thus, sensitivity to 1-methyl-L-tryptophan in the upper epithelium could act as a sign that the odour source is close, and this could be important in inducing food intake. Preliminary studies showed that both water entering the upper and lower olfactory cavities are ejected by the upper exhalant nostril (Velez *et al.*, unpublished). This means that water entering the lower olfactory cavity also contacts the upper olfactory epithelium. At low concentrations of 1-methyl-L-.tryptophan, only the lower epithelium is sensitive to this stimulus. However, when its concentration increases, the upper olfactory epithelium can also detect it. Olfactory sensitivity to this specific compound may be a sign that fish is close to the source of the odorant and thus it may induce food intake. De Groot (1971) showed, in common sole, that plugging the lower olfactory organ has little effect on food intake, whereas blocking the upper olfactory organ prevented it completely. This suggests that the upper olfactory nostril is also important for food intake. A possible explanation for these observations is that the lower epithelium is important in food localization whereas

the upper is more important in inducing food intake. Thus probably, with the lower nostril blocked fish take longer to detect and localise their prey, which may not be difficult in a 200 cm tank, such as that used by De Groot: once a sole reaches its prey the upper olfactory epithelium induces feed intake. However with the upper nostril blocked, the fish may search for its prey but the upper nostril never receives the signal that it has finally reached it and thus is not stimulated to ingest. To test this hypothesis further behavioural studies are required in which a larger tank should be used and - in addition to the analysis of food intake - food search behaviour should be considered. This putative interaction between the upper and lower olfactory epithelia of the Senegalese sole is similar to that proposed between olfaction and taste for other fish; in a number of species, olfaction is required to initiate the process of feeding behaviour and taste is required for food ingestion (Hara, 2006). This does not mean that gustation is not important to the Senegalese sole; behavioural studies carried out with common sole showed that the most potent substances evoking feeding behaviour are L-phenylalanine, L-lysine, L-asparagine, inosine 5-monophosphate and glycine betaine (Knutsen, 1992). It is known that *S. senegalensis* has olfactory sensitivity to L-phenylalanine and L-asparagine, mainly with the lower epithelium (Velez, *et al.*, 2005), but it can not smell glycine betaine or inosine 5-monophosphate (Velez *et al.*,unpublished data). Thus the effect of betaine and inosine on increasing food intake may be due to taste rather than olfaction. In addition, betaine is described as one of the main taste stimuli in marine species (Kasumyan, Døving, 2003; Polat, Beklevik, 1999; Yacoob, *et al.*, 2004). Aquaculturists use high concentrations of betaine (44 mg/g; Métailler, *et al.*, 1983) to induce food intake in sole (Mackie, *et al.*, 1980). The high concentrations required may be due to the

fact that it only activates taste receptors; fish are detecting food pellets based on taste instead of olfaction. The application of olfactory stimulants that induce food search and, probably, food intake in artificial food pellets could be useful in decreasing the required concentration of betaine required. However, even with the appropriate odorants, taste stimulants may always be essential to induce food intake. Fish may reject a food item after it has been taken into the mouth cavity; such behaviour suggests that an item that is selected as food, based on olfactory cues, may not be accepted as food when tasted. Preliminary observations showed that naïve Senegalese sole were attracted to paper that had been in contact with ragworms; however, the pieces of paper were immediately rejected after being taken into the mouth cavity (Velez *et al.*; unpublished).

Taken together, our results suggest that the lower olfactory epithelia is specialised for detecting odorants released by prey. Behavioural studies are needed to evaluate the ecological importance of this phenomenon. However, we suggest that it may contribute in a number of different ways to the success of sole finding, in a dark environment, prey that are hidden within the substrate.

3.1.2 Con-specific body fluids

It was hypothesised that the upper olfactory epithelium of sole is specialised for detecting compounds found in the water column; some of these compounds could be pheromones. Data from the literature suggest that putative pheromones may be released *via* the urine (Almeida, *et al.*, 2005; Barata, *et al.*, 2007; Barata, *et al.*, 2008b; Miranda, *et al.*, 2005; Moore, *et al.*, 1994), faeces (Frade, *et al.*, 2002; Hubbard, *et al.*, 2003; Miranda, *et al.*, 2005; Zhang, *et al.*, 2001), mucus (Huertas, *et al.*, 2007; Saglio, 1982; Stabell, Selset, 1980), gonadal fluids

(Hubbard, *et al.*, 2003; Olsén, *et al.*, 2001), specialised structures (Barata, *et al.*, 2008a; Serrano, *et al.*, 2008) and across the gills (Vermeirssen, Scott, 1996). However, the majority of these studies were carried out on freshwater fish; marine fish produce and release urine at a much lower rate (e.g. Fletcher, 1990). Indeed, the urine of the gilthead seabream is much less potent as a source of odorants than the intestinal fluid (Hubbard, *et al.*, 2003). Subsequently, urine may be less important in chemical signalling in marine fish compared to freshwater fish. Given that sole produces much skin mucus which is in intimate contact with the surrounding water, and that contents of the mucus may reflect the endocrine status of the fish (Schultz, *et al.*, 2005), mucus may also be important in chemical signalling. Thus the olfactory sensitivity of sole to bile, faeces and mucus was evaluated in both olfactory epithelia. The results showed that all three body fluids are better detected by the upper olfactory epithelium than the lower (paper III). In addition, crude fractionation of these samples (solid-phase extraction with C18 and C2/ENV+ cartridges) revealed that all fractions are better detected with the upper olfactory epithelium. These results support the hypothesis of specialization of the upper olfactory epithelium for the detection of con-specific derived compounds in the water column.

Although the chemical nature of the odorants present in each body fluid remains largely unknown, solid phase extraction together with electrophysiological studies clearly demonstrate that the olfactory activity in each body fluid was likely due to a mixture of compounds (paper III). From the solid phase extraction, three fractions containing compounds with different degrees of hydrophobicity were obtained: i) a fraction containing compounds that were not retained by the C2/ENV+ cartridges and thus are relatively hydrophilic; ii) a

fraction with compounds retained by the C18 cartridges and are, therefore, relatively hydrophobic and iii) a fraction with substances that were not retain by C18 cartridge but were retained by C2/ENV+ cartridges and are therefore of intermediate hydrophobicity.

The olfactory potency of the intestinal fluid was due to both the extract and the filtrate of C18 cartridges (paper III). The odorants in the C18 filtrate were not retained by C2/ENV+ cartridges, suggesting that they are highly polar. Thus small peptides, amino acids and their derivatives are good candidates as odorants. Recently, an amino acid derivative, L-kynurenine, has been proposed to have a pheromonal role in the masu salmon (Yambe, *et al.*, 2006). In the current study, HPLC fractionation in combination with LC-MS and electrophysiological studies showed that part of the olfactory activity of the C18 extract of intestinal fluid is due to bile acids (paper IV). Furthermore, bile acids are responsible for the differential detection by the two olfactory epithelia of this fraction (paper IV). The main bile acids identified in the C18 extract were taurocholic acid and taurolithocolic acid plus a minor third, unidentified, bile acid of 544.1 Da molecular mass (paper IV).

In bile fluid, the total olfactory potency was due to the extrates of C18 and C2/ENV+ cartridges. Given that conjugation normally decreases hydrophobicity, good candidates as odorants in the C2/ENV+ extract are conjugated sterols. The main bile acids identified in intestinal fluid were also detected in the C18 extract of bile fluid.

In mucus, all the solid phase extraction fractions had olfactory potency. No attempt was made to measure bile acids in the C18 extract of mucus. However, it is possible that bile acids different from those found in intestinal fluid and bile

fluid may be present in mucus. Another possibility is the presence of sex steroids and/or prostaglandins. Olfactory and behavioural responses to steroids and prostaglandins in fish have been described for at least four orders including Perciformes, Siluriformes, Cypriniformes, and Salmoniformes (reviwed in Stacey, Sorensen, 2006). Olfactory sensitivity of marine fish to steroids or prostaglandins has not been reported; however, the high diversity of these compounds and their relative species-specificity may simply mean that the correct stimuli have not yet been tested (reviewed in Stacey, Sorensen, 2006).

Although we did not test the olfactory potency of sole-conditioned water, our results showed that the main bile acid identified in both intestinal fluid and bile fluid are also released to the water. Thus, bile acids are released to the water *via* the intestinal fluid, making them good candidates to be used in conspecific interactions. Bile acids have been described as olfactory stimuli in many species (Baker, *et al.*, 2006; Doving, *et al.*, 1980; Frade, *et al.*, 2002; Lo, *et al.*, 1994; Rolen, Caprio, 2007; Siefkes, Li, 2004; Yun, *et al.*, 2003; Zhang, *et al.*, 2001). However, only in the case of sea lamprey (*Petromyzon marinus*) has a mixture of species-specific bile acids been identified with clearly defined biological roles (Li, *et al.*, 2002; Sorensen, *et al.*, 2005). Bile acids are known to be potent odorants in fish (Doving, *et al.*, 1980; Hara, 1994; Zhang, *et al.*, 2001). The sole is, in general, highly sensitive to bile acids. Furthermore, the upper olfactory epithelium is more sensitive to these odorants than the lower (paper III). Although one of the main bile acids identified in both bile and intestinal fluid, taurolithocholic acid is poorly smelt by sole. However, they are highly sensitive to taurocholic acid (paper IV). Taurocholic acid is common among teleosts and is not species-specific. Many fish species were reported to produce, release (Baker, *et al.*, 2006; Doving, *et al.*,

1980; Zhang, *et al.*, 2001) and detect taurocholic acid (Doving, *et al.*, 1980; Li, Sorensen, 1997; Lo, *et al.*, 1994; Michel, Lubomudrov, 1995). Thus, it is unlikely to be used as a species-specific pheromone alone. Alternatively, it may be part of a "cocktail" of different compounds that constitute a pheromone. All fish sex-pheromones identified to date are multi-component (Li, 2005; Sorensen, *et al.*, 2003). Thus, it is likely that a specific behavioural or physiological response is evoked by a mixture of odorants instead of by a single odorant.

Cross-adaptation studies showed the existence of a specific receptor in the upper epithelium which responds preferentially to taurocholic acid; even cholic acid was unable to inhibit the response to taurocholic acid by more than 60% (paper V). The existence of a receptor responding specifically to taurocholic acid suggests that the detection and discrimination of this compound is important to sole. Furthermore, these results are consistent with the upper epithelium being more involved in the detection of conspecific-derived chemical signals released into the water column than the lower.

Taken together, the results suggest a different functional role for the two olfactory epithelia; the lower olfactory epithelium is specialised for detecting prey-related odorants whereas the upper epithelium is specialised for detecting conspecific-derived odorants released into the water column or – possibly - those released by other species, including predators.

We suggest that selective pressure, due to the nocturnal nature of this fish, decreased the importance of visual cues but increased the importance of chemical perception. Due to the strategic position of the lower nostril to detect prey hidden in the substrate, individuals with high sensitivity to specific odorants within the

lower nostril were more able to detect and discriminate prey. This specificity of the lower olfactory epithelium was due to the loss of olfactory sensitivity to general odorants and gain of sensitivity to odorants specific to a given prey. The specialization of the lower olfactory epithelium in detecting a lower number of odorants, probably related to food localisation, is supported by differences in gross anatomy of the two epithelia; the lower epithelium has fewer lamellae and is slightly smaller (Velez, *et al.*, 2005). In addition, except for aromatic compounds, the absolute amplitude of EOGs recorded from the lower epithelium is lower than the upper (Velez, *et al.*, 2005). This is consistent with a lower receptor density in the lower olfactory epithelium.

To compensate the loss of olfactory sensitivity of the lower olfactory epithelium individuals with high sensitivity in the upper epithelium were selected. In addition, due to the fact that the water column is sampled mainly (if not only) by the upper epithelium, individuals with higher number of receptors specialised in the detection of chemical information related to reproduction or predator detection (compounds found mainly in the water column) were more effective in reproduction and survival.

3.2 Olfactory transduction pathways in sole

The transduction pathways involved in olfaction in fish are poorly understood. The studies included in this thesis have shown that, in the olfactory system of the sole, the PLC/IP_3 transduction pathway is more important than the AC/cAMP pathway for amino acid odorants, whereas both pathways are approximately equally involved in bile acid detection (paper V). Olfactory

sensitivity to L-cysteine is mediated mainly by the PLC/IP_3 pathway (approximately 80-90%); the AC inhibitor SQ-22536 was only able to reduce the response by around 10% (paper V). The inhibitory effects of the AC and PLC inhibitors were equal in both olfactory epithelia. This is consistent with our previous observation that olfactory sensitivity to L-cysteine is equal in both epithelia (Velez, *et al.*, 2005).

PLC/IP_3 is also the main pathway involved in the olfactory perception of both 1-methyl-L-tryptophan and L-phenylalanine (paper V). However, in the upper epithelium the contribution of AC/cAMP pathway to the olfactory activity of these compounds is also considerable. Thus, the effects of both drugs on the olfactory sensitivity to L-phenylalanine and 1-methyl-L-tryptophan differed between the two epithelia; this shows that the odorant receptors responding to these stimuli are different between the two epithelia. In the lower epithelium, the two drugs applied simultaneously were unable to inhibit olfactory responses to either odorant completely, suggesting the presence of non-PLC/non-AC dependent transduction pathways. The occurrence of different receptors between the two epithelia is consistent with the cross-adaptation studies that suggested the existence of specific receptors on the lower epithelium responding preferentially to 1-methyl-L-tryptophan. Possibly, different receptors - acting *via* different transduction pathways - are responding to the same odorant in the two epithelia. It was suggested above that the activation of 1-methyl-L-tryptophan receptors in the upper olfactory epithelium (at high odorants concentration) could be involved in induction of food intake whereas the high olfactory sensitivity of the lower epithelium could be involved in food detection and/or location. The contribution of the AC/cAMP pathway in the upper epithelium and the putative contribution of

a non-PLC/non-AC pathway in the lower epithelium support this hypothesis in that it suggests that receptors responding to food-related stimuli in both epithelia are different.

The inability of simultaneous application of both drugs to completely inhibit olfactory responses to L-phenylalanine or 1-methyl-L-tryptophan could also be due to a lower efficacy of the drugs in the sole. However, the IC_{50} values for U73122 are sufficiently low as to suggest that this drug is effective in the sole as it is in other non-mammalian vertebrates (Brann, Fadool, 2006; Jung, *et al.*, 2005; Wellerdieck, *et al.*, 1997) and there were no clear differences in the IC_{50} values either between odorants or epithelia. The responses observed during simultaneous application of both drugs are more likely to be due to (a) non-PLC/non-AC pathway(s), rather than the drugs being less than 100% effective.

The literature concerning the transduction pathways involved in olfactory sensitivity to amino acids in fish is limited. In goldfish, sensitivity to amino acids is mediated by both AC/cAMP and PLC/IP_3 pathways (Sorensen, Sato, 2005) whereas in catfish, zebrafish and Atlantic salmon, it seems to be due only to the PLC/IP_3 pathway (Lo, *et al.*, 1993; Lo, *et al.*, 1994; Ma, Michel, 1998; Restrepo, *et al.*, 1993; Restrepo, *et al.*, 1990). Our results suggest that, although other pathways may be involved, PLC/IP_3 is mainly responsible for the olfactory sensitivity to amino acids in sole (paper V).

Studies carried out in sole adapted to different salinities (35‰ and 10‰) showed that the olfactory sensitivity of the upper epithelium to L-cysteine, L-phenylalanine and glycine is not dependent on environmental salinity (paper VI). However, the absence of external Na^+ greatly inhibits responses to these amino acids in fish kept at 35‰ but not those adapted to 10‰ (paper VI). This suggests

that, although the olfactory sensitivity to amino acids does not vary with salinity, the dependence of the transduction mechanism on external Na^+ is somehow less at 10‰ than 35‰. Thus, olfactory sensitivity to amino acids *per se* does not depend on salinity but the transduction mechanism must adapt to lower salinity/Na^+ availability. This capacity of the olfactory system of sole to adapt to lower salinity may be crucial; in natural conditions, sole feed mainly in estuaries, where salinity changes daily. If the olfactory system of sole could not adapt to different salinities, they would be unable to find their prey. How the olfactory system of sole adapts to lower salinities will be the subject of future studies. However, we hypothesised that the mechanisms involved in intracellular Ca^{2+} clearance after receptor neuron stimulation may be involved in the olfactory adaptation to low salinities. It is known that the chemo-electrical transduction process in olfactory receptor neurons is accompanied by a rapid and transient increase in intracellular [Ca^{2+}] (Cadiou, *et al.*, 2000; Kleene, Gesteland, 1991; Liu, *et al.*, 2006; Menini, 1999; Restrepo, *et al.*, 1990). The Ca^{2+} is then cleared, preparing the cell for the next stimulation (Noe, *et al.*, 1997). There are several candidates for Ca^{2+} clearance including the Na^+/Ca^{2+} exchanger (Danaceau, Lucero, 2000; Noe, *et al.*, 1997; Pyrski, *et al.*, 2007). This exchanger co-transports two Na^+ ions from the extracellular to the intracellular medium and one Ca^{2+} from the intracellular to the extracellular medium; in the absence of external Na^+ this mechanism cannot work. A possible explanation for the decrease of olfactory sensitivity in the absence of external Na^+ is that olfactory Ca^{2+} clearance after olfactory stimulation in sole involves the Na^+/Ca^{2+} exchanger and, in the absence of external Na^+, receptor cells do not repolarise and olfactory sensitivity decreases. A possible explanation for the adaptation to low salinity is that, due to a chronic decrease of external Na^+,

alternative mechanisms of Ca^{2+} clearance are activated. Possible candidates are the Ca^{2+}-ATPase and the endoplasmic reticulum calcium pump (Weeraratne, *et al.*, 2006). Thus, it is possible that the decrease of external [Na^{+}] induces the production of these, or other, proteins enabling cells to clear Ca^{2+} even at the absence of Na^{+}. This hypothesis remains to be tested.

In sole, the absence of external Ca^{2+} does not affect olfactory sensitivity to amino acids either in 35‰ or 10‰ seawater. This means either that the olfactory transduction pathways involved in amino acid detection are independent of external Ca^{2+}, or that there are mechanisms which maintain [Ca^{2+}] constant in the medium surrounding the cilia and microvilli. In air-breathing tetrapods, the olfactory cilia and microvilli do not penetrate directly into the medium, but into a mucous layer, the ionic composition of which can be tightly regulated without significant loss to, or gain from, the surrounding medium (Schild, Restrepo, 1998). Sole produce large amounts of mucus. However, it is not known whether, in fish, the properties and function of the mucus produced by the olfactory epithelium are similar to those of mammals. If mucus allows the maintenance of constant ion concentrations in the medium surrounding the cilia and microvilli, then decreasing external [Na^{+}] would not have any effect on olfactory responses to amino acids.

The maintenance of olfactory sensitivity independently of external ions concentration was previously described in a number of different fish species (Hubbard, *et al.*, 2000; Parker, *et al.*, 2000; Shoji, *et al.*, 1994). In the aquatic environment, ion concentrations can vary drastically, thus aquatic organism may have developed mechanisms that allow them to maintain olfactory sensitivity independently of external salinity. The mobilization of Ca^{2+} from intracellular

Ca^{2+} reservoirs instead of the extracellular medium would allow the maintenance of olfactory responses even at the absence of extracellular Ca^{2+}. That the PLC/IP_3 transduction pathway involves mobilization of Ca^{2+} from the ER gives support to this hypothesis. However, evidence suggests the existence of IP_3 dependent channels in the plasma membrane of olfactory receptor cells and IP_3-related odorants can cause Ca^{2+} influx (Cadiou, *et al.*, 2000; Munger, *et al.*, 2000; Okada, *et al.*, 1994; Restrepo, *et al.*, 1990; Schild, *et al.*, 1995). This suggests that the IP_3/Ca^{2+} channels are present in the plasma membrane (Munger, *et al.*, 2000) and that PLC/IP_3 mediated odorant transduction may involve influx of extracellular Ca^{2+}. A possible explanation for the maintenance of olfactory sensitivity in different salinities came from a study carried out with plasma membrane fractions prepared from the olfactory rosettes of Atlantic salmon (Lo, *et al.*, 1993). This showed that stimulation with a mixture of L-amino acids leads to PIP_2 hydrolysis by PLC in a concentration-dependent manner and was maximal at 1 - 10 nM free $[Ca^{2+}]_i$. At this $[Ca^{2+}]_i$, PIP_2 hydrolysis is G-protein dependent. In addition, between 100 nM and 100 μM, Ca^{2+} directly stimulated PLC activity in a concentration-dependent manner. This appeared to be G-protein independent. It was suggested that low $[Ca^{2+}]$ sensitizes olfactory PLC to G-protein dependent stimulation by amino acids, whereas direct activation of PLC by elevated $[Ca^{2+}]$ may contribute to amplification of the signal (Lo, *et al.*, 1993). This could permit olfactory sensitivity even in an environment with low external $[Ca^{2+}]$. Even if external $[Ca^{2+}]$ is low, and the influx of Ca^{2+} is not enough to generate an action potential, it may activate PLC directly. However, this cannot explain the maintenance of olfactory response in the complete absence of Ca^{2+}. A possible explanation is that action potentials in the absence of Ca^{2+} are mediated by influx

of Na^+ ions instead of Ca^{2+} ions. *In vivo* recordings of multiunit activity in channel catfish showed that lowering external $[Ca^{2+}]$ enhances olfactory responses to amino acids (Parker, *et al.*, 2000). The authors proposed that a reduced external $[Ca^{2+}]$ may result in the increased excitability of olfactory receptor cells due either to i) lessening the Ca^{2+} block on olfactory receptor cyclic nucleotide-gated channels and/or ii) by lowering of the surface potential of neurons, shifting ion channel activation to a more negative potential (Parker, *et al.*, 2000). Thus, under lowered external $[Ca^{2+}]$, sodium ions entering through ciliary and microvillous IP_3 channels could be sufficient to generate an action potential (Parker, *et al.*, 2000). Indeed, IP_3 regulated non-specific cation channels and non-specific cation conductances were reported in channel catfish (Restrepo, *et al.*, 1990), lobster (Hatt, Ache, 1994), *Xenopus laevis* (Schild, *et al.*, 1995) and rat (Restrepo, *et al.*, 1992). The involvement of non-specific cation channels in IP_3 mediated olfactory responses gives support to the hypothesis that responses in the absence of Ca^{2+} occur *via* influx of Na^+ ions. In natural conditions, sole are unlikely to face an environment without Ca^{2+}; it can not survive in full freshwater. However, sole penetrate estuarine environments, where salinity may vary widely and rapidly, to feed and reproduce. As salinity decreases, both $[Na^+]$ and $[Ca^{2+}]$ decrease at the same rate. However, the $[Na^+]$ of seawater is much higher (460mM) than $[Ca^{2+}]$ (10mM). In 10‰ seawater, the $[Ca^{2+}]$ is around 2.9 mM while $[Na^+]$ is about 130 mM. IP_3 channels are blocked by Ca^{2+}; increasing $[Ca^{2+}]$ decreases their permeability to other cations (Schild, Restrepo, 1998). Thus, decreases in salinity increase the permeability of these channels to Na^+. The environmental $[Na^+]$ is much higher than $[Ca^{2+}]$; even if (due to low concentration) the influx of Ca^{2+} is not enough to induce action potential, the influx of Na^+ may be. In addition, Ca^{2+}

entering the cell may directly stimulate PLC and thus amplify the signal. These hypothetical mechanisms could explain the maintenance of olfactory sensitivity at different salinities and in the absence of external Ca^{2+}. However, further studies are required.

Olfactory sensitivity to bile acids in the sole is mediated by both AC/cAMP and PLC/IP_3 pathways (paper V). In the upper epithelium - and in contrast to the amino acid odorants - the effects of U73122 and SQ-22536 were approximately equal. Given that the upper epithelium is more sensitive to bile acids (Velez, *et al.*, 2005; Velez, *et al.*, 2007), this suggests that the AC/cAMP transduction pathway is of equal importance to the PLC/IP_3 pathway. However, the effect of either drug differs between the two olfactory epithelia. The AC/cAMP pathway contributes more to the olfactory sensitivity to bile acids in the upper epithelium, whereas PLC/IP_3 pathway contributes more in the lower (paper V). This suggests the existence of different receptors between the two epithelia and is consistent with the existence of specific receptors on the upper nostril responding to bile acids (as suggested by cross-adaptation studies).

Olfactory responses to TCH and CHO during application of both drugs simultaneously are considerable; 20-40% of control responses remained. Again, this suggests the presence of non-PLC/non-AC dependent olfactory transduction pathways in the sole. There are several candidates for these. Some mechanisms that do not appear to be consistent with the AC/cAMP or PLC/IP_3 mediated transduction cascades are the odorant-induced decrease in $[Ca^{2+}]_i$ recorded in human (Rawson, *et al.*, 1997) and channel catfish (Restrepo, *et al.*, 1992) olfactory receptor neurons and the odorant induced blockage of K^+ channels observed in the mudpuppy *Necturus maculosus* (Dubin, Dionne, 1993). Another

possible mechanism is the involvement of guanylate cyclase (GC). Olfactory receptor neurons that express a membrane-bound GC and a cGMP-stimulated phosphodiesterase (PDE) but neither an AC or a cAMP-depended PDE have been identified (Juilfs, *et al.*, 1997). In addition, some studies suggest the involvement of GC and cGMP- stimulated PDE in olfaction (Borisy, *et al.*, 1992; Breer, *et al.*, 1992; Juilfs, *et al.*, 1997; Meyer, *et al.*, 2000; Moon, *et al.*, 1998; Verma, *et al.*, 1993). Cyclic GMP is produced by two different classes of enzyme: cytosolic GC (soluble), and membrane-bound receptor GC (particulate). Particulate GCs contain a transmembrane domain and are activated by extracellular ligands (such as atrial natriuretic peptide) whereas cytosolic GC can be activated by nitric oxide or carbon monoxide. The initial evidence of the contribution of GC pathway in olfaction suggested that odorant-induced cGMP production is not involved in initial signalling events but in desensitization or other longer-term responses (Moon, *et al.*, 1998). However, a recent study showed that a small number of olfactory neurons specifically express cGMP-signalling components, namely GC and a cGMP-stimulated phosphodiesterase (PDE_2) (Meyer, *et al.*, 2000). This subset also expresses a cGMP-specific cyclic nucleotide-gated channel previously identified in cone photoreceptors. Furthermore, components of the prototypical AC/cAMP signalling pathway could not be detected, suggesting that these neurons use cGMP as the intracellular messenger to open cGMP-gated channels (Meyer, *et al.*, 2000). Thus, the cGMP pathway could be the third pathway involved in the olfactory signal transduction in sole. Alternatively, olfactory response during both AC and PLC inhibition could be due to direct activation of channels, without the involvement of G proteins (Labarca, *et al.*, 1988) or an

isoform of PLC and/or AC which is unaffected by the drugs. Further studies are needed in order to clarify this issue.

Another curious observation is that the sum of the inhibition caused by both drugs tested independently is close to 100% for both TCH and CHO in the two epithelia (paper V). However, the application of both drugs simultaneously failed to completely inhibit the sensitivity to bile acids in both epithelia. These results suggest some plasticity of the response; when both pathways are inhibited, an alternative pathway is activated in order to maintain sensitivity. Or it may suggest the existence of a cross-talk between AC/cAMP and PLC/IP_3 pathways. Assuming the existence of a third pathway, and if both AC/cAMP and PLC/IP_3 were working independently, the sum of the inhibition caused by both drugs tested independently should be less than 100%. Thus our results suggest that inhibiting one pathway is some how decreasing the activation of the other pathway. A functional interaction between pathways has already been proposed in salamanders and rats (Chen, *et al.*, 2000; Vogl, *et al.*, 2000). In isolated salamander olfactory neurons, the responses to cAMP-mediated odorants and IP_3-mediated odorants are not mutually exclusive but coexist in the same cell. The currents induced by IP_3-odorants exhibit identical biophysical properties as those induced by cAMP-odorants or direct activation of the cAMP cascade. By disrupting AC to block cAMP formation, it was shown that this step is necessary for the transduction of both AC and IP_3 odorant classes (Chen, *et al.*, 2000).

In rats, inhibition of PLC in isolated olfactory cilia led to an augmentation of odorant-induced cAMP signalling, whereas activation of the IP_3 pathway resulted in attenuation of odorant-induced cAMP formation (Vogl, *et al.*, 2000). Furthermore, elevated cAMP levels cause suppression of odorant-induced IP_3

signalling, whereas inhibition of AC results in potentiation of odorant-induced IP_3 formation. These results provide evidence that the two second messenger pathways do not work independently but rather show a functional antagonism. The mechanisms involved in this cross-talk between pathways are not known. Nevertheless, this suggests that odorant coding starts at the level of the olfactory epithelium.

3.3 Olfactory sensitivity to changes in environmental [Na^+] and [Ca^{2+}] and putative mechanisms involved

Marine organisms live in an environment rich in many inorganic ions, thus the capacity to estimate the levels of physiologically important ions would represent an advantage. The current work showed that the sole is sensitive to decreases in [Ca^{2+}] and increases in [Na^+] (paper VI). Previous work showed that the gilthead seabream (a marine teleost) is also sensitive to reductions in external [Ca^{2+}] (Hubbard, *et al.*, 2000); in contrast freshwater-reared sockeye salmon and goldfish are sensitive to increases in [Ca^{2+}] (Bodznick, 1978; Hubbard, *et al.*, 2002). In addition, the goldfish (Hubbard, Canário, 2007; Hubbard, *et al.*, 2002) is sensitive to increases in [Na^+]. These results suggest that i) freshwater fish are sensitive to increases of [Ca^{2+}], ii) marine fish are sensitive to decreases in [Ca^{2+}] and iii) both marine and freshwater fish have olfactory sensitivity to increases in [Na^+]. However, to confirm this hypothesis, the olfactory sensitivity to changes in cation concentration should be tested in a number of other freshwater and marine species.

It has been suggested that the olfactory sensitivity to changes in external [Ca^{2+}] both in goldfish (Hubbard, *et al.*, 2002) and gilthead seabream (Hubbard, *et al.*, 2003) is mediated by an extracellular Ca^{2+}-sensing receptor (Ca^{2+}SR) similar to that initially cloned from bovine parathyroid gland (Brown, *et al.*, 1993). The Ca^{2+}SR is highly expressed in tissues involved in regulating extracellular [Ca^{2+}]. However, it is also found in a number of other tissues, which do not have well established roles in the control of extracellular [Ca^{2+}]. In fish, this receptor is expressed by a number of tissues; gills, gut, operculum, gallbladder (Flanagan, *et al.*, 2002), pituitary (Abbink, *et al.*, 2006; Flanagan, *et al.*, 2002), olfactory lamellae (Hubbard, *et al.*, 2002), intestine, kidney, urinary bladder, central nervous system, corpuscles of Stannius, heart and bone (Loretz, 2008). The Ca^{2+}SR is a member of family C of the G-protein-coupled seven transmembrane receptors (Brown, *et al.*, 1993). Elevations in extracellular [Ca^{2+}] elicit increases in intracellular [Ca^{2+}] through interactions between the Ca^{2+}SR and PLC, which are mediated by G proteins $G_{q\alpha}$ or $G_{11\alpha}$. This activation of PLC results in the hydrolysis of PIP_2 to form IP_3 and DAG. Another consequence of $G_{q\alpha}$/PLC activation through the Ca^{2+}SR is the stimulation of cytosolic pholipase A_2 (PLA_2), which results in the production of arachidonic acid and its metabolites (reviewed in Hofer, Brown, 2003). In parallel, the Ca^{2+}SR also activates phosphatidylinositol 4-kinase, which is one of the enzymes in the biosynthetic pathway that leads to the replenishment of PIP_2. The Ca^{2+}SR interacts directly not only with $G_{q\alpha}$ and $G_{11\alpha}$, but also with $G_{i\alpha}$, which results in the inhibition of AC and a reduction in cellular cAMP levels (reviewed in Hofer, Brown, 2003). The Ca^{2+}SR is also involved in the activation of some MAP kinases, such as the extracellular signal-regulated kinases (ERKs) and jun amino-terminal kinase (JNK), which might

account for some of the actions of $Ca^{2+}SR$ agonists on cellular proliferation (Figure 3.1; reviewed in Hofer, Brown, 2003).

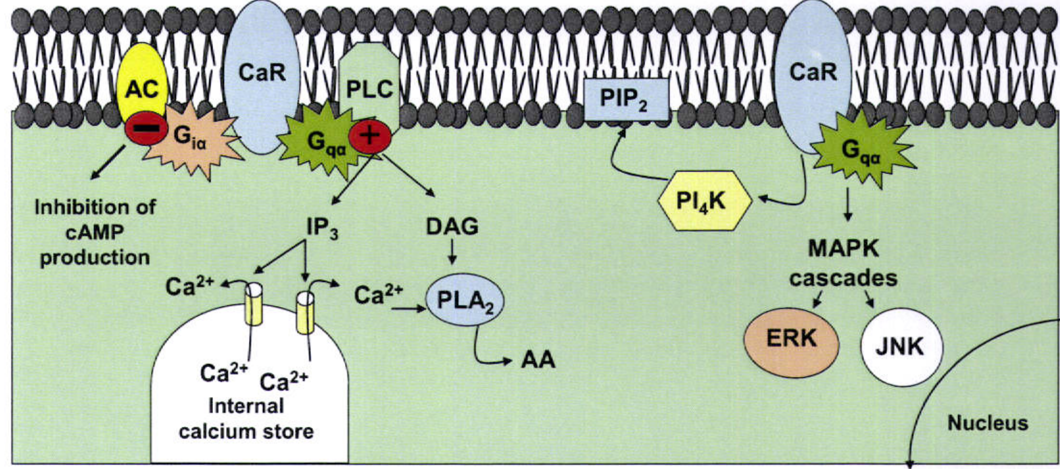


Figure 3.1 - Mechanisms of olfactory transduction involved in the activation of CaSRs. (AA) arachidonic acid; (AC) adenylate cyclise; (CaSR) calcium sensing receptor; (DAG) diacylglycerol; (ERK) extracellular signal-regulated kinases; (IP3) inosotol-1, 4, 5-trisphosphate; (JNK) jun amino-terminal kinase; (MAPK) MAP kinases; (PLC) phospholipase C; (Giα and Gqα) G protein subunities; (PIP2) phosphatidynositol-4, 5-bisphosphate; (PI4K) phosphatidylinositol 4-kinase (based on Hofer, Brown, 2003).

Calcium is not the only ligand for the $Ca^{2+}SR$; it also binds many other divalent and trivalent cations, L-amino acids, polyamines, ionic strength, H^+, drugs such as aminoglycoside antibiotics and the so-called calcilytics and calcimimetics (reviewed in Hofer, Brown, 2003). Inorganic cations mimic Ca^{2+} in activating $Ca^{2+}SR$; based on their potencies, they can be categorized into three groups: i) strong agonists: Gd^{3+}, La^{3+} and Yt^{3+}, ii) medium-strength agonists: Ca^{2+}, Ba^{2+}, Sr^{2+}, Cd^{2+} and Pb^{2+}, and iii) weak agonists: Mg^{2+}, Ga^{3+}, Fe^{3+} and Na^+ (reviewed in Chang, Shoback, 2004). The potency of cations appears to depend on two parameters; the charge number and ionic radius of the cations (reviewed in Chang, Shoback, 2004). Although the size of Na^+ is similar to that of Ca^{2+}, low charge density makes it an ineffective $Ca^{2+}SR$ ligand. In fact, Na^+ has negative effects on the activation of $Ca^{2+}SR$ (Quinn, *et al.*, 1998).

The sole has olfactory sensitivity to decreases in environmental $[Ca^{2+}]$, this effectively means that the putative receptor involved is activated by the loss of

Ca^{2+} (*i.e.* it is inactive in the bound state). It is known that when Ca^{2+} binds to the Ca^{2+}SR, PLC is activated and AC is inhibited (reviewed in Hofer, Brown, 2003). Assuming that responses to Ca^{2+} are mediated by a Ca^{2+}SR, olfactory responses to Ca^{2+} in sole could be due to the activation of AC.

The olfactory sensitivity to changes in environmental [Ca^{2+}] is higher in fish kept at 35‰ than those adapted to 10 ‰ sea water. Conversely, the olfactory sensitivity to increases in [Na^{+}] is higher in fish adapted to 10 ‰ sea water that those kept at 35‰. At the first glace, these results suggest an adaptation of the olfactory system to different environmental salinities; however, our results showed that decreasing external [Ca^{2+}] increases olfactory sensitivity to Na^{+} and decreasing [Na^{+}] decreases olfactory sensitivity to Ca^{2+} (paper VI). Furthermore, the absence of external Na^{+} completely abolishes responses to changes in [Ca^{2+}] whereas even at high [Ca^{2+}] (10mM) olfactory responses to Na^{+} are not completely abolished (paper VI). These results suggest that different sensitivities to ions are not due to adaptation of the olfactory system to low salinity *per se*, but rather to the fact that [Ca^{2+}] modulates olfactory responses to Na^{+} and environmental [Na^{+}] modulates responses to Ca^{2+} directly. Thus, [Ca^{2+}] and [Na^{+}] decrease with environmental salinity and, as a direct consequence, the olfactory sensitivity to Ca^{2+} decreases and sensitivity to Na^{+} increases. The mechanisms involved in this apparent modulation are currently unknown. However, the loss of sensitivity to Ca^{2+} when [Na^{+}] decreases supports the hypothesis of the involvement of a Ca^{2+}SR. In the mammalian Ca^{2+}SR, Na^{+} ions compete with Ca^{2+} ions for the Ca^{2+}-binding site (reviewed in Hofer, Brown, 2003), Given that the putative Ca^{2+} receptors in sole are inactive in the bound state, decreasing [Na^{+}] leads to an increase of Ca^{2+} binding and a consequent loss of olfactory sensitivity

to Ca^{2+}. This could explain how decreasing environmental [Na^+] attenuates sensitivity to changes in [Ca^{2+}]; however, it can not explain the loss of response to Ca^{2+} in the absence of Na^+.

In the sole, olfactory sensitivity to Na^+ appears to be independent of that of Ca^{2+}. The concentration/response curve to changes in [Ca^{2+}] is sigmoidal, suggesting the contribution of a receptor mechanism, whereas the concentration/response curve to Na^+ does not reach a maximum, suggesting that it may be channel-mediated (paper VI). We propose that olfactory sensitivity to Na^+ is due to a channel that opens in the presence of this ion allowing the influx of Na^+ and possibly other cations. Ca^{2+} ions could block this channel. The existence of Na^+-activated non-selective cation channels was demonstrated in the olfactory receptor neurons of lobster (Zhainazarov, Ache, 1995; , 1998); these Na^+-activated channels are equally permeable to Na^+, K^+, and Li^+. Both Ca^{2+} and Mg^{2+} reversibly inhibit channel activity in a concentration-dependent manner (Armstrong, Cota, 1999; Zhainazarov, Ache, 1995). Olfactory sensitivity of sole to changes in [Na^+] could be due to the activation of a channel similar to that previously described. Given that these channels are blocked by Ca^{2+} ions, olfactory responses to Na^+ would increase as [Ca^{2+}] decreases. This model could explain the fact that, in the absence of environmental Ca^{2+}, the olfactory response to Na^+ is larger, and it could also explain the fact that sensitivity to Na^+ is higher at 10‰ seawater than 35‰.

Given that, in the natural environment, whilst moving from seawater to freshwater, the concentration of both ions will co-vary, it is possible that Ca^{2+} sensitivity is used to warn the fish that it is reaching the limit of its salinity tolerance, whereas Na^+ sensitivity may work as a guide that allows fish to find his

way back to an environment of higher salinity. Thus, in 35‰ seawater, CaSR would bind Ca^{2+} and Na^+ channels would be blocked due to the high external [Ca^{2+}]. When salinity decreases, Ca^{2+} is released, Ca^{2+}SRs are activated leading to cell depolarisation. As the salinity decreases, olfactory sensitivity to Ca^{2+} decreases due to the lower [Na^+], whereas olfactory sensitivity to Na^+ increases because of the low [Ca^{2+}] (the blockage of Na^+ channels by Ca^{2+} ions is weaker).

The models proposed are hypothetical; further studies are clearly needed. Nevertheless, the current study shows that, in sole, as in goldfish, Ca^{2+} and Na^+ are detected by distinct and separate cellular mechanisms. However, there seems to be a degree of overlap between the two mechanisms; decreasing environmental [Na^+] reduces olfactory response to Ca^{2+} and decreasing environmental [Ca^{2+}] increases responses to Na^+.

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Chapter IV

Concluding remarks and perspectives

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Chemical identification of food-related odorants in Senegalese sole provided important information that could be used in aquaculture: to evoke higher food intake and, possibly, faster growth in cultivated sole. The work in the current thesis strongly supports the hypothesis of specialization of the lower olfactory epithelium in detecting cues released by prey.

The specific conclusions that can be taken are:

- Odorants present in ragworm macerate are different from those released into the water by living ragworms. Therefore, future studies on chemical identification of food related odorants in fish should use prey-conditioned water rather than macerates.
- The main odorants present in ragworm macerate are amino acids.
- The majority of the olfactory potency of water conditioned by ragworms is due to an amino acid derivative, 1-methyl-L-tryptophan.
- Both worm-conditioned water and 1-methyl-tryptophan are more potent at the lower olfactory epithelium than the upper.
- Olfactory sensitivity of the lower olfactory epithelium to 1-methyl-L-tryptophan is mediated by (a) specific receptor(s).
- 1-methyl-tryptophan is a prey-related odorant in sole.
- The lower olfactory epithelium is specialised for detecting 1-methyl-L-tryptophan.

Although the results suggest that 1-methyl-L-tryptophan is a food-related odorant, behavioural studies are needed to define its exact role. In conjunction

with other amino acids (*e.g.* L-phenyalanine) it is a good candidate to be used as a feeding stimulant. Nevertheless, further studies are required to test the behavioural effects of these odorants alone and as a mixture.

The functional importance of bile acid olfaction in sole is not yet clear. Chemical identification of odorants present in conspecific body-fluids provided strong evidence that bile acids may be involved in inter- and/or intra-specific interactions. The main conclusions that can be taken are:

- The total olfactory potency of skin mucus and intestinal and bile fluids is due to a mixture of compounds with different chemical properties.
- All con-specific body fluids tested and their SPE fractions are better detected by the upper olfactory epithelium.
- Bile acids are responsible for the preferential detection of the C18 extract of intestinal fluid by the upper olfactory epithelium.
- The main bile acids detected in intestinal fluid are taurocholic acid and taurolithocholic acid plus a minor, unidentified, bile acid of 544.1 Da molecular mass.
- The contribution of bile acids to the olfactory potency of intestinal fluid is due mainly to taurocholic acid.
- Taurocholic acid was also detected in bile fluid and sole-conditioned water.
- The concentration of taurocholic acid in sole-conditioned water is well above the calculated olfactory threshold of detection.
- Taurocholic acid is a good candidate to be involved in intra-specific interactions.

- The upper olfactory epithelium is more sensitive to taurocholic acid than the lower.
- There are specific receptors in the upper olfactory epithelium which respond preferentially to taurocholic acid
- The upper olfactory epithelium is specialised for the detection of conspecific derivative odorants.

In the current study, we only evaluated the contribution of bile acids to the olfactory potency of bile and intestinal fluid. Taurocholic acid is a good candidate to be involved in intra-specific interactions. However, pheromones are more likely to be multi-components rather than a single compound; the current study showed that the olfactory potency of body fluids is due to a mixture of compounds with different chemical properties. However, the chemical identification of pheromones in sole requires further work.

The olfactory mechanisms involved in odorant detection in fish are poorly studied. The current study provides a contribution to redress this lack of information. The main conclusions that can be taken are:

- The PLC/IP_3 transduction pathway is more important than AC/cAMP pathway in amino acid detection.
- Both PLC/IP_3 and AC/cAMP pathways are equally involved in bile acid detection
- The PLC/IP_3 pathway is predominant in olfactory sensitivity to L-cysteine in both olfactory epithelia.

- The effects of U73122 and SQ-22536 on the olfactory sensitivity to L-phenylalanine, 1-methyl-L-tryptophan and bile acids differ between the two epithelia
- Both amino acid and bile acid detection appears to involve the contribution of a non-PLC/non-AC dependent pathway.
- Olfactory sensitivity to amino acids does not depend on external salinity. However the maintenance of olfactory sensitivity at low salinity requires an adaptation to low [Na^+].
- Olfactory sensitivity to amino acids is independent of external [Ca^{2+}].

The results suggest the involvement of a non-PLC/non-AC pathway as well as an interaction between the PLC/IP_3 and AC/cAMP pathways in olfactory transduction in sole. Both the involvement of other pathways and the possible cross-talk between the PLC/IP_3 and AC/cAMP pathways have been proposed in other studies. However, the mechanisms involved are yet unknown. Further studies using different drugs may identify the non-PLC/nor-AC pathway. Alternatively, measurement of intracellular second messenger concentration (cAMP, IP3 and cGMP) could also be used. Such an approach would also be useful to evaluate whether different pathways are activated in the same cell. Patch-clamp studies together with pharmacological agents could help to understand the mechanism(s) behind the possible cross-talk between the PLC/IP_3 and AC/cAMP pathways.

Another two questions raised by the results are: i) which mechanisms are involved in the maintenance of olfactory sensitivity in the absence of external Ca^{2+} and Na^+ and ii) how do olfactory receptor neurons adapt to low environmental salinity. The measurement of odorant-induced current generation

in individual olfactory receptor neurons would allow the evaluation of the mechanisms involved in neuronal depolarization in the presence and absence of external cations. In addition, molecular biology studies could be use to evaluate differences between gene expression in the olfactory system of fish kept at different salinities.

Increasing evidence suggests that fish can detect changes in environmental ion concentrations. However, the mechanisms involved and the functional importance of this sensitivity are unknown. The main conclusions that can be taken about the olfactory sensitivity of sole to changes in [Ca^{2+}] and [Na^{+}] are:

- The sole is sensitive to decreases in [Ca^{2+}] and increases in [Na^{+}].
- Adaptation of fish to low salinity (10‰) decreases olfactory sensitivity to Ca^{2+} and increases sensitivity to Na^{+}.
- Environmental [Ca^{2+}] modulates olfactory responses to changes in [Na^{+}] and environmental [Na^{+}] modulates responses to changes in [Ca^{2+}].
- Ca^{2+} and Na^{+} are detected by distinct and separate cellular mechanisms. However, there is some degree of overlap between the two mechanisms.

We hypothesise that responses to decreases in [Ca^{2+}] are due to the activation of a receptor similar to the Ca^{2+}SR, whereas sensitivity to increases in [Na^{+}] is channel-mediated. Pharmacological studies similar to those used to evaluate the metabolic pathways involved in amino acid and bile acid detection will be useful in the evaluation whether olfactory responses to cations are due to G-protein coupled receptors, channels or other mechanisms. Identification and isolation of the possible receptors and/or channels using molecular biology techniques could also be tried.

The current study clearly shows that the two olfactory epithelia of sole are specialised in detecting different odorants. Such specialization occurs both by the occurrence of different receptors and activation of different transduction pathways in the two olfactory epithelia; olfactory discrimination starts at the level of the olfactory epithelium.

Although the precise functional importance of both olfactory epithelia of sole can not be drawn, this study provides strong evidence that the upper olfactory epithelium is specialised in detecting stimuli involved in intra-specific interactions whereas the lower is specialised in the detection of prey-related olfactory cues.

The current work has opened a range of questions that need to be answered in the future; "Finding solutions always generates new problems".