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Electroencephalogram recordings from the olfactory bulb of juvenile (0 year) Atlantic cod in response to amino acids

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Olfactory sensitivity of juvenile (0 year) Atlantic cod *Gadus morhua* to 20 L-amino acids was studied by recording electroencephalograms (EEG) from the olfactory bulb. Leucine, methionine, asparagine, glutamine, alanine and threonine were highly stimulatory; proline, phenylalanine, aspartic acid and tryptophan were the least stimulatory. Threshold concentrations determined for four amino acids were 10^{-8} M for alanine, 10^{-7} M for arginine and leucine and 10^{-6} M for glutamic acid.

Key words: amino acids; concentration-response relations; EEG; gadoids; olfaction; relative stimulatory effectiveness.

Atlantic cod *Gadus morhua* L. feed on prey belonging to many taxonomic groups, dominated by copepods during their early life stages, and decapods and fishes as adults (Mattson, 1990; Bromley *et al*., 1997). They feed throughout the day, including at low light intensities and in the dark (Bromley *et al*., 1997; Løkkeborg, 1998). Løkkeborg (1998) suggested that Atlantic cod locate prey using vision during the day and chemoreception at night. Klemesten (1982) noted that Atlantic cod consumed as much prey during the darkness of polar winter as they did during other periods of the year, suggesting that sensory modalities other than vision (*i.e*. mechano-reception and chemoreception) are involved in prey detection (Janssen, 2004). In a telemetric field study, Løkkeborg (1998) observed that Atlantic cod oriented towards fish bait located as far as 698 m away, mostly when they encountered the bait's odour plume. Further, Atlantic cod swam upstream towards squid *Loligo* sp. extracts introduced into a flume (Pawson, 1977), and shrimp *Pandalus borealis* extract induced benthic-oriented search behaviour (Ellingsen & Døving, 1986). Finally, feeding behaviour can be released in Atlantic cod by electrically stimulating the olfactory tract (Døving & Selset, 1980). Taken together, these studies indicate the possible

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role of the olfactory organs in the detection of prey-related chemical stimuli by Atlantic cod.

Electrophysiological techniques have been applied to characterize the olfactory sensitivity and specificity of several fish species (Sutterlin & Sutterlin, 1971; Hara, 1975; Goh & Tamura, 1980; Silver, 1982; Kobayashi & Fujiwara, 1987; Johnsen *et al.*, 1988; Ishida & Kobayashi, 1992). In some cases, the specific receptor types that underlie these responses have also been identified (Hara, 1992). Although still relatively sparse, these studies provide a baseline against which the olfactory sensitivity of other fishes, differing in phylogenetic origin, feeding niche and foraging behaviour, can be compared. Studies of olfactory responses in adult marine fishes are rare (Hara, 1992) and virtually nonexistent for small 0 year juveniles, despite this being a critical period of the life history during which feeding habits change rapidly. Further, as far as is known, the sensitivity of the olfactory organs to individual amino acids has never been reported in any gadid species, of any age. In the present study, the sensitivity of small juvenile (0 year) Atlantic cod to 20 amino acids was assessed by recording electroencephalograms (EEG) from the olfactory bulb (OB).

Experiments were performed on 25 juvenile Atlantic cod between February and May 2003. Fish were obtained from the hatchery at the Institute of Marine Research-Austevoll, Norway. The fish were reared in 1500 l tanks at 8–12° C, and were fed with a commercial dry diet using automatic feeders. Individuals of 6–8 cm total length (L_T) (8–11 g) were used because it is at approximately this size that Atlantic cod juveniles migrate to deeper water and their diet changes from zooplankton to macro-invertebrates and fishes (Bromley *et al.*, 1997).

The Atlantic cod tested were anaesthetized with an intraperitoneal injection of alphaxolone-alphadolone (Saffan; 12 mg kg^{-1} body mass). Once anaesthesia was achieved (as judged by the absence of opercular ventilatory movement), fish were immobilized using pancuronium bromide (0·6 mg kg^{-1} body mass). The specimen was then secured on a block of plastic clay, molded to the shape of the fish, and placed in a flow-through plastic tray. The gills were perfused through the mouth with filtered sea water; the water used for this was drawn from the same source as that which supplied the rearing units in which juveniles were maintained. Perfusion water was held in a header tank at 10° C. The protocol used in this preparation adhered to current animal care guidelines as set out by the European Commission, and were approved by the Institute of Marine Research animal welfare oversight committee.

Juvenile Atlantic cod have a very well-developed oval olfactory rosette with numerous lamellae. It is connected to the OB by a short olfactory nerve. Given the small size of the test fish, it was necessary to prevent sea water from spilling into the cranial cavity. This was accomplished by filling the cavity with mineral oil, which provided sufficient isolation of the recording site from the sea water bathing the olfactory rosette. To access the OB, a small patch of skin, connective tissue and a portion of the underlying cranium were removed from the region immediately posterior to the nares and the cranial fluid surrounding the OB was gently aspirated out. The EEG was recorded differentially using teflon-insulated silver wire electrodes, 0·13 mm in diameter (Stoelting Co., Wood Dale, IL, U.S.A.). The recording electrode was placed on the OB and the reference electrode on the cranial muscles. After placing the electrodes, the

cranial cavity was filled with mineral oil. The fish were grounded through a stainless steel electrode inserted into the flank musculature. In each preparation, the recording electrode was positioned in a location at which the response to a standard chemical (L-alanine) was maximum; this was the dorso-posterior surface of the OB in all preparations.

The EEG signals were amplified with an AC preamplifier (P55; Astro-Med, Inc., West Warwick, RI, U.S.A.) under a band-pass filter setting of 3–300 Hz. The amplifier output was monitored on an oscilloscope (Tektronix), digitized (National Instruments A/D board) and saved on a PC using custom software (JASCO Research, Victoria, British Columbia, Canada). The induced EEG responses were integrated (time constant = 0·5 s) using Chart software (AD Instruments Pty Ltd., Castle Hill, Australia) and their amplitude was measured as the maximum of the integrated trace, in mV. (see Fig. 1 for an example of an integrated trace).

The skin covering the nares was removed and the olfactory epithelium was perfused with natural sea water at a constant rate of 4·0 ml min^{-1}; the stimulus,

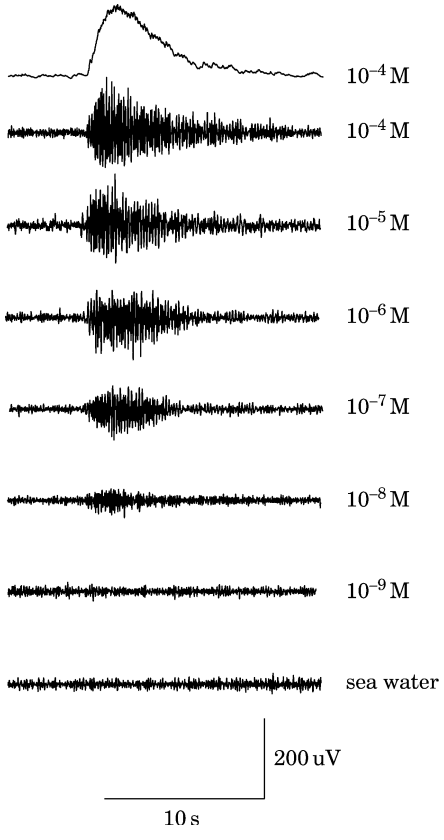

FIG. 1. A series of EEG responses to logarithmically increasing concentrations of L-alanine in juvenile *Gadus morhua*. The response threshold is between 10^{-9} and 10^{-8} M. The upper trace for the concentration 10^{-4} M is the integrated form of the lower (neural) trace. The magnitude of responses was measured as the maximum of integrated trace.

dissolved in the same water, was added to this flow using a three-way solenoid valve, controlled by an electronic time switch. For stimulation, the valve was opened for 3 s, delivering a stimulus volume of 0·5 ml. Photometric dye tests showed that the stimulus arrived at the olfactory epithelium 5 s after the activation of the valve, reached a peak concentration of 40% of that applied by 7 s, and decayed to a baseline level after 12 s. The concentrations reported have not been corrected for dilution. An inter-stimulus interval of at least 120 s was allowed, by which time responses to successive stimulations were completely recovered from adaptation effects. The temperatures of the perfusing water and stimuli were identical.

The EEG responses to 20 amino acids were recorded at 10^{-4} M. Pure sea water was used as a control. Stock solutions of 10^{-2} M stimulants (Sigma-Aldrich, Schnelldorf, Germany) were prepared weekly and stored in a refrigerator. They were diluted as necessary on the day of testing. Each chemical was tested at least twice (thrice in most cases) in the same preparation. After each amino acid, response to a standard (10^{-4} M L-alanine) was tested to ensure that the preparation remained stable. Controls were undertaken in between amino acid presentations to eliminate the possibility of cross contamination. Response magnitude of each chemical from all fish were averaged and expressed as a percentage of that of 10^{-4} M L-alanine (relative stimulatory effectiveness; RSE). Thresholds (concentration necessary to produce an integrated response greater than that of control) for four amino acids namely L-alanine (short side chain), L-leucine (long side chain), L-arginine (basic) and L-glutamic (acidic) were also determined. These groups of amino acids are known to react with different receptor types (Hara, 1992).

Application of the seawater control to the olfactory epithelium produced no responses, but the amino acid solutions induced high amplitude rhythmic oscillatory responses in the OB (Fig. 1). Integrated responses showed that it took *c.* 2 s to reach maximum response from the base line and that the responses gradually declined back to the base line within *c.* 10 s (Fig. 1). The magnitude of responses varied depending upon the quality and concentration of the stimulus. The responses to the test stimulants, measured as the maximum of the integrated traces and expressed as RSE, are shown in Fig. 2. Based upon the RSE, amino acids could be generally grouped as follows: highly sensitive (RSE *c.* 133–100%; leucine, methionine, asparagine, glutamine, alanine and threonine); least sensitive (RSE <50%; proline, phenylalanine, aspartic acid and tryptophan); moderately sensitive (RSE *c.* 60–90%; rest of the amino acids). Threshold levels for the response were determined by studying the concentration-response (C-R) characteristics. Fig. 1 presents an example of typical EEG responses to alanine from 10^{-9} to 10^{-4} M. The thresholds were located between the following concentrations: 10^{-9}–10^{-8} M for alanine, 10^{-8}–10^{-7} M for arginine and leucine and 10^{-7}–10^{-6} M for glutamic acid (Fig. 3). Responses to all four amino acids continued to increase at supra-threshold stimulus concentrations. The slope of the C-R function for leucine increased more steeply than that for the three other amino acids and saturated at 10^{-3} M. In one preparation, responses to 10^{-2} M leucine were smaller than those at 10^{-3} M (Fig. 3).

In characterizing the sensitivity of fishes to odours, EEGs compare very well with other techniques such as electro-olfactograms (EOG), olfactory nerve twig

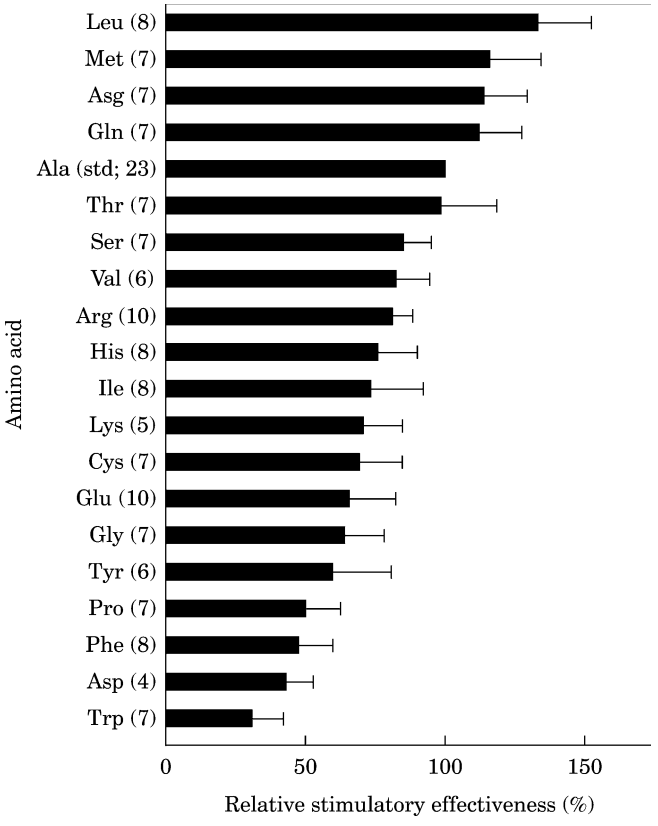

FIG. 2. Relative stimulatory effectiveness of 20 amino acids [Leu, leucine; Met, methionine; Asg, asparagine; Gln, glutamine; Ala, alanine; Thr, threonine; Ser, serine; Val, valine; Arg, arginine; His, histidine; Ile, isoleucine; Lys, lysine; Cys, cysteine; Glu, glutamic acid; Gly, glycine; Tyr, tyrosine; Pro, proline; Phe, phenylalanine; Asp, aspartic acid; Trp, tryptophan (all L-isomers)] at 10^{-4} M in eliciting EEG responses in juvenile *Gadus morhua*. The values + S.D. are expressed as a percentage of the responses to alanine (standard). The number of fish tested is given in parenthesis.

recordings (NTR) and mucosal neural recording (MNR) (Silver, 1982; Kobayashi & Goh, 1985). The neural inputs from different classes of stimuli (*e.g.* bile acids, nucleotides and amino acids) are mapped onto discrete areas of the OB, with possibly even finer-scale mapping within each class of stimuli (Nikonov & Caprio, 2001). Such chemotopy may result in recording area-specific responsiveness (to different classes of stimuli) within the OB. While this may be the case when making single-unit recordings from the OB, the multi-unit EEGs reported here (recorded from amino acid sensitive zone of OB and expressed as RSEs) are indicative of the gross responsiveness of olfactory epithelium to a given amino acid. Combined with C-Rs, the RSE has been used to determine olfactory sensitivity in most of the electrophysiological studies so far conducted on fishes.

Of the chemicals that fish olfactory receptor neurons are capable of detecting, those associated with feeding are mostly the amino acids (Carr, 1982). Hence, amino acids, particularly naturally occurring L-enantiomers were tested. Structure-activity studies demonstrate that the most stimulatory amino acids are linear

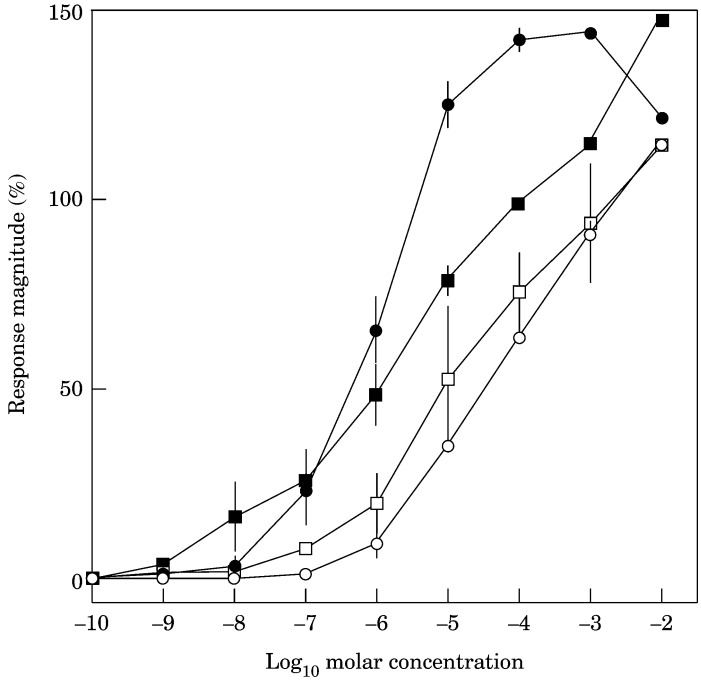

FIG. 3. The concentration and EEG response relationship for four amino acids [leucine (●), alanine (■), arginine (□) and glutamic acid (○)] in juvenile *Gadus morhua*. The response magnitudes ± S.D. ($n = 3$) are expressed as a percentage of responses to 10^{-4} M alanine.

L-amino acids with three to six carbon atoms and an uncharged side chain (Hara, 1975; Silver, 1982). The present study is in general agreement with this: neutral amino acids with different numbers of carbon atoms such as leucine, methionine, asparagine, glutamine, alanine and threonine elicited the strongest responses. Among the charged amino acids, those that are positively charged (basic: arginine, histidine and lysine) were only moderately effective and those that are negatively charged (acidic: aspartic and glutamic acids) were even less effective. Aromatic amino acids such as tryptophan and phenylalanine were the least effective odorants tested.

Generally, RSE of amino acids in eliciting electrical olfactory responses is similar across different species. Nevertheless, there are some species-specific differences: at 10^{-4} M, RSE of arginine and cystine in grass carp *Ctenopharyngodon idella* (Valenciennes) (Johnsen *et al.*, 1988) were 2·7 and 2·0 times higher respectively than those in rainbow trout *Oncorhynchus mykiss* (Walbaum) (Hara, 1975). Caprio (1980) noted that the EOG response of the marine catfish *Arius felis* (L.) to L-glutamine was only 86% of that of alanine, while it was 137% of that in the freshwater catfish, *Ictalurus serracanthus* Yerger & Relyea. In this respect, it is noteworthy that the RSE of leucine in Atlantic cod is just 1·2 times higher than in rainbow trout (Hara, 1975), and as much as 1·9 times higher than in rabbitfish *Siganus fuscescens* (Houttuyn) (Ishida & Kobayashi, 1992); all of these data were obtained from bulbar EEGs using 10^{-4} M presentations of the stimulus. The reasons for such differences are not

currently known, but may be associated with species specificity in diet, or in social, agonistic and reproductive behaviours.

The detection thresholds for amino acids in Atlantic cod (10^{-8} to 10^{-6} M) are comparable to those for other fishes (Hara, 1992). Johnstone (1980), however, reported that the threshold for leucine was $2{\cdot}05 \times 10^{-5}$ M in adult Atlantic cod, and it was the amino acid with the highest threshold among the eight amino acids tested. In contrast, in the present study the threshold for leucine was at 10^{-7} M (Fig. 3). The basis for this inconsistency is unclear, although it may be related to methodological differences and to the age of the test fish: Johnstone (1980) studied adult Atlantic cod (29–39 cm L_T) using heart rate conditioning, whereas the present electrophysiological study targeted 0 year juveniles 6–8 cm L_T. Although limited, there is evidence of age and life-history stage-related changes in olfactory sensitivity. In an EOG study on European eel *Anguilla anguilla* (L.), Crnjar *et al.* (1992) observed that leucine was significantly more stimulatory to elver stage animals than to glass eels. Further, when comparing their results on juvenile eels to those of Silver (1982) on adults, these authors also noted that juveniles were much more sensitive to asparagine than were adults. Additional studies on possible age-related changes in olfactory sensitivity at different life stages would be useful.

In addition to RSE and threshold values, the range of concentrations over which olfactory neural responses increase appears to differ amongst amino acids and is probably related to their molecular structure (Ishida & Kobayashi, 1992). In this study, the range of concentrations over which the four amino acids tested responded was (in $\log_{10}$ units): seven for alanine, six for arginine, five for glutamic acid and four for leucine (Fig. 3). The dynamic range of C-R may be crucial for fish in detecting concentration gradients and navigating towards the source of chemicals.

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