

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

Zélia Velez ^{a,b,c}, Peter C. Hubbard ^{a,*}, Jörg D. Hardege ^b,
Eduardo N. Barata ^{a,c}, Adelino V.M. Canário ^a

^a Centro de Ciências do Mar, Universidade do Algarve, Faro, Portugal

^b Department of Biological Sciences, University of Hull, Hull, UK

^c Departamento de Biologia, Universidade de Évora, Évora, Portugal

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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 chemo-sensory food location in the sole.

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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

* Corresponding author. Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. Tel.: +351 289 800100x7880 or 7389; fax: +351 289 800069.

E-mail address: [phubbard@ualg.pt](mailto:p Hubbard@ualg.pt) (P.C. Hubbard).

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 Centriprep-10 tubes, 3 kDa Amicon Centriprep-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 performed 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 \times 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 $^{\circ}\text{C}$, followed by an increase to 150 $^{\circ}\text{C}$ at 10 $^{\circ}\text{C min}^{-1}$ and then to 300 $^{\circ}\text{C}$ at 15 $^{\circ}\text{C min}^{-1}$ and this final temperature was maintained for 5 min. The temperature of the injection port was set at 250 $^{\circ}\text{C}$ and that of the detector was set at 280 $^{\circ}\text{C}$. 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 $\mu\text{g ml}^{-1}$, 40 $\mu\text{g ml}^{-1}$, 10 $\mu\text{g ml}^{-1}$ and 5 $\mu\text{g ml}^{-1}$) 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

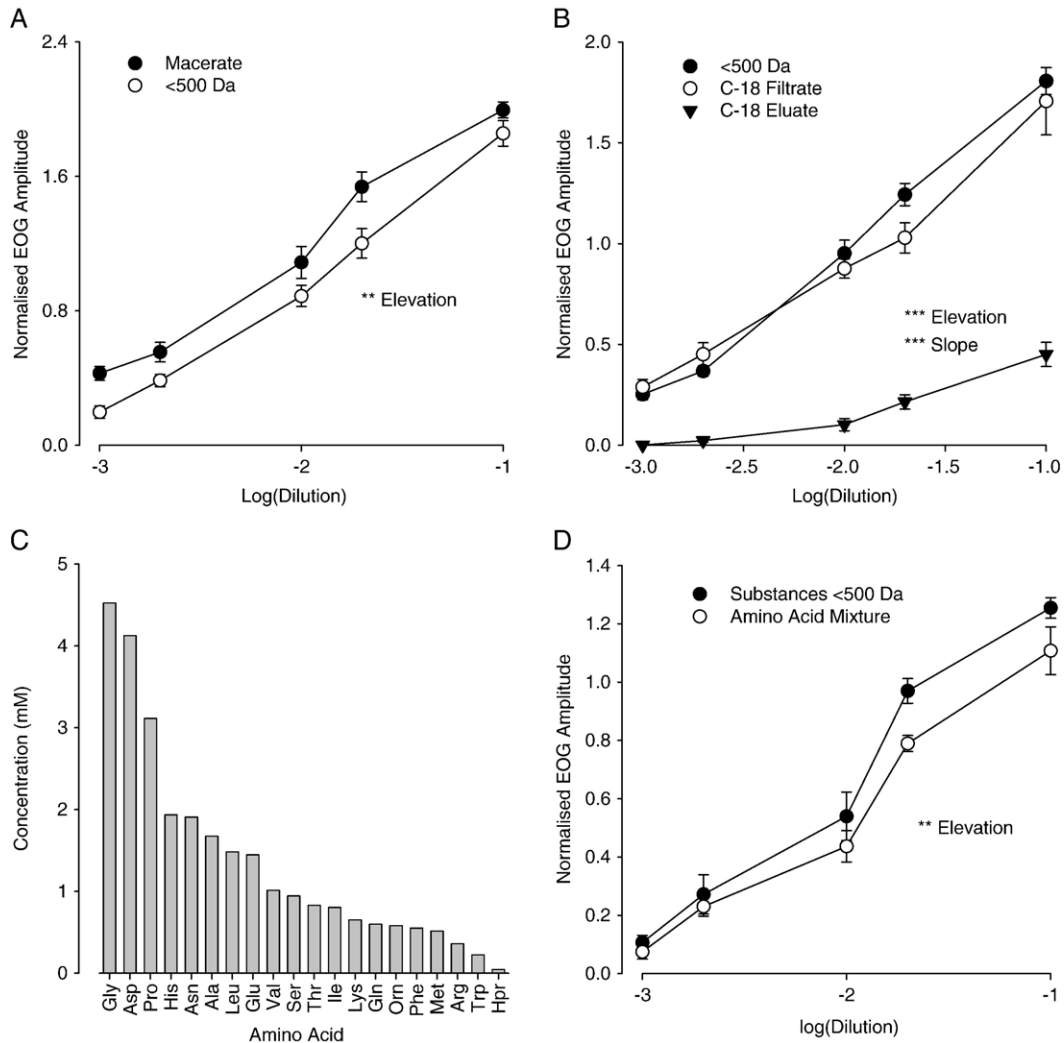


Fig. 1. Olfactory responses of the Senegalese sole (*Solea senegalensis*) to a homogenate of *H. diversicolor*. A. Semi-logarithmic plot of normalised EOG amplitude recorded to the original homogenate (filled circles) and the fraction containing compounds smaller than 500 Da (open circles). Data are shown as mean \pm 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 \pm 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 \pm 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

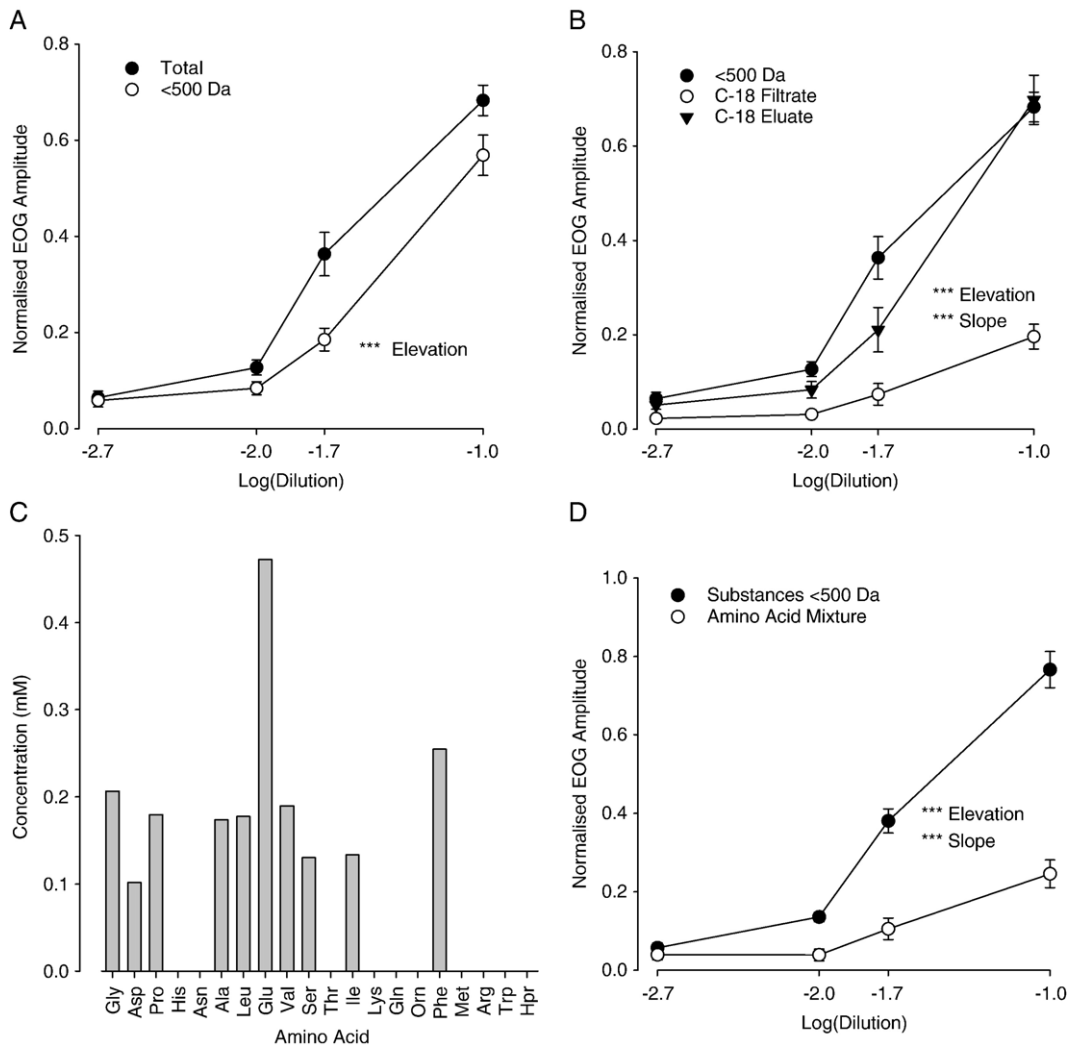


Fig. 2. Olfactory responses of the Senegalese sole (*Solea senegalensis*) to compounds released by living *H. diversicolor*. A. Semi-logarithmic plot of normalised EOG amplitude recorded in response to original sample (filled circles) and the fraction containing compounds smaller than 500 Da (open circles). Data are shown as mean \pm 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 \pm 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 \pm 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; Kubitzka et al., 1997; Mackie et al., 1980; Papatryphon and Soares, 2000; Reig et al., 2003). Most of these studies were performed 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.

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