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Extracellular Multi-Unit Recording from the Olfactory Nerve of Teleosts

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Dr Xiaoyan Cao
Review Editor - Chemistry | Biochemistry
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Dear Dr Cao,

Thank you very much for your recent communication about our manuscript (JoVE60962R1) submitted for publication in the *Journal of Visualized Experiments*. We have followed the suggestions given on the manuscript, and detailed replies are also given on the manuscript. Nevertheless, we have also included a brief description in the "Replies to Editorial comments"

Thank you very much for your consideration. We look forward to hearing the editorial decision in due course.

Yours sincerely,

Two handwritten signatures in blue ink are shown. The first signature on the left is "Peter Hubbard" and the second signature on the right is "Zélia Velez".

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TITLE:**Extracellular Multi-Unit Recording from the Olfactory Nerve of Teleosts****AUTHORS AND AFFILIATIONS:**Peter C. Hubbard¹, Zélia Velez¹¹Centro de Ciências do Mar (CCMAR), Universidade do Algarve, Campus de Gambelas, Faro, Portugal**Corresponding Author:**

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KEYWORDS:

olfaction, sensitivity, nerve, fish, acidification, amino acid, electrophysiology, extracellular

SUMMARY:

Extracellular multi-unit recording from the olfactory nerve is a sensitive, robust and reproducible method for assessing olfactory sensitivity in marine fish. It records primary sensory input and is independent of external salinity.

ABSTRACT:

Recent studies have shown that ocean acidification affects olfactory-driven behavior in fish. This may be due in part to a reduction in olfactory sensitivity in high PCO_2 /low pH water. To assess the effects of ocean acidification, or olfactory sensitivity in marine fish in general, we propose that extracellular multi-unit recording from the olfactory nerve is the method of choice. Although invasive, it is sensitive, robust, reproducible and independent of external salinity (unlike the electro-olfactogram [EOG], for example). Furthermore, it records a primary sensory input into the CNS, prior to any central processing. We show that this method can show a reduction in olfactory sensitivity that is both temporary and odorant-dependent, using a range of amino acids to construct concentration-response curves and calculate the thresholds of detection.

INTRODUCTION:

Fish rely heavily on olfaction for many aspects of their lives including finding food, avoiding predators, assessing potential mates and migration, among others¹⁻³. Therefore, assessing olfactory sensitivity in fish (What do they smell? How sensitive are they to these compounds?) is vital to fully understand these processes. Furthermore, anthropogenic effects on the environment, such as ocean acidification and pollution, may have profound effects on the olfactory system, even at sublethal levels, because it is necessarily in intimate contact with the surrounding water⁴. In vivo electrophysiology is the experimental approach of choice to assess olfactory sensitivity in fish. Three main techniques are available: the electro-olfactogram (EOG), the electro-encephalogram (EEG) recorded from the olfactory bulb, and multi-unit recording

from the olfactory nerve⁵.

The EOG is the most widely used of these three⁶. It is a direct current (DC) field potential recorded above the olfactory epithelium and is believed to be the summed generator potentials of those olfactory receptor neurons (ORNs) responding to a given odorant. However, as it is recorded in the water, rather than inside the fish, the amplitude of response is not only dependent on the signal generated by the fish, but also on the conductivity of the surrounding water; the higher the conductivity (or the lower the resistance), the lower will be the amplitude. This may mean that the EOG is a less sensitive method in seawater than freshwater⁷.

The EEG recorded from the olfactory bulb is also widely used in investigation of olfaction in fish. However, the olfactory bulb is the first-order processing center for olfactory sensory input⁸; it is highly organized into glomeruli, and consequently the response recorded depends heavily on the position of the recording electrodes. For example, the input from ORNs detecting amino acids is processed by glomeruli in the lateral region of the olfactory bulbs, whereas that from conspecific-derived chemicals is directed to the medial region⁹⁻¹². Pheromonal input may be directed to highly localized glomeruli within the olfactory bulb. Depending also on the anatomy of the species in question, the ideal recording position for a given odorant may not be easily accessible.

Multi-unit recording from the olfactory nerve circumvents the main problems with the EOG and EEG outlined above. As it records action potentials passing down the axons of the ORNs from the epithelium to the bulb, it is a primary sensory signal. And as it is recorded inside the fish, the amplitude of response is independent of external salinity. Nevertheless, of course it has some disadvantages. Firstly, depending on the anatomy of the species, more extensive surgery is required to expose the olfactory nerve than for the EOG. Secondly, because the signal is smaller than the EOG, it requires slightly more sophisticated, and therefore expensive, equipment. A general description of other experimental approaches is given by John Caprio⁵. The aim of this article is to outline how to record extracellular multi-unit responses from the olfactory nerve of the seabream (*Sparus aurata*) in vivo to amino acid odorants as an example of this technique, and how to identify, and overcome, some of the more common problems encountered in such an experiment.

PROTOCOL:

Animal maintenance and experimentation was carried out in certified experimental facilities and followed Portuguese national legislation (DL 113/2013) under a 'group-1' license by the Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal. As this protocol involves animal handling, it has to be approved by the local and/or national body that regulates welfare of animals used in scientific experiments, in addition researchers need to have the appropriate training and licenses to carry out such procedures.

1. Stimulus preparation

NOTE: Most fish have highly sensitive olfactory system, thus great care must be taken when

preparing the olfactory stimuli to be used in the experiment. The glassware used to make up the stimuli should be washed in 5% bleach (sodium hypochlorite), rinsed thoroughly with tap water and dried. Immediately prior to use rinse the glassware thoroughly with seawater (the same water used to make the stimulus dilutions). Take care that none of this water comes into contact with bare skin.

1.1. Make 100 mL of 10^{-2} M L-glutamine, L-leucine and L-serine; store 1 mL aliquots of each at -20 °C until use.

1.2. On the day of the experiment, prepare from these aliquots, 10^{-3} M to 10^{-7} M solutions (in steps of x10 dilution) using both control and high CO₂ seawater.

NOTE: L-serine (10^{-3} M) will be used as a positive control, or standard. Water used to make up the dilutions of the stimuli, and treated in exactly the same way as the stimuli but without addition of any odorant, will be used as the negative control or blank.

2. Preparation of control and high CO₂ water

2.1. Prepare control water by collecting 1 L of charcoal-filtered seawater.

2.1.1. Using a pH probe, check the pH; it should be around 8.2. If not, bubble with atmospheric air until this pH is reached.

2.1.2. Using an alkalinity titrator measure the alkalinity of the water.

2.1.3. Measure the water temperature and salinity.

2.2. Prepare high CO₂ water by filtering 1 L of sea water, then bubble CO₂ until the desired pH is reached.

2.2.1. Using a pH probe, check the pH; it should be around 7.7.

2.2.2. Using an alkalinity titrator, measure the alkalinity of the water.

2.2.3. Measure the water temperature and salinity.

2.3. Determine CO₂ pressure in both control and high CO₂ water using a software designed to calculate CO₂ parameters in water (e.g., CO2Calc software¹³).

2.3.1. In the **input** window add the values of water pH, temperature, salinity and total alkalinity (**Figure 1**).

2.3.2. Select the constants, units and scales (see the recommended values in **Figure 2**).

2.3.3. Press the button **process** to determine CO₂ pressure.

NOTE: **Figure 3** shows an example of a result sheet.

3. Preparation of the fish

NOTE: A seabream of 200–400 g is used in this protocol.

3.1. Anesthetize the fish by immersion in aerated natural seawater containing MS222 (ethyl-3-aminobenzoate methanesulfonate salt). When response to a tail pinch has stopped, inject into the flank muscle the neuromuscular blocker gallamine triethiodide (10 mg·kg⁻¹ in physiological saline).

NOTE: The concentration of anesthetic used varies among species; for a seabream of 200–400 g, use 200 mg·L⁻¹ buffered with 400 mg·L⁻¹ NaHCO₃.

3.2. Place the anesthetized fish on a cushioned support. The exact shape and size depend on the model species; for seabream (200–400 g), use a padded V-shaped support, made in house.

3.3. Place a silicon tube (diameter = 10 mm) in the mouth, connect the tube to a submersible pump in a reservoir of anesthetic-containing, aerated seawater, and pump water over the gills at ~100 mL·min⁻¹·kg⁻¹.

NOTE: The size of the silicon tube used will depend on the size of the fish.

3.4. Insert the earthing pin into the flank muscle and connect it to the head-stage of the amplifier).

3.5. Cover the fish with damp cloth (or paper towel) with only the head exposed, ensuring that the covering does not impede the exit of water from the gills.

NOTE: The eyes can be covered with pieces of damp paper/cloth or black plastic.

3.6. Position the tube of the stimulus-delivery system, i.e., the glass tube connected to a supply of background seawater, into the nostril.

NOTE: Micro-hematocrit tubes can be used (length = 75 mm, ID = 1.15 mm, OD = 1.55 mm); these can be pulled to a finer point on an electrode puller for use with smaller fish. It is important to ensure that the olfactory epithelium is kept wet during the surgery (described below).

3.7. Expose the olfactory nerves by removing the skin and bone of the skull between the eyes (the olfactory nerves usually run together between the eyes) with the aid of a dental (ideally) or hobby (e.g., Dremel) drill or jeweler's polisher (with dental drill-bits) under a dissecting microscope (inside a Faraday cage).

3.7.1. In the seabream, remove the part of the skull immediately above the eyes, with a circular saw, from just anterior to the eyes to just posterior to them. Then, using a drill bit, remove the bone between the eyes; the olfactory nerves lie between the eyes.

3.8. Once sufficient bone has been cleared, remove the fat and connective tissue overlying the nerves using fine forceps; take care to not damage the nerves or puncture any blood vessels.

NOTE: Experience will help to refine the dissection; the smaller the dissection, the more stable the preparation will be. However, sufficient tissue must be cleared; for the unexperienced, when the olfactory bulbs are just visible, clear slightly more anteriorly to expose the part of the nerves as they join the bulbs to allow the correct positioning of the electrodes.

3.9. Clean the electrodes prior to use by connecting them to negative pole of a 3V DC source (e.g., two AA batteries in series) and placing the tip in physiological saline (or seawater diluted 1:3 in freshwater) for 20–30 s; a steady stream of small bubbles should be seen coming from the tip.

3.10. Once the olfactory nerves are exposed, insert the recording electrodes (held on micro-manipulators) in a position that gives a maximal response to the standard (e.g., 10^{-3} M L-serine), and a minimal response to the blank. Use parylene-coated tungsten electrodes (**Table of Materials**) connected to the head-stage of an alternating current (AC) preamplifier.

NOTE: In the seabream, the strongest responses to amino acids are usually seen with the electrodes placed in the lateral side of the nerve, close to where it joins the olfactory bulb. This may hold true for other species, as the glomerular organization of the bulb is broadly similar among species. However, experience is always the best teacher.

4. Electrophysiological recording

NOTE: As with most electrophysiology, multi-unit recording needs to take place within a Faraday cage. However, extracellular recording does not usually require an anti-vibration table; most movement will come from the fish. Nevertheless, a strong, stable table is required with a metal surface to secure the magnetic bases of the micro-manipulator stands.

4.1. Set up a stimulus delivery system to allow the rapid switching from clean background water to stimulus-containing water, e.g., by using a solenoid-operated three-way valve. Connect the common outlet to the tube carrying water to the olfactory rosette, and place one line in a seawater reservoir and the other in the test solution.

NOTE: When the valve is switched (by passing DC current), the water flow switches from background water to that containing the odorant. The stimulus should be given for long enough to see a clear peak in the integrated response, followed by a period of accommodation; the time used in the current protocol is 4 s, but longer time may be necessary depending on the species.

4.2. Connect the valve driver to the trigger of an analogue-digital converter (e.g., Digidata); when the valve is switched from background to stimulus-containing line, this will start the recording of the data. Configure the software to start recording at the trigger event and continue for a predetermined period (e.g., 10 s).

NOTE: Ten seconds should be enough, but this can be shortened or lengthened, depending on the experimental question.

4.3. Check the stability of the preparation by testing (recording and measuring the amplitude of the integrated response) repeatedly with the standard, 10^{-3} M L-serine in this case, and allowing 1 min to elapse between successive stimuli.

NOTE: Depending to some extent on species and odorant, the responses should have an amplitude within 10% of each other (as a rule of thumb), and should have a rapid onset, rise to maximum activity, and return to baseline after the stimulus is absent (**Figure 4**).

4.4. Record the olfactory nerve responses to amino acids in control sea water (from the lowest to the highest concentration) and allow 1 min to elapse between successive stimuli.

NOTE: It is possible that, for some species and/or some odorants, more time is necessary. But for amino acids and seabream, 1 min is sufficient.

4.5. Record the response to 10^{-3} M serine and a control water blank solution.

4.6. Change the background water from control seawater to high CO_2 seawater, by placing the background line into the bottle with high CO_2 seawater.

NOTE: It is advisable to insert another hematocrit tube (or equivalent) into the end of the stimulus and background lines to avoid touching the water and ensure the end of the tube remains in the water.

4.7. Prior to testing the response of the olfactory nerve to amino acids in high CO_2 seawater, condition the olfactory epithelium with high CO_2 water by following the high CO_2 water over the olfactory epithelium for a few minutes.

NOTE: Experience has shown that, for seabream, 5 min is sufficient.

4.8. Record the olfactory nerve responses to amino acids in high CO_2 seawater (from the lowest to the highest concentration).

4.9. Record the response to a high CO_2 water blank solution.

4.10. Record the response to 10^{-3} M serine and a control water blank solution.

NOTE: The raw signal (nerve activity) should be filtered (low pass around 2,000–5,000 Hz, high pass 50–300 Hz) and passed to an analogue-digital converter (**Table of Materials**). For easier quantification of nerve activity, the raw signal can also be integrated using a leaky integrator (**Table of Materials**) and passed to the analogue-digital converter, and from there, both raw and integrated signals to a computer running the appropriate software (e.g., Axoscope).

5. Data analysis

5.1. Subtract the amplitude of the integrated response to the blank (in mV) from the amplitude of the integrated responses to all stimuli.

5.2. Normalize responses to stimuli by dividing the amplitude of the previous response to the standard (10^{-3} M serine); this reduces inter- and intra-fish variability.

5.3. Calculate the thresholds of detection by linear regression of the concentration-response curves (on a semi-logarithmic plot), according to the formula $\log(N + 1.5) = a \log C + B$, where C is the molar concentration, N is the normalized response amplitude, and a and b are constants^{7,14}.

NOTE: The threshold of detection is then the value for x where $y = 0.1761$ (i.e., $\log 1.5$; $N = 0$); the concentration above which a response will be seen (i.e., the fish can smell it). Some odorants evoke sigmoidal concentration-response curves when plotted semi-logarithmically (e.g., calcium^{15,16}; in this case, the normalized data can be fitted to a three-parameter Hill plot which will give the maximum response amplitude and the EC_{50} (i.e., [odorant] that gives half-maximum response; also a measure of sensitivity).

5.4. Compare the thresholds of detection and/or the maximum response amplitude and the EC_{50} of stimuli tested in control water and those tested in high CO_2 water.

REPRESENTATIVE RESULTS:

A typical response to the positive control (10^{-3} M L-serine; **Figure 4A**) and negative control (blank; **Figure 4B**) recorded from the olfactory nerve of a seabream is shown in **Figure 4**. In the presence of the stimulus (black horizontal bar; in the olfactory cavity, in contact with the olfactory epithelium), note the rapid increase in activity (reflected in the upward deflection of the integrated signal) to a peak within about one second of stimulus onset, followed by a period of accommodation (while the stimulus is still present), and a return to baseline activity once the stimulus has ended. The absolute amplitude of the response is highly dependent on electrode position; if a low amplitude response is recorded, try changing the electrode positions. A slower rise to peak activity may be due to the tube carrying the stimulus-containing water to the olfactory epithelium being placed too far away from the epithelium; try moving the nose-tube closer to (but not touching) the epithelium. Note that, in contrast, the blank evokes little or no response. A significant positive response (i.e., increase in activity) to the blank may indicate contamination of the water used to make the dilutions of the stimuli; making fresh dilutions with clean water (and glassware) should resolve this. If not, a more thorough cleaning of the water

system (including activated charcoal filters) may be necessary. A negative response (i.e., decrease in activity) may indicate a slight change in flow rate when the valve is switched due to, for example, a blockage in the valve.

A typical concentration-response curve (plotted semi-logarithmically), in this case to L-leucine (10^{-7} M to 10^{-3} M), is shown in **Figure 5A**. Note that increasing concentrations of the odorant evoke increasingly large increases in activity, and therefore, in the amplitude of the integrated responses. A plot of the normalized data, and their corresponding linear regression, is shown in **Figure 5B**. The estimated threshold of detection can be calculated from the value of x when $y = 0.1761$ (i.e., $\log 1.5$; where $N = 0$). In this case, this value is -7.48 ; that is, the calculated threshold for L-leucine in this fish is $10^{-7.48}$ M. The exponent α can similarly be estimated from linear regression of the normalized data on a log-log plot; $\log N = \alpha \log[\text{odorant}] + \text{constant}$. The factor γ then gives the increase in odorant concentration required to increase the response amplitude by one log unit; that is, it is an estimate of the steepness of the concentration-response curve¹⁷. In this example, $\alpha = 0.277$ and $\gamma = 3.61$; therefore, to increase the response amplitude ten-fold (i.e., one log unit; $\log 10 = 1$), the stimulus concentration needs to be increased by $10^{3.61}$ -fold (4,074-fold).

A typical sigmoidal concentration-response curve (**Figure 6A**) when plotted semi-logarithmically, in this case to L-glutamine, is shown in **Figure 6B**. A similar concentration-dependent increase in response amplitude is seen; however, at the higher concentrations, this increase becomes less so that the response amplitude reaches a maximum (N_{max}). This allows the data to be fitted to a three parameter Hill equation:

$$\frac{N}{N_{\text{max}}} = \frac{[\text{odorant}]^n}{(EC_{50}^n + [\text{odorant}]^n)}$$

This way, the EC_{50} (the odorant concentration at which a 50% maximal response is evoked) and the Hill co-efficient (a measure of the steepness of the slope of the linear part of the sigmoidal curve) can be calculated.

FIGURE LEGENDS:

Figure 1: Software screenshot showing the input window from the program CO2Calc. Highlighted (red boxes) are the fields required for carbonate parameter calculation.

Figure 2: Software screenshot showing the input window for the appropriate constants, units and scales. Values shown are recommended for conditions under which the described experiments were carried out; they may change.

Figure 3: Software screenshot showing the results window.

Figure 4: Typical multi-unit responses recorded extracellularly from the olfactory nerve of seabream in vivo in response to 10^{-3} M L-serine (A) and blank (B). Upper traces show the

integrated responses and lower traces show the raw (nerve) signal. Stimuli were applied to the olfactory epithelium (horizontal bars). Note the rapid increase in activity during the 1 s of exposure, a peak in activity, followed by a period of accommodation (while the odorant was still delivered to the epithelium) and a return to baseline levels once the odorant delivery has ceased. Little or no increase in activity is seen following stimulation with water treated the same way as odorant dilutions, with the exception of adding any odorant (blank).

Figure 5: Typical concentration-response curve for L-leucine recorded extracellularly from the olfactory nerve in vivo. (A) As the concentration of L-leucine applied to the olfactory epithelium (horizontal bars) increases from 10^{-7} M to 10^{-3} M, a concomitant increase in activity is seen in the nerve. Upper traces show the integrated responses and lower traces show the raw (nerve) signal. (B) Linear regression ($R^2 = 0.97$) of normalized data plotted semi-logarithmically to calculate threshold of detection as the value for $\log[\text{L-leucine}]$ when $\log(N + 1.5) = 0.1761$ (i.e., where $N = 0$). In this example, this value is -7.48; the estimated threshold of detection for L-leucine in this fish is therefore $10^{-7.48}$ M.

Figure 6: Typical concentration-response curve for L-glutamine recorded extracellularly from the olfactory nerve in vivo. (A) As the concentration of L-glutamine applied to the olfactory epithelium (horizontal bars) increases from 10^{-7} M to 10^{-3} M, a concomitant increase in activity is seen in the nerve. Upper traces show the integrated responses and lower traces show the raw (nerve) signal. (B) Semi-logarithmic plot of normalized data fitted to a three-parameter Hill equation ($R^2 = 0.99$). For this example, the calculated $EC_{50} = 3.11$ μ M, and the Hill co-efficient = 0.565).

DISCUSSION:

The current study describes the use of multi-unit (extracellular) recording from the olfactory nerve of the seabream (*S. aurata*), a marine sparid of great importance in aquaculture. However, this experimental approach can be broadly applied to other fish; the surgery and exact placement of electrodes will clearly depend on the anatomy of the olfactory system, and the choice and concentration of anesthetic may depend on the species under study. For example, the olfactory nerve of the goldfish (*Carassius auratus*) is short; in this case, recording the EEG from the olfactory bulb would be easier. The choice of odorant may also depend, to some extent, on the species. The current study used amino acids. As far as the authors are aware, all fish species investigated to date have olfactory sensitivity to amino acids^{1,18}. This sensitivity has been implicated in diverse processes such as food location, chemical communication and recognition of natal waters¹⁹⁻²³. However, the sensitivities of different species are, broadly speaking, rather similar and do not depend on lifestyle or habitat. They are also well-defined molecules and are cheaply and easily available. These reasons make them ideal test stimuli for studies on olfaction in fish, especially those investigating the effects of anthropogenic disturbances (e.g., acidification or pollution), where results can be easily compared across species²⁴.

Depending on the species in question, preparations for multi-unit recording can remain stable for several hours; the amplitude of response to the internal standard (10^{-3} M L-serine in the current study) should not vary by more than 10% between successive tests. Any significant

deviation from this rule of thumb could be due to: (i) movement of the fish, and therefore displacement of the electrodes and/or nose-tube; (ii) contamination of the water, for example, by coming into contact with the experimenter's hands (especially if lower concentrations of a given odorant give larger responses than higher concentrations); or (iii) deterioration of the health of the preparation). In case (i), the fish should be checked for having moved; if so, reposition it, and add more anesthetic to the water and/or give another dose of gallamine triethiodide. Allow 5 min and retest the standard. If the response is still smaller, then reposition the electrodes and/or nose-tube until a sufficiently large response is recorded. In case (ii), simply remake a fresh dilution series of the odorant, using clean glassware and water. In case (iii), check that the flow of water over the fish's gills is adequate, that the water is flowing over the gills (i.e., exiting via the opercula, rather than the mouth), and the water is well-aerated. Different fish species have widely different temperature preferences; ensure that the laboratory temperature (and that of the water in contact with the fish) is as close to the temperature that the fish are kept at as possible. Ensure, too, that the fish are not stressed, and avoid moving them (even from one tank to another) for at least a week prior to recording. Electrical noise is, of course, the bane of an electrophysiologist's life; however, the current article is not the appropriate medium to discuss how to overcome/reduce this. Nevertheless, 'The Axon Guide' (available freely as a pdf for download from the manufacturer's website) is a source of practical advice on noise minimization. Once a large, stable response is evoked by the standard stimulus, and a concentration series gives a concentration-dependent increase in amplitude, with minimal response to the blank, recording responses to test stimuli can begin. Some authors give the same stimulus three times, and calculate the arithmetic mean for subsequent data analysis. However, these are technical replicates, and this approach will increase the time a recording session takes by three-fold. The current authors prefer to test a given odorant once, but always part of a concentration-response curve. This not only allows the calculation of the threshold of detection or EC_{50} (as described), but also ensures that concentrations close to those that the fish would experience in its natural environment are tested (this is not always known). Furthermore, any outlier responses, due to contamination for example, are easier to spot; these can then be repeated using a freshly made sample if necessary.

Multi-unit recording from the olfactory nerve may be invasive, but it is more sensitive than the EOG when recorded in seawater⁷, as it is independent of external salinity. It can therefore be used to assess olfactory sensitivity to odorants, such as calcium and sodium, changes in the concentrations of which would also affect conductivity and consequently voltages recorded¹⁵. As an estimate of the number of ORNs responding to a given odor (i.e., action potentials travelling along ORN axons from the olfactory epithelium to the bulb), it represents a raw, unprocessed signal (initial processing of olfactory input begins in the bulbs). Therefore, it is a better parameter to assess the direct effects of pollutants, such as heavy metals, and environmental changes, such as pH, on the olfactory system than the EOG or EEG^{24,25}. Recording from the olfactory bulb in seawater with high PCO_2 (and therefore low pH) may be affected by central effects of pH on neural processing; the 'GABA_A receptor theory' of ocean acidification²⁶, whereby reduction in water pH causes a redistribution of Cl^- and HCO_3^- ion in the CSF and a consequent shift of GABAergic activation from inhibitory (hyperpolarizing) to excitatory (depolarizing). Furthermore, in such studies, it is important to assess the effects of acidification or pollutants using odorant

concentrations similar to those the fish is likely to encounter in its natural environment. For amino acids, this is in the nano to micromolar range²⁷⁻²⁹; close to the threshold of detection of these compounds in fish^{1,18}. Estimation of the threshold of detection for a given odorant can give some idea of the importance and/or biological role of the olfactory sensitivity. For example, the sea lamprey (*Petromyzon marinus*) has high olfactory sensitivity to specific bile acids released by larvae down to a threshold of 10^{-13} M³⁰; this sensitivity allows adults to locate and identify suitable spawning grounds, and therefore act as a migratory pheromone over long distance. Similarly, ripe female sea lamprey have high olfactory sensitivity to spermine (threshold 10^{-14} M), a polyamine released in the milt by males, which then attracts them to the nests of spermiating males³¹. Other fish also have olfactory sensitivity to polyamines³²⁻³⁵, but not with sufficiently low thresholds of detection to support a similar pheromonal role; instead, avoidance of decaying fish is suggested. Nevertheless, with such high olfactory sensitivities, it is possible to imagine that a slight reduction in sensitivity (i.e., increase in threshold), even when the response amplitude is not dramatically reduced, could cause severe problems for fish²⁴.

When plotted semi-logarithmically, concentration-response curves to odorants can be exponential, linear or sigmoidal¹⁸. In the case of amino acids, such semi-logarithmic concentration-response curves are either linear (i.e., logarithmic), sigmoidal or power functions⁷. That no saturation of the response is seen (i.e., no plateau in the concentration-response curve, even at supra-environmental concentrations) is probably due to several receptors binding to individual amino acids, depending on their concentration, rather than each amino acid binding to a specific receptor; as the concentration of a given amino acid increases, more receptors are able to bind it and therefore respond. Nevertheless, fish can distinguish between mixtures of amino acids³⁶⁻³⁹; this is likely due to combinatorial patterns of activity evoked in the olfactory bulbs^{12,40}; the axons of all ORNs expressing the same receptor protein terminate at the same glomeruli in the olfactory bulbs^{41,42}, and one amino acid can activate more than one glomerulus.

However, highly specific odorants, such as pheromones, may evoke sigmoidal or quasi-sigmoidal concentration-response curves^{43,44}. The inference, although not empirically tested, is that these olfactory responses are due to highly specific receptors which bind the pheromone molecule and little else. Therefore, above a given concentration, all receptors are occupied, and further increases will evoke no further responses in other ORNs. Therefore, these data can be fitted to a three-parameter Hill plot, and the maximal response, EC₅₀ and Hill co-efficient can be calculated^{15,45,46}. This can give valuable information, such as apparent affinity and apparent receptor number, that linear or exponential concentration-response curves cannot give.

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DISCLOSURES:

The authors have nothing to disclose.

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Manual Calculation: Input

Input

Results

Sample Metadata (Optional)

Name Date Time Latitude Longitude

Comment

Sample Data

Physical Data (All Fields Required)

Salinity
 Temperature (°C)
 Pressure (dbars)

Adjusted Conditions (Optional)

Temperature (°C)
 Pressure (dbars)

Carbonate Data (Two Fields Required)

Enter any two fields except TA and CO3 or fCO2 and pCO2 together

TA ($\mu\text{mol/kgSW}$)
 TCO2 ($\mu\text{mol/kgSW}$)
 pH (chosen scale)
 fCO2 water (μatm)
 pCO2 water (μatm)
 CO3 ($\mu\text{mol/kgSW}$)

Nutrient Data (Optional)

Total P ($\mu\text{mol/kgSW}$)
 Total Si ($\mu\text{mol/kgSW}$)

Additional Data (Optional)

Total Ca (choose units)

Air-Sea Flux Data (Optional)

pCO2 Air (μatm)
 Windspeed (choose units)

Calculation Preferences

CO2 Constant	KHSO4	
<div>K1, K2 from Mehrbach et al., 1973 refit by Dickson and Millero, 1987</div>	<div>Dickson, 1990</div>	
pH Scale	Total Boron	Air-Sea Flux
<div>Total scale (mol/kg-SW)</div>	<div>Uppstrom, 1974</div>	<div>Raymond and Cole, (2001)</div>

File Capture

☐ Record

Output File:

Browse

Clear All Data

Process

Input Conditions

Computed Constants

Physical Parameters

Salinity

35,000

Temperature (°C)

22,000

Pressure (dbars)

10,000

Density (kg m⁻³)

1024,219

Nutrient Data

Total P (μmol/kgSW)

0,000

Total Si (μmol/kgSW)

0,000

Air-Sea CO₂ FluxFlux (mmol/m²/d)

Carbonate

TA (μmol/kgSW)

2855,000

TCO₂ (μmol/kgSW)

2423,010

pH (chosen scale)

8,200

fCO₂ water (μatm)

320,330

pCO₂ water (μatm)

321,393

CO₃ (μmol/kgSW)

322,319

Calcium

Calculated (μmol/kgSW)

10284,570

User Value

Auxiliary Results

HCO₃ (μmol/kgSW)

2090,859

CO₂ (μmol/kgSW)

9,832

B Alk (μmol/kgSW)

112,228

OH (μmol/kgSW)

7,281

P Alk (μmol/kgSW)

0,000

Si Alk (μmol/kgSW)

0,000

Rvelle

9,101

xCO₂ (dry @ 1 atm)

(ppm)

329,831

Ω Ca (Using:

Ca from Salinity

CO₃ from Carbonates)

7,714

Ω Ar (Using:

Ca from Salinity

CO₃ from Carbonates)

5,041

Ω Ca (Using User Values)

Ω Ar (Using User Values)

Calculation Preferences

CO₂ Constant: K1, K2 from Mehrbach et al., 1973 refit by Dickson and Millero, 1987

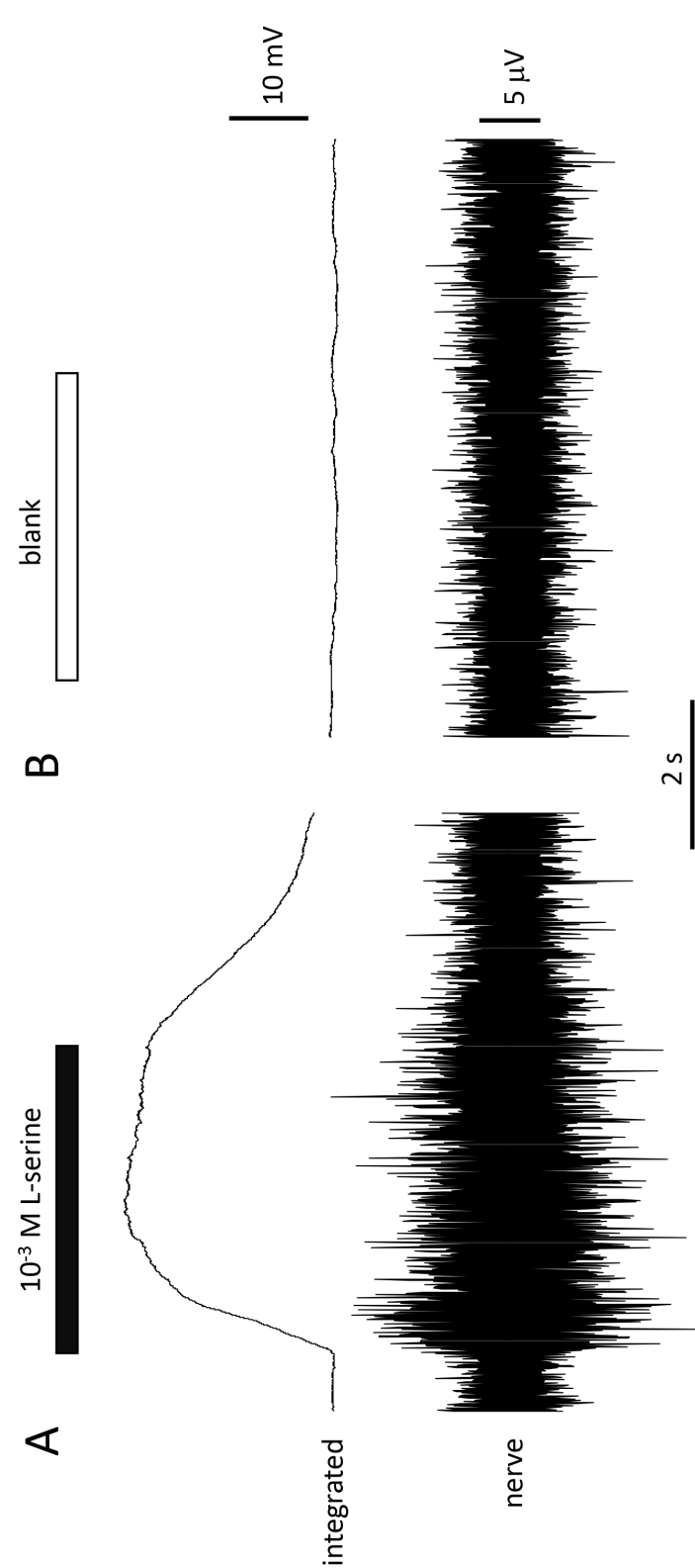
pH Scale: Total scale (mol/kg-SW)

KHSO₄: Dickson, 1990

Total Boron: Uppstrom, 1974

Air-Sea Flux:

Figure 4



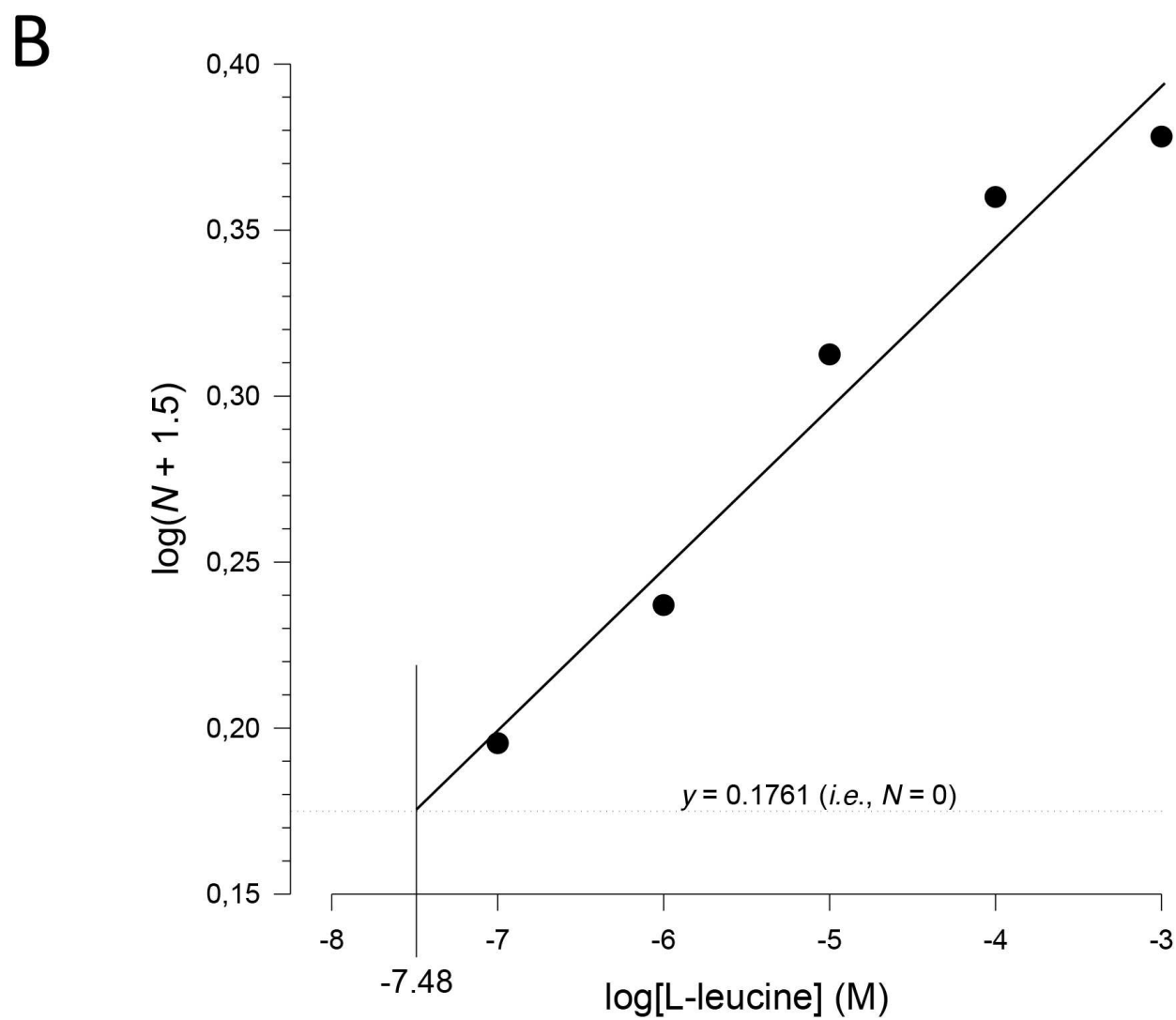
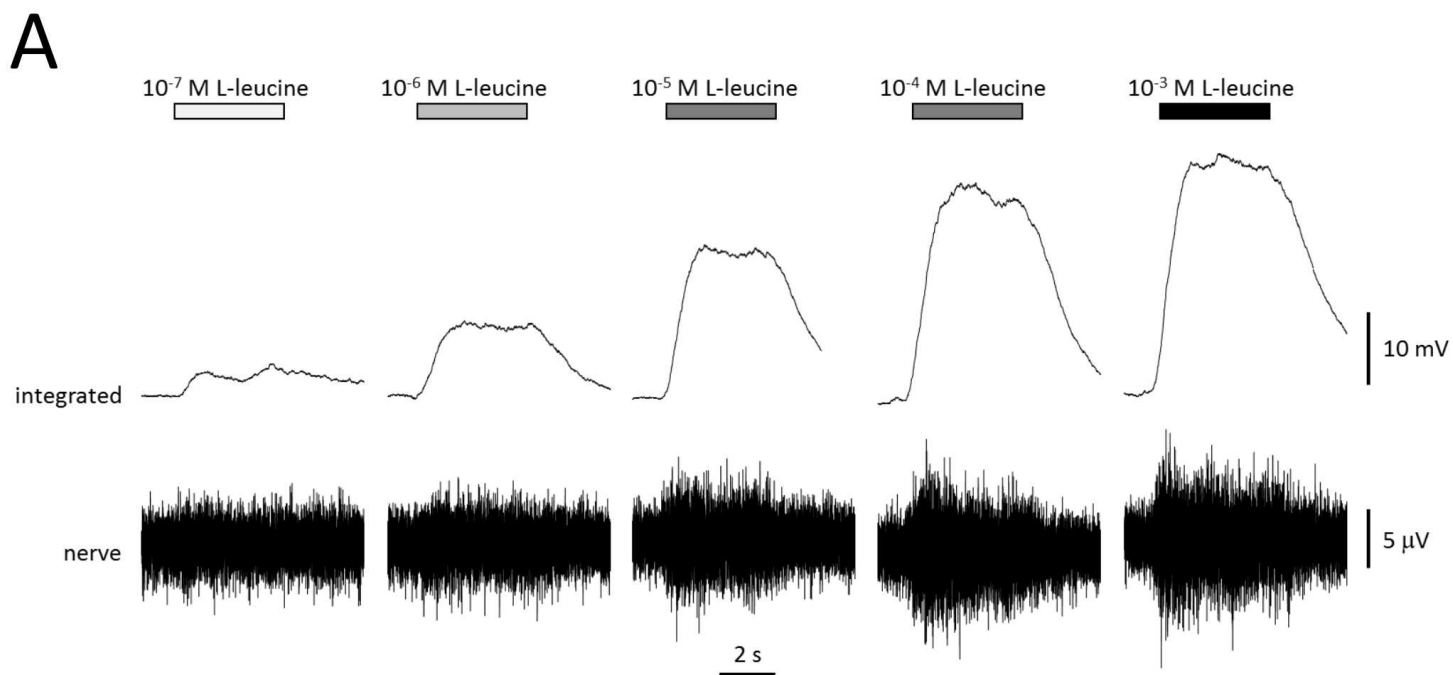
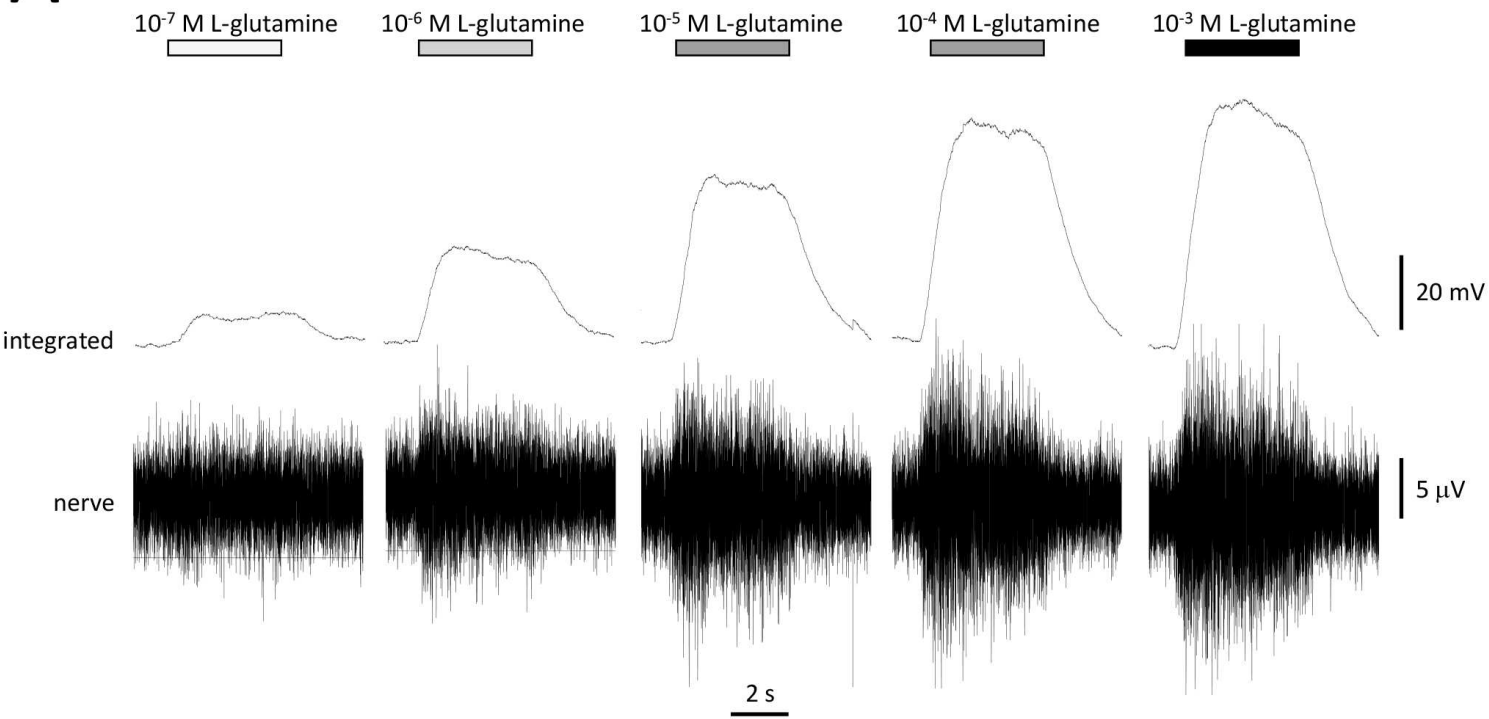
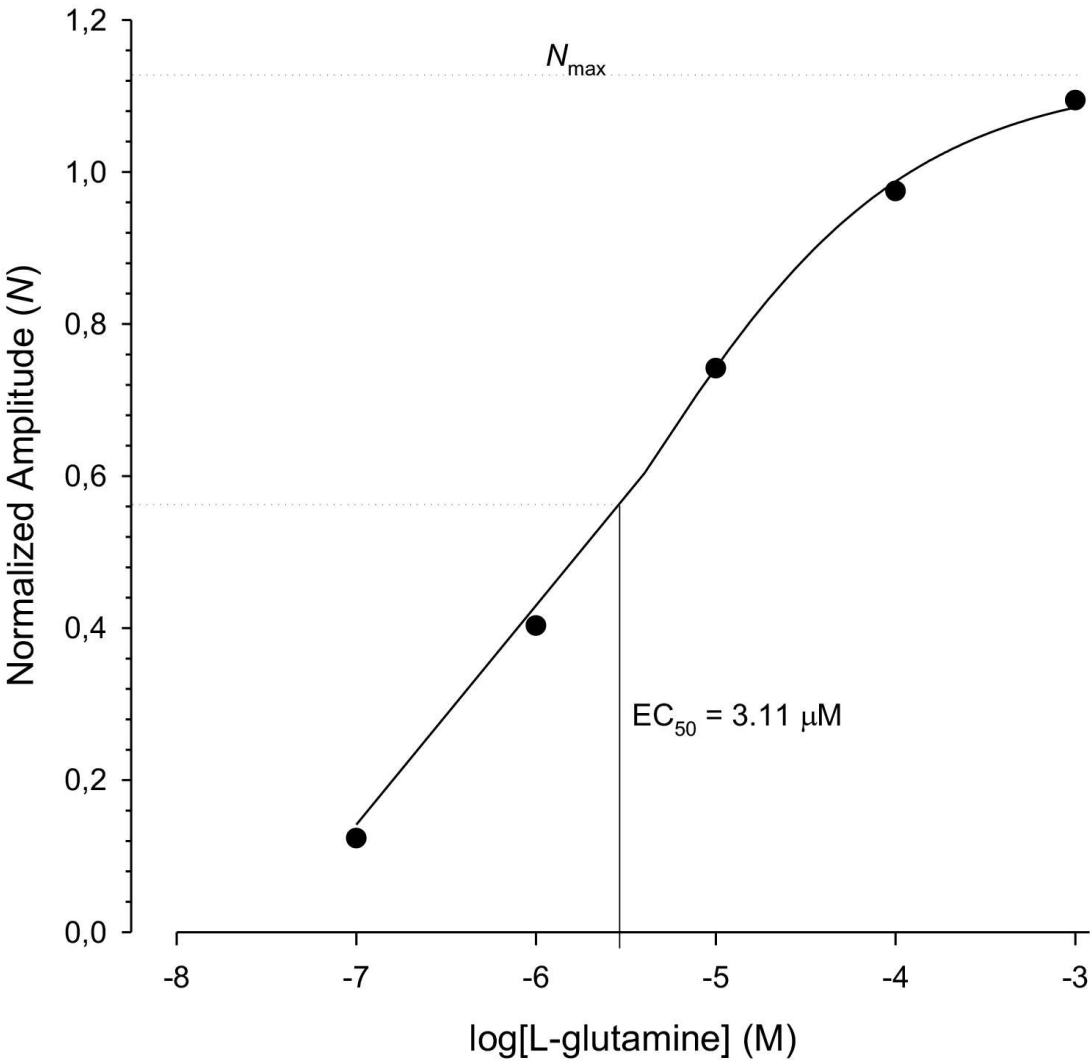


Figure 6

A



B



Name of Material/Equipment	Company	Catalog Number
AC pre-amplifier	Digitimer Ltd (Welwyn Garden City, UK)	NL104
Digidata	Molecular Devices, LLC. (San Jose, CA, USA)	1440A
EMG Integrator	Digitimer Ltd (Welwyn Garden City, UK)	NL703
Faraday cage	Made in-house	
Filter	Digitimer Ltd (Welwyn Garden City, UK)	NL125/6
Gallamine triethiodide	Sigma-Aldrich (Portugal)	G8134
L-glutamine	Sigma-Aldrich (Portugal)	G3126
L-leucine	Sigma-Aldrich (Portugal)	L80000
L-serine	Sigma-Aldrich (Portugal)	S4500
Metalic base-plate	Any	
Micro-hematocrit tubes	Any	
Micro-manipulators	Narishige International Ltd (London, UK)	M-152
MS222 (ethyl-3-aminobenzoate methanesulfonate salt)	Sigma-Aldrich (Portugal)	E10505
pH probe	Hanna instruments (Póvoa de Varzim, Portugal)	HI12302
Refractometer	Hanna instruments (Póvoa de Varzim, Portugal)	HI96822
Sodium chloride	Sigma-Aldrich (Portugal)	746398
Solenoid valves	The Lee Co. (Essex, CT, USA)	LFAA1201618H
Stereo-microscope	Zeiss, Leica, Olympus	Any suitable model.
Titration	Hanna instruments (Póvoa de Varzim, Portugal)	HI84531
Tungsten micro-electrodes 0.1 MΩ	World Precision Instruments (Hitchin, UK)	TM31A10
Valve Driver	Made in-house	
Water pump (submersible)	Any	

Comments/Description

Neurolog pre-amplifier specifically designed for this type of recording.

Analogue-digital converter.

Leaky' electrical integrator to integrate raw activity of the nerve.
To reduce electrical noise.

Filter module for electrophysiological recording.

Neuromuscular blocker

Amino acid used as odorant

Amino acid used as odorant

Amino acid used as odorant

Provides base for micro-manipulators.

To position water supply to the olfactory epithelium

Position electrodes

Anesthetic

Probe to measure pH of water.

Refractometer to measure water salinity

For saline solution

For switching between background water and stimulus solutions (no longer available)

For dissection and placement of electrodes.

Titration to measure water alkalinity, pH and temperature.

Extracellular electrodes.

12 V DC source for operating solenoid valves.

To supply anesthetic-containing water to the gills of the fish.

Replies to Editorial comments:

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.

Reply: The replies are given on the manuscript; for the most part, these have been complied with as suggested.

2. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps (in the imperative tense) contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

Reply: The Protocol has been changed to comply with the suggestions of the Editor. In particular, the instructions concerning the stimulus delivery system has been expanded, and the section discussing stability, and what to do when the preparation is not stable, has been moved to the Discussion.

3. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Reply: Several of the steps have been changed, with this suggestion in mind. For details, please see comments on the manuscript.

4. Please organize the sections/steps properly so that the protocol can be followed in chronological order (see some specific comments in the manuscript).

Reply: The order has been changed slightly, and is now in chronological order. Again, please see replies to comments on the manuscript.