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DO FISH SNIFF? A NEW MECHANISM OF OLFACTORY SAMPLING IN PLEURONECTID FLOUNDERS

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Summary

Although olfaction is known to be a highly developed sense in a variety of fishes, little information is available about behavioral mechanisms by which fishes sample the olfactory environment. This study was undertaken to assess the role of spontaneous jaw protrusion ('coughing') as a potential mechanism for olfactory sampling in pleuronectid flounders.

Investigations were carried out using a combination of physiological, morphological and behavioral techniques. Physiological results show (1) that typical respirations are coupled to measurable olfactory sac pressure fluctuations and (2) that during a coughing event, water is rapidly sucked into the olfactory sac. Morphological results indicate (3) a direct linkage system between the protrusion apparatus and the olfactory or associated accessory sacs, and (4) that coughing is associated with a rapid expansion or stretching of these sacs. Lastly, behavioral studies demonstrate (5) that coughing rates increase significantly over background activity when flounders are presented with attractive food odorants. From these results, I propose that coughing in pleuronectid flounders represents a behavior truly analogous to sniffing in certain air-breathing organisms.

Introduction

Olfactory systems of fish are among the most highly developed olfactory senses of vertebrates (Kleerekoper, 1969). Field and laboratory studies have repeatedly demonstrated the acute sensitivity of many fishes to a variety of odorants commonly occurring in the aquatic environment (for reviews, see Little, 1978; Tucker, 1978; Hara, 1982; Hasler and Scholz, 1983; Caprio, 1988). However, it is not yet clear what physiological or behavioral mechanisms fishes use to enhance detection of biologically important signals such as food odorants (e.g. Atema *et al.* 1980; Mackie *et al.* 1980; Carr, 1988) or pheromones (Von Frisch, 1941; Liley, 1982; Stacey and Kyle, 1983; Sorenson *et al.* 1987).

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In general, odorant molecules absorbed in mucus bind to ciliated and microvillar-type receptor cells which give rise to fibers of the olfactory nerve. These receptor cells are embedded in a mucus-coated, ciliated epithelium which may be folded into lamellae to form a rosette arrangement, resulting in an increased receptive surface area. In teleostean fishes, the olfactory epithelium is typically located in two blind sacs situated on the dorso-anterior aspect of the head (Burne, 1909). These structures maintain open communication with the environment by way of one or two nares serving as avenues of incurrent and excurrent exchange (for a review, see Kleerekoper, 1969; Finger, 1988).

Olfactory sampling in fishes has been thought to be a relatively involuntary behavior. Døving *et al.* (1977) have classified fishes into two major groups distinguished by the mode of water transport through the olfactory chamber. In one group (isosmates), water movement is achieved largely by the ciliary action of the apical support cells of the olfactory epithelium. In these fishes, an increase in water flow over the olfactory tissue may be achieved by increasing swimming speeds (see also Copeland, 1912; Bashor *et al.* 1974). In other, primarily benthic, species (cyclosmates), water circulation is created by a pumping mechanism in which volume changes of accessory nasal chambers occur synchronously with respiratory movements, provide a passive, rhythmic irrigation of the olfactory rosette (Burne, 1909; Pipping, 1927; Van den Berghe, 1929; Kleerekoper and Van Erkel, 1960; Johnson and Brown, 1962; for reviews, see Kleerekoper, 1969; Yamamoto, 1982).

Evidence for a specifically voluntary olfactory sampling behavior in either group is lacking. Interestingly, several investigations have interpreted jaw movements or coughing as positive indicators of olfactory responsiveness (e.g. Tavolga, 1956; Haynes *et al.* 1967; Sutterlin, 1975; Pawson, 1977; Atema *et al.* 1980; Døving, 1989), but the possibility that coughing is employed by fish as an active olfactory sampling behavior directly analogous to sniffing in mammals has never been demonstrated. During a coughing event, the pressure gradient over the gills is reversed and water is shunted from the opercular cavity to the buccal cavity (Ballintijn, 1969; Hughes and Adeney, 1977). This behavior cleans the gills (Hughes, 1975) and may also serve to eject particulate matter from the olfactory chamber (Kapoor and Ojha, 1972; Døving *et al.* 1977). However, the sensory inputs that trigger coughing are not understood in detail since many species, particularly cyclosmates, are known to cough in clean, well-aerated water (Ballintijn, 1985).

The study described here was undertaken to assess the role of coughing as a voluntary sampling behavior used by pleuronectid flounders. The results show that coughing in representatives of this cyclosmate group is accompanied by a rapid drop in pressure in the olfactory chamber qualitatively and quantitatively different from pressure changes during normal respiration. A mechanism by which jaw protrusion is linked to water suction into the olfactory chamber is proposed. Lastly, I provide behavioral evidence suggesting that coughing behavior can be elicited in flounders by olfactory stimuli.

Materials and methods

This study was performed during two visits to Friday Harbor Laboratories, University of Washington, Friday Harbor, Washington. All observations were made on pleuronectid flounders (primarily rock sole, *Lepidopsetta bilineata* Ayres or starry flounder, *Platichthys stellatus* Pallas). Since olfactory structures on the eyed side are functionally dominant (Kapoor and Ojha, 1973; Yamamoto and Ueda, 1979; Rao and Finger, 1984), the investigations described here were limited to the eyed side unless otherwise stated.

Animal collection and maintenance

Large animals

For anatomical and physiological experiments, large animals (30–70 cm in length) were captured by otter trawl during June and July 1985 and 1989 in the North Puget Sound near Friday Harbor Laboratories. Animals were maintained either in large (3 m) circular tanks or in individual flow-through seawater tables (60 cm×80 cm×25 cm deep; 7–10 °C) with a sandy substratum for 2–5 weeks prior to experimentation. Fish were fed clams (*Macoma nasuta*) and shrimp (*Pandalus danae*) daily.

Small animals

For behavioral experiments, small fish (2–5 cm) were used for ease of handling in the laboratory. Animals were collected near the laboratory using a two-person beach seine, identified to species, and maintained together in a large (130 cm×80 cm×12 cm deep), flow-through seawater table kept at ambient temperature (7–10 °C) and provided with a sandy substratum. Animals were kept for 1 month prior to experimentation, and fed clams (*M. nasuta*) and polycheate worms collected each day from a mud flat near the site where the flounders were seined.

Functional observations

High-speed cinematography

Because coughing is rapid, high-speed cinematography was used to record this behavior. To capture an event reliably, coughing was induced by exposing the fish to a stream of dilute vinegar. All filming was carried out using a Locam 5103 (Redlake) at 100 frames s^{-1} on 16 mm Ektachrome 7250 color positive film. Illumination was provided by two fiber optic lights (Dolan–Jenner) oriented at 45° to the plane of focus. The room was otherwise darkened to prevent the fish from seeing out of the aquarium. Film was analyzed on a freeze-frame analysis projector (Nac).

Dye observations

Nasal currents were visualized using fluorescein dye. For these observations, large animals lightly anesthetized with MS222 were placed in individual shallow Plexiglas chambers big enough for the fish to turn around easily. Dyes were

applied using a 1 ml syringe fitted to a no. 25 needle directed over the anterior naris on the eyed side. Water was exchanged using a syphon hose 1–2 min following application of dyes.

Nasal casts

To test whether coughing resulted in a detectable increase in nasal sac volume, casts of nasal sacs were made of fish in the coughing (experimental) and abducted (control) positions. Ten of eleven large *P. stellatus* were paired by size; all were decapitated. In six experimental fish, the jaws were secured in a protruded position and latex (Carolina Biological Supply) was slowly injected through the eyed-side posterior naris to fill the nasal sac. This procedure was repeated on the blind-side nasal sac using a different colored latex. Casts of nasal sacs of five control fish were made in a similar fashion, except that the jaws were secured in an abducted position during injections. Immediately following latex injections, the heads were fixed in 70 % ethanol. Once set, casts were carefully dissected from the nasal sacs, allowed to dry overnight, and weighed.

Pressure measurements

Surgery

All surgery was conducted on fish that had been anesthetized with MS222 at concentrations such that animals failed to respond to tactile stimulation applied to the caudal peduncle. Pressure transducers were then implanted into the fish as described below. During experiments, flounders were lightly anesthetized and kept in sand-free Plexiglas tanks large enough to allow free movement. Between experiments, cannulated flounders were allowed to swim freely or bury themselves in sand.

Buccal cannula implantations

Cannulae were inserted into the buccal cavities of five fish. Cannulae (7–15 cm in length) were constructed from polyethylene tubing (PE 200 intramedic tubing, 0.062 cm×2.5 cm inner diameter; 0.082 cm×2.5 cm outer diameter) fashioned into a flare at one end. For each fish, a Foredom F series-RB high-speed drill equipped with Foredom SR-1 speed control was used to puncture a small hole in the posterior margin of the preoperculum. A cannula was then guided into the buccal cavity using a no. 12 needle. The flared end was securely positioned against the back of the buccal cavity and stabilized with plastic clamps.

Nasal cannula implantations

Cannulae were inserted into the eyed-side anterior naris and positioned directly over the olfactory rosette of the same animals as above. Nasal cannulae were constructed from 10–15 cm lengths of intramedic PE 200 tubing fitted with a short (4 cm) length of narrower tubing (PE 100, intramedic: inner diameter, 0.034 cm×2.5 cm; outer diameter, 0.060 cm×2.5 cm) to allow easy access. Cannu-

lae were stabilized with 3.0 gauge silk suture in such a way as to permit unhindered opercular movement. Nasal cannulae were removed following each experiment.

Pressure recordings

Pressure changes in the nasal and buccal cavities were recorded using Milar Mikro-tip catheter pressure transducers (1–3F pressure sensor model no. P-249). These transducers were threaded into the cannulae described above to within 5 cm of the preoperculum. Catheters were filled with water using a syringe, air bubbles were removed from the system, and cannulae were sealed with gum. Pressure transducers were connected in series with Milar transducer control units (model no. TCB-100), which were in turn connected to Grass pre-amplifiers and Grass regulated power supplies. Signal outputs were read on a Gould Brush 220 two-pen chart recorder.

Behavioral experiments

Flow tank specifications

All behavioral experiments were conducted using a 31 l flow-through tank (30 cm × 84 cm × 12.5 cm deep), the floor of which was ruled with a grid to provide a measurement scale. Uniformity of flow was achieved using two honeycomb-shaped flow straighteners (Hexcell Corp.) spaced 6.5 cm apart, 25 cm downstream of the influx hose. Volume flow rate was adjusted to be at least 4 l min^{-1} at the start of each session.

Olfactory stimuli

Crude odorant stimuli were prepared daily by macerating 2–3 *M. nasuta* in 1 l of water. Control seawater and odorant solutions (pH 7.8) were filtered (5 μm), chilled to the ambient flow tank temperature (8°C) and randomly colored with a vegetable dye marker (Schilling) to which flounders showed no behavioral response during preliminary trials. Solutions were transferred to Nalgene bottles fitted with a manually controlled drip system and located out of sight of the experimental arena. Bottles were kept chilled throughout the experiment. Solutions were introduced into the flow tank 1 cm behind the flow straightener immediately upstream of the experimental arena at a rate of 80 ml min^{-1}. This configuration provided a distinct stimulus stream in the experimental arena when solutions were released.

Experimental protocol

Experimental trials were conducted either at dawn or at dusk when fish appeared to be most active in the laboratory. Each trial was continuously videotaped using a Panasonic color video camera mounted above the experimental arena. Videotaping was conducted using standard room lighting. During trials, observations of the arena could be made from a remote monitor, so as not to disturb the animals. Flounders were tested individually in all but one trial in which two fish were videotaped at the same time.

Approximately 2 h before each trial, individuals were placed in the flow tank and allowed to adjust to the experimental arena. Each animal was videotaped for 5–10 min to monitor background activity levels. At the end of this period, odorant solution or filtered sea water was randomly introduced into the arena. At 2–3 min, the solution was turned off. Once the stimulus had cleared (5 min), the procedure was repeated roughly 10 times. Each individual was recorded for only one trial, and each trial lasted approximately 90 min.

Analysis

Videotaped trials were analyzed for responsiveness to stimuli such that the person scoring the behavior did not know whether control or odorant stimulus was being presented. Coughing and biting were recorded independently as a function of time for each sequence within a trial. A spontaneous jaw protrusion was defined as a coughing event. Biting was defined as coughing in conjunction with a forward lunge and/or particulate matter entering or exiting the mouth. Both coughing and biting rates were determined for each sequence (baseline, filtered sea water, odorant); sequences in which fish showed obvious signs of stress (e.g. burying or escape behavior) were omitted. For statistical analysis, coughing and biting rates for the two treatments (filtered sea water or odorant) were averaged across a trial. Coughing and biting events were generally easy to distinguish; however, to ensure against possible sampling error, trials were analyzed in two ways: including and excluding biting events. Baseline coughing and biting rates were determined from the first intertreatment sequence in which fish did not show signs of stress.

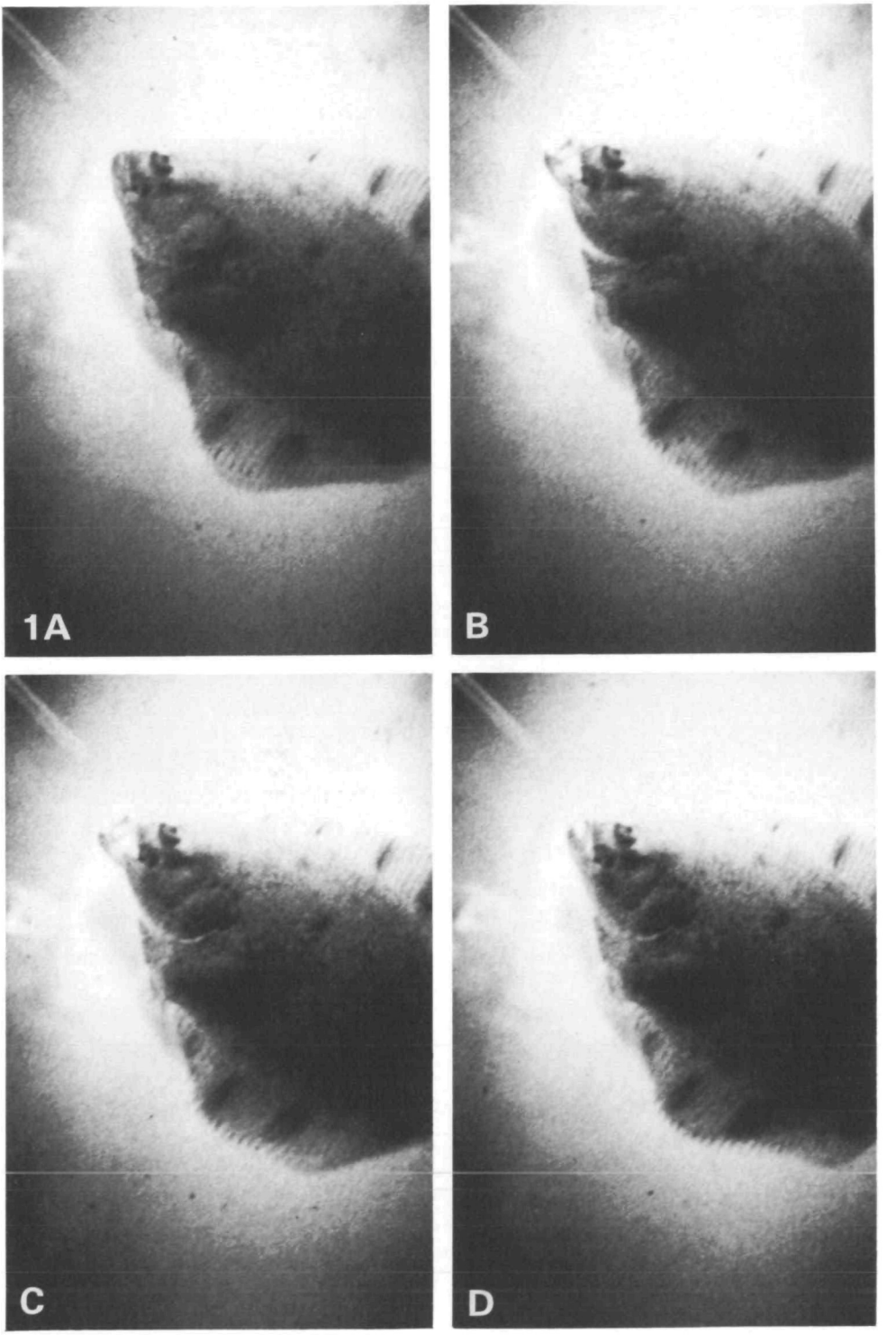
Results

General observations

When fluorescein dye was injected into the water directly above the naris of a rock sole, excurrent pulses could easily be observed. Pulses were emitted in a rhythmic pattern tied to respiration. During each inhalation, water entered the olfactory chamber exclusively from the anterior naris due to closing of the posterior nasal flap. Inhalations pulled the external nares slightly together, presumably due to swelling of the olfactory chambers. Water was primarily emitted through the posterior naris.

Fig. 1 shows a sequence of frames taken from a high-speed movie of a flounder coughing. The coughing event is characterized by a rapid protrusion of the jaw (Fig. 1A,B), and expulsion of water from the mouth (Fig. 1C), during which time water is sucked into the olfactory chambers through the anterior naris. As the mouth closes (Fig. 1D), water is rapidly emitted through both nares.

Fig. 1. High-speed recordings of a flounder coughing. This series of frames (A–D) shows a flounder coughing in response to an olfactory stimulus delivered by the pipet pictured in the upper left-hand corner of each frame. Frames are separated by 50 ms. The fish was approximately 5 cm in length.


1A
B
C
D

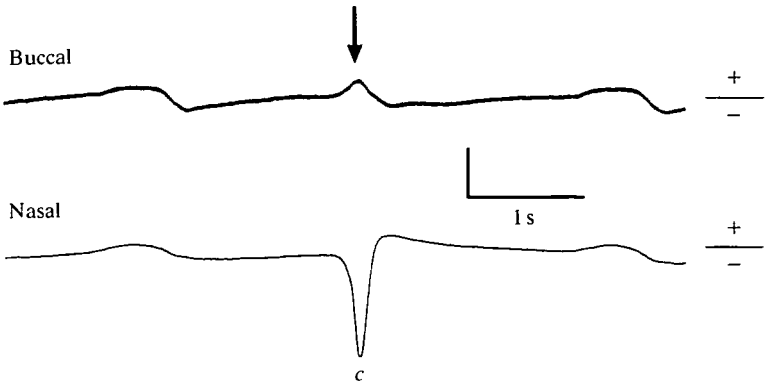


Fig. 2. Buccal cavity and nasal sac pressures during normal respiration and coughing. The upper trace shows buccal cavity pressures recorded during two normal respirations interrupted by a coughing event. Nasal sac pressure fluctuations (bottom) follow respirations rhythmically. The coughing event is denoted by a slight increase in positive buccal cavity pressure (arrow), which forces water out of the buccal cavity, and a sharp decrease in nasal sac pressure (*c*) which causes water to be sucked into the nasal cavity. See discussion in text (recorded from *Platichthys stellatus*). Vertical scale is 133 Pa for buccal pressure and 67 Pa for nasal pressure.

Pressure recordings

Fig. 2 illustrates a typical pressure response coincident with normal respiration and a cough. As an animal is quietly breathing on the floor of a holding tank, prolonged incurrent (negative deflection) and more pronounced excurrent (positive deflections) pulses of water circulate through the nasal sac. During a coughing event (labeled *c*), water is rapidly forced out of the mouth. At this moment, buccal cavity pressure increased by 24.5±3.6% (S.E.) relative to peak respiration pressure measured immediately before a cough (N=15; significantly different from 0; P<0.01, paired t-test, Zar, 1984). This increase was accompanied by a definite sharpening of the waveform (Fig. 2, arrow).

In contrast to buccal cavity pressure, nasal pressure dropped dramatically during a coughing event, averaging 6.27±0.38 (S.E.) times more negative than the lowest nasal pressures observed during typical respiration (N=15; significantly different from 0; P<0.001, paired t-test). Nasal sac pressure reached a peak negative deflection at an average delay of 0.038±0.0064 s after the buccal cavity pressure peak (N=20) and was quicker to reverse, showing peak positive values an average of 0.061±0.00734 s before the buccal cavity pressure attained its maximal negative value (N=20).

Latex casts

Latex casts of olfactory chambers were significantly larger when jaws were protruded, indicating an expansion of the chamber from the abducted position (Fig. 3A). This relationship was also observed in latex casts of the blind side (Fig. 3B), but the absolute difference in volume was less.

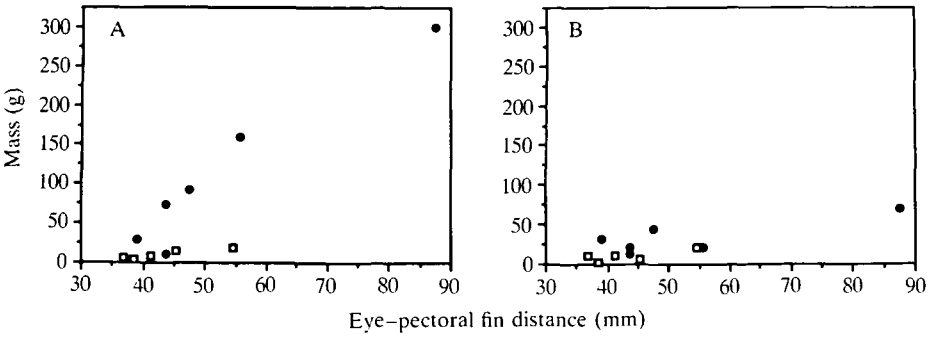


Fig. 3. The volume of the nasal sac as a function of jaw protrusion. (A) Masses of latex casts of nasal sacs on the eyed side from size-paired fish with jaws fixed in either protruded (circles) or abducted (squares) positions, as a function of head size. Differences are significant by a Mann–Whitney U test ($0.01<P<0.05$; $N_a=5$, $N_b=5$; $U=2$; largest, unpaired fish not included in the analysis). (B) Masses of casts from the nasal sac on the blind side as a function of head size. Symbols as in A. Differences are also significant ($0.01<P<0.05$; $N_a=5$, $N_b=5$; $U=2$).

Functional anatomy

Anatomical investigation revealed a linkage system by which jaw protrusion is tied to volume fluctuations in the large lachrymal and possibly the smaller ethmoidal accessory olfactory sacs. Dissection of four specimens (two *L. bilineata* and two *P. stellatus*) revealed a bifurcation in the primordial ligament, which typically runs between the lateral posterior face of the mandible and the dorso-lateral surface of the maxilla in other acanthopterygian fishes. Fig. 4A illustrates a second ligament departing from the posterior margin of the mandible to insert near the ventral margin of the lachrymal bone. A second set of ligaments attaches to the dorso-lateral tip of the maxilla, to the most dorsal process of the lachrymal bone, and to the dorso-lateral edge of the maxilla. No direct ligamental connection to the nasal bone was observed.

The combination of these four ligaments provides a linkage system by which even the slightest rotation of the maxilla results in a displacement of the lachrymal bone sufficient to cause a visibly dramatic compression or expansion of the accessory olfactory sac (Fig. 4B). Preliminary experiments suggest, however, that cutting the maxillary–lachrymal or maxillary–mandibular ligaments does not permanently abolish the normal pressure fluctuations observed during respiration and coughing shown in Fig. 5A. Fig. 5B shows buccal and nasal pressure recordings immediately after the maxillary–lachrymal ligament had been cut. Although buccal pressure fluctuations persisted, nasal pressure fluctuations were virtually absent and coughing was reduced. Moreover, activity was not appreciably reduced further by eliminating the maxillary–mandibular ligament (Fig. 5C). Coughing ability returned to this fish within an hour (Fig. 5D), suggesting that additional ligaments contribute to the maintenance of this mechanism.

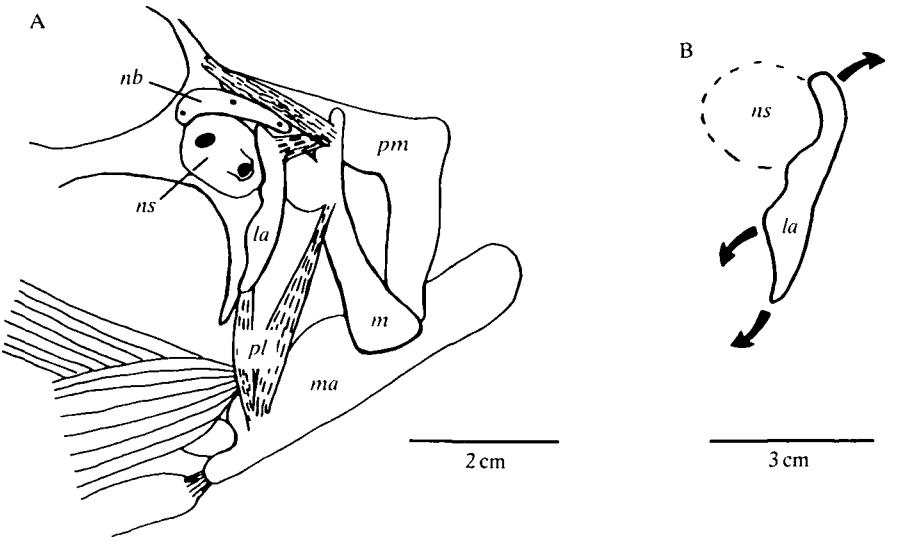


Fig. 4. Anatomical relationship between the jaw apparatus and the olfactory sacs in a pleuronectid flounder. (A) Simplified diagram showing the position of the nasal sac (*ns*) in relation to the maxillary (*m*), lachrymal (*la*) and nasal bones (*nb*). Note the bifurcation of the primordial ligament (*pl*) as described in the text. *ma*, mandible; *pm*, premaxilla. (B) Diagrammatic sketch showing rotational forces acting on the lachrymal bone as a result of jaw movements during protrusion. The lachrymal bone is thus free to squeeze or pull against the nasal sac (see text; for more detailed anatomical descriptions see Kyle, 1921; Flüchter, 1963; Yazdani, 1969; reviewed by Kleerekoper, 1969). Abbreviations are as in A.

Behavior

Besides responding to odorant stimulation, flounders sometimes coughed spontaneously or in response to particulate matter or fluorescein dye introduced directly into the mouth. These latter methods of stimulation were invariably associated with a hasty 180° pivot away from the stimulus and/or burying. However, olfactory stimuli did not provoke these adverse reactions, but rather predictably elicited a stereotypic behavior pattern: in addition to coughing, flounders were observed to lift their heads off the substratum and initiate searching behavior (i.e. tightly pivoting or scuttling in the direction of the odorant stimulus).

Behavioral trials showed that the coughing rates of flounders increased when they were presented with an odorant stream (Fig. 6). Flounders showed a greater tendency to cough when presented with an attractive odorant stimulus (Fig. 6A), than when presented with filtered sea water. Differences were significant when odorant and seawater treatments were compared (background subtracted, paired t-test, $P<0.05$), but not between background and seawater treatment coughing levels (Student's t-test, $P=0.265$). When biting events were included in the analysis (Fig. 6B), differences between seawater and odorant treatments persisted

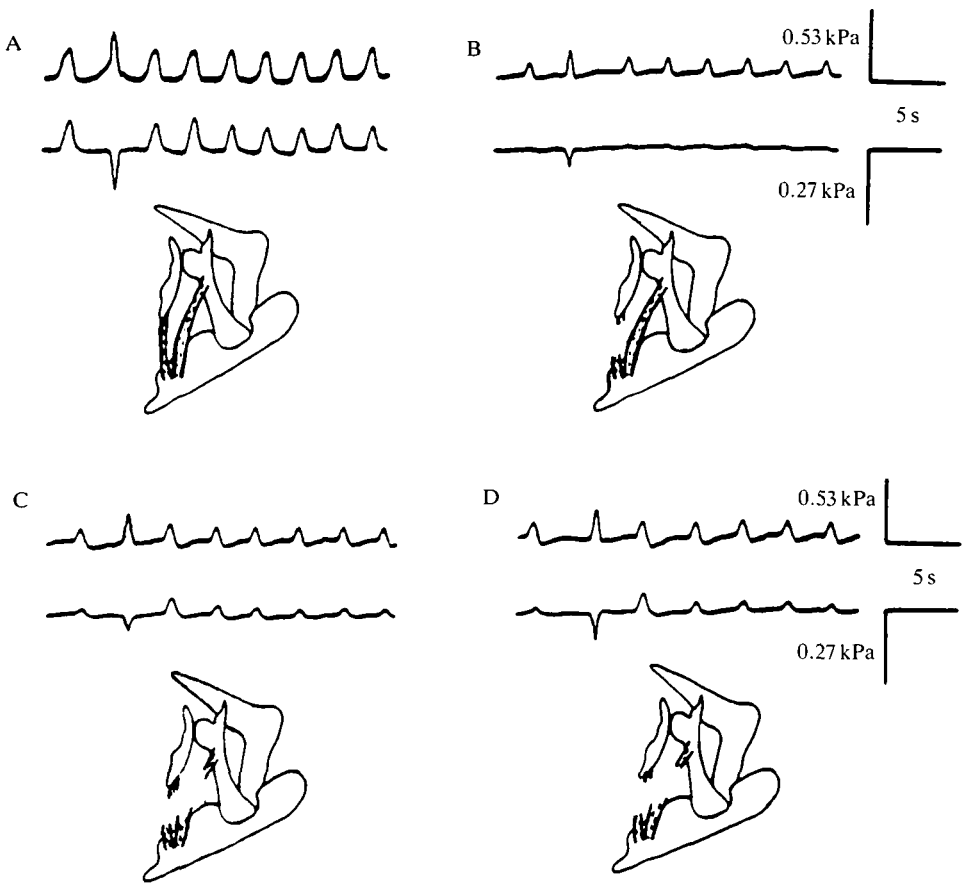


Fig. 5. Effect of cutting the lachrymal–mandibular and maxillary–mandibular ligaments on pressure fluctuations. Top, buccal pressure; bottom, nasal pressure. (A) Pressure fluctuations accompanying normal respiration and a cough recorded in an intact fish (*Lepidopsetta bilineata*). (B) Pressure fluctuations recorded from the same fish 10 min after the lachrymal–mandibular ligament had been cut (see diagram below). 12 min after this cut was made, the maxillary–mandibular ligament was also cut. (C) Pressure fluctuations recorded 10 min after the maxillary–mandibular ligament had been cut. (D) Pressure fluctuations recorded 30 min after the maxillary mandibular ligament had been cut, indicating that the peak pressure recorded during a spontaneous coughing event has recovered to within 50 % of its original value.

(background subtracted, paired t-test, $P<0.025$), while activity during seawater treatments was not significantly different from background activity levels ($P=0.276$).

Discussion

Functional significance of coughing

Results presented here provide clear evidence of physiological and morphological mechanisms by which flounders are capable of deliberately sampling odorants

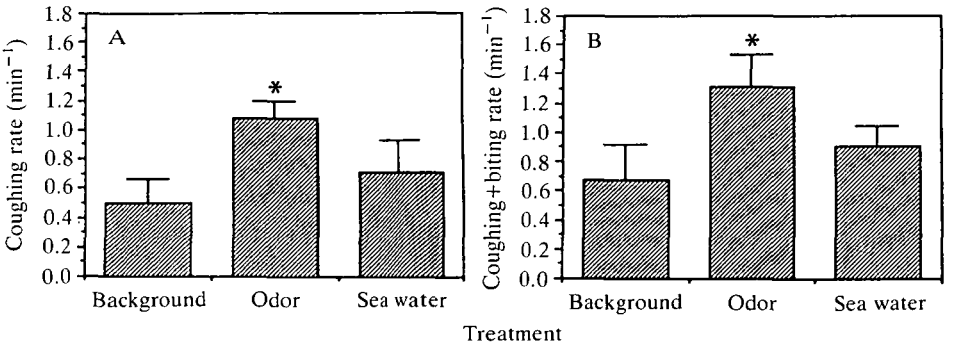


Fig. 6. Behavioral responses to olfactory stimuli. (A) Coughing rates in the absence of a stimulus (background) or when either sea water or an odorant stimulus was presented. The significantly higher rate is marked with an asterisk (see text). (B) Combined coughing and biting rates under the same conditions as in A. The significantly higher rate is marked with an asterisk (see text). Values are mean +S.E. (N=9).

in surrounding water. Although the presumption that fish can sniff is not novel (Pipping, 1927; Døving *et al.* 1977; Atema *et al.* 1980), this is the first study that rigorously demonstrates pressure fluctuations in the olfactory sac resulting from a grossly detectable behavior: spontaneous jaw protrusion or 'coughing.'

Physiological results indicate that water is sucked into the olfactory chamber during a cough and that this response is produced by rapid expansion or stretching of the nasal sac. Furthermore, typical respirations are coupled to measurable nasal sac pressure fluctuations with minimal delays. These findings indicate a tight coupling of energy transmission between the protrusion apparatus and the olfactory system.

Anatomical observations support this direct linkage system. Latex injection studies show that simple movements of the maxilla and mandible can result in substantial deformations of the nasal sacs, but precise mechanisms underlying volume changes during coughing require further demonstration. For example, the relative contributions of lachrymal and ethmoidal accessory sacs in coughing are not investigated in this study. Once determined, however, it should be possible to cut ligaments systematically and then ascertain quantitatively how specific accessory nasal sac pressures change. Preliminary studies indicate that cutting the maxillary–lachrymal and maxillary–mandibular ligaments only temporarily diminishes the nasal pressure fluctuations associated with normal respiration and reduces the magnitude of the pressure drop during coughing events. That this reduction is not permanent suggests that some redundancy must exist to maintain water fluctuation through the accessory olfactory sacs. This hypothesis is strengthened by preliminary data collected from the blind side that also show decreases in pressures during coughing events, yet this side shows no bifurcation in the primordial ligament (G. A. Nevitt, unpublished observation; see also Yazdani, 1969).

Behavioral studies presented here clearly indicate that flounders cough preferentially when presented with food odorants. The observation that coughing is accompanied by a suite of searching behaviors substantiates the hypothesis that coughing serves an olfactory function in addition to its function as a means of ejecting foreign material from the olfactory chambers or gills (Kenshalo and Isaac, 1977; Kleerekoper, 1967; for a review, see Kleerekoper, 1982). The use of filtered, pH-controlled test solutions further reduces the possibility that coughing was used to clear debris or irritants from the gills or olfactory sac specifically during odorant stimulation.

Odorant transport: physiological and physical constraints

I have shown that coughing increases the volume flux rate of water through the olfactory sac; however, other factors also increase olfactory sampling efficiency. Once inside the olfactory chamber, odors dissolved in water are first trapped by olfactory mucus coating the olfactory epithelium before arriving at olfactory receptor cells (see Tucker, 1978). Olfactory binding proteins have been identified in olfactory mucus or olfactory receptor lymph in organisms as diverse as cows (Pevsner *et al.* 1986) and moths (Vogt and Riddiford, 1981), respectively. These proteins bind odorant molecules and transport them to or away from olfactory receptor cells. Bachtin and Filiushina (1974) have also suggested that mucopolysaccharide and lipoprotein components of fish mucus form complexes that carry odorant molecules to olfactory receptor cells.

What is more, stimulatory frequencies of olfactory receptor cells are bounded by physiological constraints such as stimulus adaptation and habituation to odorants (e.g. Hara, 1982). Therefore, to activate a population of receptor cells maximally and consequently to maximize sampling efficiency, pulses of odorant must be delivered at a frequency with a period of roughly the recovery time for these receptor cells. This phenomenon has been most convincingly demonstrated in the spiny lobster *Panulirus argus*, in which antennal flick frequency matches the refractory period of the olfactory receptor cells being stimulated with each flick (Schmitt and Ache, 1979).

In addition to physiological limitations, odorant sampling is subject to physical constraints on flow through the olfactory chamber. At this size, wall effects exerted by the three-dimensionality of the chamber and associated lamellae will greatly alter flow characteristics, making conclusions drawn from purely *in vitro* studies questionable. For example, results from studies of excised lamellae have suggested that ciliary movements are the primary mechanism by which water is transported over olfactory lamellae in fish. Velocities of dye fronts moving over excised lamellae of garfish placed in Ringer's solution have been reported to be as high as $2.9\times10^{-3}\,m\,s^{-1}$ (Bashor *et al.* 1974). These authors concluded that ciliary activity causes the highest velocity of fluid flow in the olfactory chamber to be at the surface of the epithelium. However, because motile olfactory cilia also transport mucus, a substance considerably more viscous than Ringer's solution, these surface flow velocities are overestimated.

The precise role played by cilia in transporting mucus and water over the olfactory epithelium is not yet clear, but it is likely that large-amplitude pressure fluctuations in the olfactory chamber constitute the major driving force of water flow across the lamellae. The magnitude of the resulting velocity gradient of flow will therefore determine the flux of odorant molecules to the lamellar surface, and thus requires further characterization.

Generally, the flow characteristics of a given fluid moving through a pipe can be described by a Reynolds number, (*Re*):

$$Re = lU/v,$$

where l is the pipe diameter, U is the average flow velocity and v is the kinematic viscosity of the fluid. This dimensionless number provides a means of comparing flow, and therefore odorant transport, under different conditions. For example, if *Re* is small (<1), viscous forces will predominate, and flow will be laminar. Alternatively, if *Re* is high (>1000), inertial forces will predominate and turbulence may result.

In the simplest hypothetical case, water flow over a lamella of the olfactory epithelium has a low *Re* ($\ll 1$). Flow is steady, unidirectional and laminar. Owing to the no-slip condition, flow velocity is zero at the surface of the mucus-coated tissue, creating a velocity gradient between the surface and the maximal velocity some distance above the surface (Vogel, 1981). This velocity gradient effectively insulates the mucus layer from odorant molecules in the fluid flowing past it. Because diffusion of even small molecules is slow (Berg, 1983), any mechanism that enhances delivery of odorant molecules close to the mucus layer would improve odorant absorption by mucus and possibly increase sampling of odorants by olfactory receptor cells.

In a more realistic situation, such as during normal respiration, flow of water through the olfactory chambers is rhythmic (Pipping, 1927; Van den Berghe, 1929; Kleerekoper and Van Erkel, 1960; Døving *et al.* 1977). *Re* is still small but, since the velocity gradient is shallower (Batchelor, 1967), diffusional exchange between the mucus layer and the water flowing past it is improved. This situation should consequently enhance olfaction by delivering more odorant molecules to the mucus layer and by clearing away molecules within a reasonable time to render a given olfactory receptor cell excitable for further stimulation.

Convective enhancement of odorant transport by coughing

Theoretically, transport of odorant molecules to the mucus layer can be enhanced by modifying convective transport in at least two ways. First, flow of odorants to the mucus layer is increased by increasing the velocity of laminar flow, and thus *Re*. This effectively decreases the thickness of the boundary layer surrounding the mucus-coated olfactory epithelium and, consequently, reduces the distance odorants must diffuse in order to be absorbed by the mucus layer. This condition also leads to an increase in the absorption rate of odorants owing to the higher velocity of flow closer to the mucus layer. This mechanism may be used

during normal respiration in flounders or as a means to enhance olfactory efficiency in rapidly swimming isosmate fishes.

Second, during a coughing event, the impulsive acceleration of water through a narrow, tubular opening into the larger olfactory chamber will increase the vorticity of flow in the chamber (Vogel, 1981). However, using data collected by Kux *et al.* (1978; naril opening 0.06 mm^2; peak velocity through opening 0.015 $m\,s^{-1}$), Atema (1988) has suggested that flow over the lamellae in a fish nose is strictly laminar, $0.2<Re<2$. From the volume change of the nasal sac (0.03–0.34 ml) and the time of a coughing event (250 ms), the volume flow rate of water through the naril opening (0.2–1.8 mm^2) and the olfactory chamber of a flounder can also be determined. Estimating the cross-sectional area of the olfactory chamber through which the volume of water must pass (3–7 mm^2) gives the average fluid velocity during a cough. This calculation suggests an average velocity through the olfactory chamber of 0.06–0.6 $m\,s^{-1}$ and a corresponding *Re* of the order of 100–1000 during a coughing event. Data further suggest that *Re* is conserved well within an order of magnitude for large (30–70 cm) fish, implying that allometric constraints underlying the physics of coughing remain relatively constant, at least within the size range of fish examined (Fig. 3A).

As is the case with increasing laminar flow velocity, introducing vortices could result in a steeper velocity gradient over the olfactory epithelium, due to a higher flow velocity closer to the surface of the mucus layer. At intermediate *Re*, there is a likelihood that some components of flow will be heading in the direction of the mucus layer. This is in sharp contrast to the laminar condition, in which bulk flow is both unidirectional and parallel to the epithelium and odorants must diffuse over a greater distance to reach the mucus layer. If vortices can be initiated, the net result of coughing would be to increase transport of odorant to the mucus layer.

Chemoreception in a patchy environment

Diffusion of odorants is limited to short distances in water. Consequently, fish are not typically presented with simple odorant gradients to follow, but must instead rely on information derived from constantly changing odorant patches (Atema, 1988). The ability to cough allows fish to sample odorant patches efficiently and thus acquire specific and often ephemeral olfactory information. When odorant patches are small, as in the case of local and distinct pheromonal communication (Von Frisch, 1941; Quinn, 1980; Liley, 1982), coughing would constitute a more effective means of sampling odors than from the continuous circulation of water over the olfactory epithelium that accompanies respiration. Like sniffing in mammals, coughing would allow fish voluntarily to sample patches specifically and frequently. Gobies (*Bathygobius soporator*), for example, have been observed to cough predictably during courtship (Tavolga, 1956). However, the precise spatial or temporal olfactory information that is made available to these and other fishes through coughing remains to be shown.

In summary, the results presented here provide a variety of evidence suggesting

that flounders are capable of actively sampling odorants by coughing. Coughing thus provides an olfactory sampling mechanism in addition to water intake resulting from forward motion, ciliary motion or continuous pumping action tied to respiration. From the combined physiological, anatomical and behavioral investigations presented here, I further propose that coughing represents a behavior analogous to sniffing in certain air-breathing organisms. Whether olfactory coughing is limited to cyclosmate fishes, or extends to isosmate groups with limited accessory olfactory sacs, remains to be shown.

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