Prey odour enhances swimming activity and feed intake in the Senegalese sole

Eduardo N. Barata, François Hubert, Luis E.C. Conceição, Zélia Velez, Paulo Rema, Peter C. Hubbard, Adelino V.M. Canário

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ABSTRACT

Olfaction is important in many aspects of the life-history of fishes including feeding, and more so in nocturnal benthic feeders. In the current study we assessed the importance of olfaction in food-search behaviour of the Senegalese sole, an economically important marine species both as farmed and wild-caught. Whole-body homogenates of the polychaete Diopatra neapolitana were fractionated by solid-phase extraction (SPE) using C18 cartridges and the olfactory potency of the resultant fractions (hydrophobic eluate and hydrophilic filtrate) was assessed by the electro-olfactogram in juvenile sole. In addition, the effect of both the homogenate and SPE fractions on sole locomotion was assessed in a flow-through tank (fluviarium). Finally, whole-body homogenate was added to commercial feed pellets and tested whether it could enhance food consumption by sole. The SPE hydrophilic filtrate contained the majority of the olfactory activity found in the whole-body homogenate. Both the homogenate and filtrate, but not the eluate, increased number of movements, time moving, linear velocity, distance travelled and time swimming upstream of sole in the fluviarium; ablation of the olfactory epithelia disrupted these behavioural responses to the homogenate. Intact sole consumed more pellets flavoured with worm homogonate than those without. These results show that olfaction plays an important role in food-search behaviour of the Senegalese sole and that the hydrophilic fraction of D. neapolitana whole-body homogenate contains key substances affecting sole search behaviour; moreover, ingestion by sole was enhanced by addition of worm homogonate to the dry feed pellets.

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1. Introduction

Fishes rely upon sensory information for food detection, recognition and selection. In general, feeding shows a stereotyped sequence of behavioural components: arousal, searching, food uptake and ingestion. The arousal phase is mediated mainly by olfaction. In the searching phase, the relative importance of different sensory modalities depends upon the feeding strategy and ecological niche. Feeding behaviour is completed by food uptake and ingestion, triggered by chemical stimuli [De Groot, 1971; Hara, 1993]. Fish chemical senses (reviewed by Caprio and Derby, 2008) are important in feeding behaviour, and more so in nocturnal benthic feeders.

The Senegalese sole (Solea senegalensis Kaup 1858; hereafter 'sole'), is a marine flat-fish of high economic value both as farmed and wild-caught (Imsland et al., 2003). This benthic species is more active during the night and displays a typical creeping behaviour along the bottom towards the feed (pers. obs.). In the natural environment, sole feeds mainly on invertebrates living in the sediment, such as polychaetes, bivalves and crustaceans (Cabral, 2000). As fish do not form part of the sole’s natural diet, commercial fishmeal-based diets are not attractive (Reig et al., 2003). The importance of chemoreception in feeding of soleids has been highlighted by the difficulties in weaning juvenile S. senegalensis and S. solea from live feed to artificial diets (Dinis et al., 1999; Howell, 1997). A mixture of inert and live feed may increase the weaning success of sole fry (Imsland et al., 2003), and consumption of inert feed by both fry and juveniles may be enhanced by addition of natural food-related odorants.

The Senegalese sole is a good model species to investigate the importance of olfaction in feeding behaviour, due to its feeding strategy and its well-developed olfactory system which is accessible to electrophysiological recordings (Velez et al., 2005, 2007). The aims of this study were three-fold. Firstly, to assess the olfactory potency of the polychaete Diopatra neapolitana in juvenile sole, using the electro-
olfactogram (EOG). Whole-body homogenate of *D. neapolitana* and fractions (hydrophobic extract or eluate and hydrophilic filtrate) obtained by solid-phase extraction (SPE; C-18 cartridges) were tested. The worm species chosen is common in the mudflats of *Ria Formosa* (Algarve, Portugal), and is commonly used as fish bait and as feed for sole broodstock. Secondly, to investigate the effect of the worm homogenate and SPE fractions (eluate and filtrate) on the swimming behaviour of sole in a flow-through tank and whether behaviour responses are mediated by olfaction. Finally, to assess whether addition of worm homogenate to commercial feed pellets enhances their ingestion by juvenile sole.

2. Materials and methods

2.1. Polychaete homogenate and solid-phase extraction

Live *D. neapolitana* caught in the mudflats of *Ria Formosa* (Algarve, Portugal) were homogenised in distilled water (100 g l$^{-1}$) and passed sequentially through 100 $\mu$m, 50 $\mu$m, 10 $\mu$m, and 1.2 $\mu$m filters (Whatman GF/C). The homogenate was then passed through solid-phase extraction (SPE) C18 cartridges (previously activated with 2 ml methanol and then washed with 2 ml bi-distilled water; IST – International Sorbent Technology, Hengoed, U.K.), and substances retained were eluted with 2 ml ethanol (eluate containing mostly non-polar hydrophobic compounds). Aliquots of the unfraccionated homogenate, eluate and filtrate were stored at −20 °C until use.

2.2. Recording of the electro-olfactogram (EOG)

The EOG was recorded, as previously described (Velez et al., 2005), in six sole (100–300 g of body weight) reared according to standard procedures (see Conceição et al., 2007 for references) at the Experimental Station of Ramalhete (Universidade do Algarve, Faro, Portugal). Briefly, after gradual adaptation to dilute seawater (12%) over several days, anaesthetized fish (intra-peritoneal injection of 300 µl100 g$^{-1}$) Saffan, Schering-Plough Animal Health, Welwyn Garden City, UK) were placed on a padded surface with aerated water pumped over the gills. The recording electrode was placed over the exposed olfactory rosette at a position that resulted in the largest response to $10^{-3}$ M l-cysteine (standard stimulus) and the reference electrode was placed lightly on the skin of the head. Standard stimulus and substances from *D. neapolitana* (unfractionated worm homogenate, and SPE eluate and filtrate fractions) were dissolved in dilute seawater (12‰); ethanol in the eluate was evaporated before dilution. The stimuli were given in a randomized order between experiments, but the same type of stimulus was presented in order of increasing concentration; at least 1 min was allowed between successive stimuli. Standard (1-cysteine $10^{-3}$ M) and “blank” stimuli (water treated in the same way except for the addition of odorant) were applied regularly. Once all stimuli had been applied to one epithelium, the fish was turned over and the same stimuli were similarly applied to the opposite epithelium. The EOG amplitude was blank-subtracted and normalized to that of the standard. Normalized EOG amplitudes $[\log_{10} (n + 1.5)]$-transformed data were compared among worm homogenate, SPE eluate and filtrate, and between the upper and lower epithelia by three-way repeated measures ANOVA. The Fisher LSD test was used to compare mean responses elicited among stimuli within each concentration and olfactory epithelium.

2.3. Behavioural assays

Juvenile sole of the same stock as above were kept in 50 l fibreglass tanks (up to 9 fish per tank) under 12 h photoperiod for at least 2 weeks before use. The tanks (of yellowish colour on the inner side) were in a semi-closed circuit of aerated seawater (18–23 °C) with hourly water renewal. Fish were fed (4–5% body weight per day) on commercial pellets (Aquasoja 2–3.5 mm, Sorgal SA, Portugal) delivered automatically (Fishmate F14, Petmate Ltd, UK) six times per day (3 meals during the light and dark phases with 3 h intervals).

Behavioural assays were carried out in a flow-through tank or fluvium made of glass (1250 × 30 × 170 mm), similar to that described by Serrano et al. (2008). The test area (450 × 320 mm) with a 120-mm deep water column was limited upstream and downstream by two plastic nets (5-mm mesh) and covered with a glass plate. Particle- and charcoal-filtered natural seawater was pumped into the fluvium forming a continuous laminar flow (12 l min$^{-1}$) through the test area which was illuminated from below with infrared light. A black and white infrared-sensitive CCD camera (Ikegami ICD 47/47E IR) fitted with a 4- to 10-mm zoom lens fitted with an infrared filter was placed above the test area and connected to a computer for video-tracking (25 frames s$^{-1}$) of position (every 2 s) and movement of single fish using the EthoVision Pro 3.1 system (Noldus Information Technology, Wageningen, The Netherlands). Data filtering was applied to separate genuine locomotion from slight body movement. To calculate mean linear velocity (cm s$^{-1}$), movement was defined as the fish moving at least two thirds of its standard length between two consecutive data points. To discriminate between the states of motion and no motion (to calculate the time in motion and number of movements), transition from no motion to motion was defined as when linear velocity exceeded one-third body length per second, whereas the reverse transition was defined as when linear velocity decreased to below one-sixth body length per second.

2.3.1. Daily activity

The activity of six fish (standard length, S.L. = 147 ± 14 mm, mean ± S.D.; weight = 47 ± 17 g placed singly in the test area was monitored continuously, unfed, for 3 days. Sole showed significantly higher frequency of movement during the dark phase (14.7 ± 2.6 h$^{-1}$, mean ± S.E. M.) than during the light phase (2.6 ± 0.8 h$^{-1}$; Student’s t test for paired samples, $t_{5} = 4.51, P < 0.01$; Fig. 1), confirming previous results in different rearing conditions (Bayarri et al., 2004). Therefore, all behavioural assays described below were carried out during the dark phase (starting 2 h after lights-off).

2.3.2. Effect of test substances on locomotion of intact and anosmic sole

Whole-body homogenate of *D. neapolitana* and its SPE fractions were diluted in seawater (collected at the inflow to the fluvium) at 1, 10 and 100 µg l$^{-1}$ in a glass reservoir. Test and control stimuli were delivered from the glass reservoir into the test area with a peristaltic pump (10 ml min$^{-1}$), via two polyvinyl chloride (PVC) tubes (7 cm

Fig. 1. Daily activity of sole (number of movements per hour, mean ± S.E.M., N = 6) measured in a fluvium over 3 days under 12 h light:dark cycle; filled horizontal bar indicate dark phase.
Six groups of six tagged fish were kept in flow-through tanks (50 × 50 cm) with 40 l of seawater (20–22 °C), aeration, and 11 h photoperiod. During 7 days before the experiment, fish were handfed one meal (2% of fish weight in each tank) consisting of pellets without Ballotini microbeads, at 0.5 h after lights-off; a second identical meal was given during the light phase (3 h after lights-on). During this period, all fish were manipulated as they would be during the experiment (see below) to create habituation and reduce acute handling stress. In the three following days, feed pellets with microbeads were given to all fish starting at 0.5 h after lights-off. In each of the three experimental days, the water inlet of a tank was closed and feed pellets were given by hand. Fifteen minutes later, all fish in the tank were anaesthetized in phenoxyethanol (400 ppm) and X-radiographies were taken to visualize and count the number of ingested microbeads; the procedure was repeated for each tank every 15 min. Three groups of fish received treated feed pellets and the other three groups received control feed pellets sprayed with distilled water; the concentration of test homogenate in the tanks was estimated to be 1 mg l⁻¹ (assuming simultaneous immediate dilution from the pellets). The size and weight of fish were similar between treatment (N = 18) and control (N = 18) groups (treatment: S.L. = 176 ± 6 mm, mean ± S.D., weight = 85 ± 7 g; control: S.L. = 177 ± 6 mm, weight = 85 ± 2 g). To convert number of microbeads ingested by the fish to weight of ingested feed, X-ray photographs of known feed pellet weights containing microbeads were taken and a regression line was calculated: pellets weight = 0.0118 × number of microbeads (R² = 0.958; N = 160; P < 0.05). Percentage of feed intake for each fish was normalized to its own weight [% feed intake = ingested food (g)/fish weight⁻¹ (g) × 100] and averaged per day. The Student’s t test (one-sided) was used on arcsin-transformed data to compare percentage feed intake between treatment and control fish. One fish in the treatment group did not ingest any pellets and, therefore, was excluded from the analysis.

3. Results

3.1. EOG responses to worm substances

The form of the EOG recorded from upper and lower epithelia was very similar and typical of fish EOGs in general (Fig. 2A). The worm homogenate and its SPE filtrate were potent olfactory stimuli evoking olfactory responses down to 0.1 mg ml⁻¹ in both olfactory epithelia (Fig. 2B and C); the filtrate evoked as large EOG responses as the homogenate except for the highest concentration at which the EOG responses were smaller than those evoked by the homogenate. The EOG responses of both epithelia to each concentration of the SPE eluate were less than those evoked by both the homogenate and filtrate; only at 10 mg ml⁻¹ did the EOG amplitudes evoked by the eluate accounted for any fraction of the olfactory potency of the homogenate (Fig. 2B and C). The EOG amplitudes evoked by the homogenate, filtrate and eluate in each olfactory epithelium were not different between the two epithelia at any concentration (univariate F tests: homogenate upper vs. lower epithelium, F₁,₅ = 0.831, P = 0.404; filtrate upper vs. lower epithelium, F₁,₅ = 1.038; P = 0.355; eluate upper vs. lower epithelium, F₁,₅ = 1.151; P = 0.332).

3.2. Behavioural responses to worm odour

Sole swam throughout the test area of the fluviumarium, mostly close to the bottom, sometimes with movements oriented towards the nets limiting the test area upstream and downstream. Ablation of the olfactory epithelia clearly affected the behavioural responses to the worm homogenate. Both intact and sham-operated fish swam for longer towards the upstream net in the test area with increasing concentration of worm homogenate; this was not seen in anosmic
fish. At 100 µg l⁻¹ homogenate both intact and sham-operated fish swam upstream for longer than anosmic fish (Fig. 3A). In contrast, the time swimming towards the downstream net (Fig. 3B) was not significantly different among intact, sham-operated and anosmic fish ($F_{2,24} = 0.691, P = 0.511$) at any concentration ($F_{2,48} = 2.552, P = 0.089$). Also, both for intact and sham-operated fish locomotion parameter values increased with increasing concentration of worm homogenate, whereas locomotion of anosmic was not affected by worm homogenate; at 100 µg l⁻¹ both intact and sham-operated fish moved more frequently, spent more time moving with higher linear velocity and, consequently, travelled longer distance than anosmic fish (Fig. 4A–D).

**Fig. 2.** Electro-olfactograms (EOGs) recorded from the sole. (A) Typical EOGs recorded from the upper olfactory epithelium in response to (horizontal bars) $10^{-3}$ M l-cysteine, and *D. neapolitana* whole-body homogenate and corresponding C18 SPE fractions (filtrate and eluate) at 1 mg ml⁻¹. (B and C) Semi-logarithmic plots of normalized EOG amplitudes (mean ± S.E.M., N = 6) in upper (B) and lower (C) olfactory epithelium in response to *D. neapolitana* whole-body homogenate (filled circles) and corresponding C18 SPE fractions (filtrate: open circles; eluate: filled triangles). Different letters indicate significant differences among stimuli within each concentration (LSD test, $P < 0.001$).

**Fig. 3.** Time (mean ± S.E.M.) that intact (open bars, N = 9), sham-operated (right-oriented trace pattern, N = 9), and anosmic (left-oriented trace pattern, N = 9) sole spent in upstream (A) and downstream (B) swim in the fluviarium, tested with control water or *D. neapolitana* whole-body homogenate at 10 and 100 µg l⁻¹. Fish were observed for 10 min. $P$ values above bar groups indicate the probability of non-significant differences between the homogenate and filtrate together and the eluate (univariate $F_{1,24}$ test for planned comparisons).
Increasing the concentration of both worm homogenate and its SPE filtrate caused an increase of time swimming towards the upstream net in intact fish, whereas this was not seen for the SPE eluate; at 100 µg l⁻¹ of both the homogenate and SPE filtrate, fish swam upstream for longer than at the same concentration of the eluate (Fig. 5A). No significant difference was found between the SPE eluate across concentrations and both control stimuli (ethanol: 22.3 ± 4.0 s, mean ± S.E.M.; distilled water: 22.5 ± 3.7 s; \( F_{1,36} = 0.402, P = 0.530 \)). In contrast, the time swimming towards the downstream net (Fig. 5B) was not affected by any stimuli (\( F_{4,36} = 0.319, P = 0.863 \)).

Fig. 4. Locomotion parameters (mean ± S.E.M.) of intact (open bars, \( N = 9 \)), sham-operated (right-oriented trace pattern, \( N = 9 \)) and anosmic (left-oriented trace pattern, \( N = 9 \)) sole in the fluviarium, tested with control water or D. neapolitana whole-body homogenate at 10 and 100 µg l⁻¹; (A) number of movements, (B) time in motion, (C) linear velocity and (D) distance travelled. Fish were observed for 10 min. P values above the bar groups indicate probability of non-significant differences between the intact and sham-operated together and the anosmic fish (univariate \( F_{3,32} \) test for planned comparisons).

Fig. 5. Time (mean ± S.E.M.) that sole spent in upstream (A) and downstream (B) swim in the fluviarium, tested with D. neapolitana whole-body homogenate (open bars, \( N = 9 \)) and corresponding C18 SPE filtrate (right-oriented trace pattern, \( N = 8 \)) or C18 SPE eluate (left-oriented trace pattern, \( N = 7 \)) at 1, 10 and 100 µg l⁻¹. Fish were observed for 10 min. P values above bar groups indicate probability of non-significant differences between the homogenate and filtrate together and the eluate (univariate \( F_{3,28} \) test for planned comparisons).
at any concentration \((F_{2,72} = 0.580, P = 0.562)\). Also, the parameters describing fish locomotion were affected by both worm homogenate and SPE filtrate but not by the SPE eluate. Increasing concentration of both homogenate and SPE filtrate induced more frequent movements, more time spent moving with higher linear velocity and, consequently, longer distance travelled, whereas increasing concentration of the SPE eluate did not affect fish locomotion; at 100 \(\mu\text{g l}^{-1}\) all locomotion parameters but linear velocity were of significantly higher value for both worm homogenate and SPE filtrate than for the SPE eluate (Fig. 6A–D). Fish locomotion parameters in control experiments (ethanol or distilled water) were not different from those seen for the SPE eluate (Table 1).

In the short-term ingestion assay carried out, sole ingested significantly more feed sprayed with the \(D.\ neapolitana\) homogenate than control feed (Fig. 7).

### Table 1

Locomotion parameters \((\text{mean} \pm \text{S.E.M.})\) of sole tested with distilled water or ethanol in the fluvium (control experiments).

<table>
<thead>
<tr>
<th>Locomotion parameter</th>
<th>Control stimuli</th>
<th>Univariate (F_{1,36}) test(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Distilled water</td>
<td>Ethanol ((N=9))</td>
</tr>
<tr>
<td>Number of movements</td>
<td>(77 \pm 11)</td>
<td>(56 \pm 0.7)</td>
</tr>
<tr>
<td>Time in motion (s)</td>
<td>(49.2 \pm 6.6)</td>
<td>(47.9 \pm 7.4)</td>
</tr>
<tr>
<td>Linear velocity (cm s(^{-1}))</td>
<td>(5.9 \pm 0.7)</td>
<td>(6.8 \pm 0.5)</td>
</tr>
<tr>
<td>Distance travelled (cm)</td>
<td>(267.7 \pm 38.8)</td>
<td>(295.6 \pm 39.9)</td>
</tr>
</tbody>
</table>

Statistical comparison with locomotion parameters with SPE eluate of \(D.\ neapolitana\) whole-body homogenate is given by univariate \(F\) test values and corresponding \(P\) values.

\(^a\) Planned comparison between SPE eluate across concentrations and both control stimuli.

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4. Discussion

Olfactory cues from *D. neapolitana*, especially hydrophobic substances, enhanced swimming behaviour of sole juveniles and may be related to the enhanced ingestion of dry-feed flavoured with worm homogenate observed in a short-term ingestion assay. This is supported by three lines of evidence in this study. Firstly, the hydrophobic fraction (SPE filtrate) accounted for the majority of the olfactory potency of *D. neapolitana* whole odour (homogenate) and both the homogenate and filtrate, but not the eluate (mostly composed of hydrophobic substances), enhanced motion frequency and time, mean linear velocity and distance travelled of sole. Moreover, upstream swimming of sole towards the odour source was also increased by both the homogenate and filtrate. These results suggest that hydrophilic *D. neapolitana* odour(s) intensify sole search behaviour and, ultimately, attract them to their prey. Secondly, such behavioural responses of sole to whole-worm odour were disrupted by ablation of their olfactory epithelia, demonstrating that olfaction is important in their food-search behaviour. Finally, addition of *D. neapolitana* homogenate to commercial feed pellets increased pellet ingestion. Olfactory cues are likely responsible for the higher consumption of worm flavoured feed pellets, although other chemical senses may also be involved in this response. This can be clarified with similar tests using anosmic fish.

The odours of *D. neapolitana* responsible for the sole search behaviour have not yet been identified. However, molecular size filtration and high pressure liquid chromatography of the hydrophilic fraction of whole-body worm homogenate together with olfactory potency assessment of chromatographic fractions by EOG, showed that the vast majority of the odours are hydrophilic compounds smaller than 500 Da, including amino acids (data not shown). Furthermore, in another study (Velez et al., 2007) using the polychaete *Hedistes diversicolor*, an artificial mixture of amino acids identified in whole-body homogenate and its SPE (C18) filtrate matched the olfactory potency of both the homogenate and its filtrate, suggesting that amino acids account for the vast majority of olfactory potency. Other types of odours, however, may also be used by sole in pre-searching, since living *H. diversicolor* release small molecular weight odorants (<500 Da) extractable by C18 SPE cartridges (Velez et al., 2007). Whether living *D. neapolitana* also release odorants other than amino acids important for chemosensory food location by sole remains to be investigated. Possibly, substances released by living polychaetes may act as olfactory attractants mediating site location (arousal and search), whereas at closer distance from the prey additional compounds including amino acids (which would be released in higher quantities by injured worms) mediate the final stages of sole feeding behaviour (i.e. local search, uptake and ingestion) through olfaction and/or other chemosensory mechanisms (e.g. taste receptors).

Supplementation of artificial feeds with feeding stimulants may enhance consumption and more so if such stimulants are derived from the natural diet of each fish species. Several studies have focused on the identification of feeding stimulants in fish (Adron and Mackie, 1978; Carr et al., 1996; Kubitza et al., 1997; Mackie et al., 1980; Papatriphon and Soares, 2000, 2001, 2002; Reig et al., 2003); however, most used commercially available chemicals without consideration of the natural feeding stimulants for each fish species. In different species, feeding behaviour is triggered by different chemical substances acting via olfaction and/or taste (Hara, 1994). Amino acids, nucleotides, nucleosides and quaternary ammonium bases have all been identified as feeding stimulants from experiments with juveniles of different species (Jones, 1992; Takeda and Takii, 1992). For example, glycine betaine, trimethylglycine and dimethylthetin have been reported as feeding stimulants for juvenile Dover sole (Mackie and Mitchell, 1982; Mackie et al., 1980). However, there appears to exist species specificity for feeding stimulants and, in general, mixtures are more effective than single compounds (Jobling, 1995; Jones, 1992).

In the present study flavouring feed pellets with *D. neapolitana* homogenate had at least a short-term positive effect on feed- ingestion, possibly involving perception of prey-related odors acting as attractants. Accordingly, we suggest that further progress in finding putative feeding stimulants for sole should aim at the chemical identification of odors from preys and subsequent evaluation of whether flavouring of dry feed with a mimic of prey odour (wherein the relative composition of odors may be important) has a sustained positive effect in feed consumption. In conclusion, the current study has shown that olfaction plays an important role in sole food-search behaviour, and that the hydrophobic fraction of *D. neapolitana* whole-body homogenate contains key substances affecting this behaviour. Further studies should aim at the chemical identification of prey odorants and assessment of their effects on intake, growth and feed conversion rate.

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