Three-dimensional structure of the nasal passageway of a hagfish and its implications for olfaction

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Running title: Olfactory implications of hagfish nasal anatomy

 Abstract

From high resolution (65 μm) data acquired by magnetic resonance imaging, we have reconstructed the nasal passageway of a hagfish (probably *Eptatretus stoutii*). We have used this reconstruction to investigate how the anatomy and morphometry of the nasal passageway influence the olfactory ability of the hagfish. We found that the long, broad section of the passageway preceding the nasal chamber will delay the response to an odour by about 1 s. Diffusion of odorant to the olfactory epithelium, upon which the olfactory sensitivity of an animal depends, will be favoured by the relatively large surface area of the olfactory epithelium (approximately 115 mm^2) and a modest expansion in the nasal chamber. Oscillating flow (0.3 - 0.4 Hz) within the narrow (65 - 130 μm) sensory channels of the nasal chamber is laminar (Reynolds number approximately five) and quasi-steady (Womersley number less than one). Distribution of flow over the olfactory epithelium may be aided by: a) a constriction prior to the nasal chamber; b) partial blockage of the nasal passageway by a protrusion on the central olfactory lamella; and c) the inward inclination of the olfactory lamellae. Unexpectedly, we found several anatomical features that may assist the monorhinal hagfish locate the source of an odour.

Introduction

Transport of odorant from the external environment to the olfactory epithelium is the first key step in animal olfaction (Craven et al., 2010, p. 933). In animals where the olfactory epithelium is housed in a nasal chamber, the transport process involves bulk movement of fluid into the nasal chamber, bulk movement of fluid over the olfactory epithelium, and finally diffusion of odorant from the moving fluid to the olfactory epithelium. Bulk movement of fluid is orchestrated by one or more pumps (e.g. Zeiske et al., 1992, p. 22).

The olfactory ability of an animal is critically dependent on the odorant transport process. For example, how quickly an animal responds to an odour will depend on how fast fluid passes through any duct linking the nasal chamber to the external environment. The sensitivity of an animal to an odour will depend on efficient capture of odorant as fluid passes over the olfactory epithelium. Factors influencing capture include: 1) the nature of the flow, i.e. whether it is laminar or turbulent (Levich, 1962, p. 39 and p. 139); 2) the degree to which flow is unsteady (Loudon and Tordesillas, 1998, p. 73); 3) the surface area of the olfactory epithelium (Schmidt-Nielsen, 1997, p. 586); 4) thorough distribution of flow over the olfactory epithelium; and 5) the time available for diffusion (LaBarbera and Vogel, 1982, p. 56). In addition, the ability of an animal to locate the source of an odour will be assisted by two nasal chambers, which will permit spatially discrete comparisons of the dispersed odorant (e.g. Gardiner and Atema, 2010).

There have been very few studies of odorant transport in animals (Craven et al., 2010, p. 934). Here, we have begun to investigate the odorant transport process in a hagfish, using a reconstruction of its nasal passageway. We chose a hagfish because it has a relatively simple, well-developed internal nasal architecture. In addition, the hagfish is an ideal subject for magnetic resonance imaging (MRI), since its narrow eel-like head will fit into a small diameter radio-frequency coil, allowing acquisition of high resolution data (Callaghan, 1991, p. 173). A hagfish has, however, only one olfactory organ, which presumably will limit its ability to detect an odour source. One unexpected finding of our study relates to this presumed limitation.

Olfaction appears to play an important part in hagfish behaviour. For example, hagfish respond quickly, and from some distance, to the arrival of food on the seabed (Foss, 1968, p. 9; Braun and Northcutt, 1998, p. 513), which they approach from downstream (Strahan, 1963, p. 18), sometimes in a zigzag fashion (Martini, 1998, p. 71), suggesting an ability to track scent (Porter et al., 2007, p. 27). On the other hand, they can have difficulty locating the source of an odour (Strahan, 1963, p. 18), a consequence perhaps of their single olfactory organ. Furthermore, the olfactory sensitivity of hagfish appears to be low (10^{-5} M; Døving and Holmberg, 1974, Fig. 2B) compared with keen-scented fish, such as sharks (10^{-10} M; Tricas et al., 2009) and eels (10^{-18}M; Teichmann, 1959, Table 12).

Both the gross anatomy and the fine structure of the olfactory organs of the two principal hagfish genera, *Myxine* and *Eptatretus*, have been outlined by Theisen (1973, 1976). The nasal passageway consists of a single nostril, a nasal duct, a nasal chamber, and a nasopharyngeal duct (SN, ND, NC and NPD, respectively, Fig. 1A).[1] The nasal

duct is supported by a series of cartilaginous rings (Johansen and Strahan, 1963, pp. 353-354). A junction region connects the nasal duct to the nasal chamber, and contains a structure described by Theisen (1973, p. 272; 1976, p. 168) as a 'valve' (JF, Figs. 1A and 1C).

The nasal chamber itself contains seven closely packed olfactory lamellae arranged in parallel (La, Fig. 1B).[2] Each lamella is attached to the roof and posterior wall of the nasal chamber. In *Eptatretus stoutii* and *Eptatretus deani*, the central lamella is described as having two ventral 'extensions' on its anterior edge (P, Fig. 1C; Theisen, 1976, p. 168 and Fig. 1A). These 'extensions' are also apparent in Figure 32.4B of Braun and Northcutt (1998). In both *Myxine* and *Eptatretus*, the olfactory epithelium lines the sides of the lamellae and the two lateral walls of the nasal chamber (OE, Fig. 1B; Theisen, 1973, p. 273; Theisen, 1976, p. 169), creating eight 'sensory' channels (SC, Fig. 1B). Olfactory epithelium is not, however, found on the free edges of the lamellae, or in the 'furrows' between lamellae (Fig. 1B; Theisen, 1973, p. 273; Theisen, 1976, p. 169). The olfactory epithelium in hagfish comprises ciliated and microvillous receptor cells interspersed among supporting cells (Theisen, 1973, Fig. 19; Theisen, 1976, pp. 169-170). Non-sensory cells bearing motile cilia are not present (Theisen, 1973, p. 281). Therefore flow through the sensory channels cannot be driven by beating cilia, as it appears to be in other fish (Bashor et al., 1974; Døving et al., 1977). Whether a mucus layer covers the olfactory epithelium is not known.

Bulk movement of fluid through the nasal passageway is generated by the furling and unfurling of two scrolls of tissue collectively known as the velum, which occupies its own chamber between the nasopharyngeal duct and the gills (V and VC, Fig. 1A;

Strahan, 1958). The velum is responsible for ventilating both the nasal chamber and the gills. In particular, the velum must, in the absence of cells bearing motile cilia, be solely responsible for driving fluid through the sensory channels of the nasal chamber.

Although hagfish have a well-developed olfactory system, they have other sensory organs too, including a 'crown' of tactile nasal and oral barbels (NB and OB, Fig. 1A; Worthington, 1905, p. 650), a pair of inner ears (Jørgensen, 1998) and a pair of poorly developed eyes (Locket and Jørgensen, 1998). In addition, *Eptatretus* has a primitive lateral line system (Braun and Northcutt, 1997). Of particular relevance to this report are the Schreiner organs, taste bud-like chemosensors found on the surface of the head, in the oral region, throughout the nasal passageway, and in the velar chamber (Braun, 1998).

We have performed a high resolution (65 μm) MRI scan on the head of a hagfish and from this scan reconstructed the three-dimensional structure of its nasal passageway. We have then used this reconstruction to develop the anatomical work of Theisen (1973, 1976) and to analyse the morphometry of the nasal passageway (e.g. volumes of individual regions, and the variation in transverse cross-sectional area). In association with known respiratory data, we have subsequently used the morphometry data to determine the nature of flow in the sensory channels. Our results have enabled us to comment on the hagfish's olfactory ability, including its response time, sensitivity and ability to locate an odour.

Materials and Methods

Specimen

The adult hagfish specimen used for MRI is from the teaching laboratory in the Department of Biology and Biochemistry at the University of Bath. The specimen has been preserved for at least 35 years. It is currently stored in an aqueous solution consisting of 4.5% propane-1, 2-diol, 2% formaldehyde and 0.5% 1-phenoxy-2-propanol. Its eye spots are no longer visible and the exact number of its slime pores is difficult to count. The total length of the specimen (Fernholm and Hubbs, 1981, p. 70) is 29 cm. There is no information on when or where the specimen was collected.

Magnetic resonance imaging

Magnetic resonance imaging was performed with the hagfish specimen immersed in the perfluoropolyether Fomblin Y04 (Solvay Solexis, Milan). Fomblin has two advantages. First, it lacks hydrogen atoms and therefore does not give a proton signal in MRI. Consequently, the field-of-view may be reduced, making data acquisition more efficient (in an aqueous medium the proton signal from the surrounding water would increase the field of view). Second, Fomblin limits interfacial artefacts arising from differences in magnetic susceptibility between the specimen and the fluid it is immersed in (Scotland et al., 1998, p. 1306).

The specimen is, however, less dense than Fomblin and therefore floats on it. To ensure full immersion, the specimen was first placed in a screw-cap cylindrical glass tube (28 mm internal diameter) half-filled with Fomblin, and pushed back into the fluid with plastic film stretched across the neck of the tube. More Fomblin was added to the upright tube through a small hole in the plastic film until the tube was full. The specimen was kept at an angle of about 45°, head upwards, for several hours to encourage release of any air bubbles trapped in the nasal passageway. The tube was then inserted at a slight angle, tail uppermost, into a horizontal birdcage radio-frequency coil (35 mm internal diameter). In this arrangement, the tail pressed against the screw cap while the head, importantly, did not touch the side of the tube (a previous MRI scan, in which the head *was* in contact with the side of the tube, revealed a distorted nasal duct).

Imaging was performed essentially as described by Holmes et al., (2008, pp. 507-508). A T_1-weighted data set was collected over 40 hours on a Bruker Biospec Avance system using a 7T horizontal 30 cm bore magnet. A Bruker micro-imaging gradient insert (BG-6, 60 mm internal diameter), was used with 100 A amplifiers, giving a maximum gradient of 1,000 mT m^{-1}, with a rise time of 50 μs. T_1-weighted images were acquired using a FLASH pulse sequence (Callaghan, 1991, p. 148) with the following parameters: echo time 3.6 ms; repetition time 40 ms; flip angle 30°; 90 averages; spectral width 100,000 Hz; field-of-view 4.4 x 1.3 x 1.3 cm^3 [L x W x H]; acquisition matrix 676 x 200 x 200. The resolution of the resultant data set was 65 x 65 x 65 μm^3. Prior to Fourier transformation the data set was zero-filled by a factor of two in the x, y and z directions. Zero-filling resulted in the appearance of artefacts in

some images (e.g. Ar, Fig. 2J). The MRI scan is available as an IMG file comprising 400 16-bit images of the hagfish's head in longitudinal section.

Regions of the nasal passageway

We define the various regions of the nasal passageway as follows (Figs. 1A and 1C). The nasal duct extends from the beginning of the nasal passageway (where a distinct duct is first formed) to the point just anterior to the dorsal spur (arrows, Figs. 1A and 1C). The junction region extends from the dorsal spur to the point just anterior to the protrusion of the central lamella (P, Fig. 1C). The nasal chamber extends from the anterior tip of this protrusion to the most posterior part of the olfactory lamellar array (the point at which the sensory channels vanish), and includes the incipient nasopharyngeal duct. The nasopharyngeal duct continues as a discrete duct posterior to the nasal chamber.

Reconstruction of the nasal passageway

The nasal passageway was reconstructed by two methods. The first gave a model (Model 1) that was used to investigate the anatomy of the nasal passageway and for some morphometric analysis. This is the model shown in Figures 3-5. The second method gave a model (Model 2) that was used exclusively for morphometric analysis. Both models span approximately the first 20 mm of the nasal passageway, and include the nasal duct, the junction region, the nasal chamber and part of the nasopharyngeal

duct. Close inspection of the models shows that they have essentially the same three-dimensional structure.

The reconstruction process for each model is described below. In both cases, the raw MRI data were converted to 8-bit images prior to processing.

a) Model 1

Reconstruction of the nasal passageway was performed using the image processing software ScanIP (Simpleware Ltd, Exeter, UK; Young et al., 2008). Six hundred and three transverse images of the raw MRI data, corresponding to the first 19.6 mm of the nasal passageway, were segmented (Soille, 1999, pp. 6-8). Segmentation was accomplished using the Threshold segmentation tool. Surrounding tissue with similar greyscale values segmented in the same step was disconnected from the nasal passageway using the Paint segmentation tool. Other regions in the head with similar greyscale values (e.g. the palatine bars, PB, Fig. 2G; Peters, 1963, Fig. 9) were disconnected using the Floodfill segmentation tool. The Paint segmentation tool was also used to fill large gaps in the lamellae that had probably been caused by metallic fragments (see 'General comments on the MRI scan', Results). Where these gaps spanned several lamellae, the bilateral symmetry of nasal passageway guided the painting process. Additionally, small gaps in the lamellae and sensory channels were filled using the Morphological Close filter, dilating and eroding (Soille, 1999, p. 49) with a structuring element one voxel thick in the x, y and z directions. The segmented data were exported as an STL file, with the pre- and post-smoothing options set to 20 and 30 iterations, respectively. Volume preservation was ensured by comparing the

original and smoothed models, which showed a negligible (0.35 %) difference in internal volume.

b) Model 2

Reconstruction was performed essentially as described by Craven et al., (2007, pp. 1328-1329) from 600 transverse MR images, corresponding to the first 19.5 mm of the nasal passageway. First, the raw MRI data were digitally processed. Noise was reduced by applying a 3 x 3 median filter (Gonzalez and Woods, 2002, pp. 123-124). Contrast was enhanced by applying a linear contrast stretch (Gonzalez and Woods, 2002, pp. 85-86) followed by a 'controlled' saturation of 10 % of the brightest pixels in each image. Next, the images were segmented. Automatic algorithmic segmentation was effective at detecting edges and contours surrounding the parts of the nasal passageway with a relatively simple geometry (the nasal and nasopharyngeal ducts). Manual segmentation was necessary, however, to correct errors caused by the algorithmic schemes in the geometrically complex parts of the nasal passageway (the junction region and the nasal chamber), and to remove artefacts in the data (see Model 1, above). The bilateral symmetry of the nasal passageway was again used as a guide to manually segment the large (probably metal-induced) gaps in the lamellae. Finally, the surface of the nasal passageway was generated from the segmented data by a modified form of the Marching Cubes algorithm (Lorensen and Cline, 1987), smoothed as described by Craven et al., (2007, p. 1329), and exported as an STL file. The original and smoothed models showed a negligible (0.12%) difference in internal volume.

Morphometric analysis

Morphometric analysis of the nasal passageway was generally performed according to the methodology of Craven et al., (2007, pp. 1329-1330). The transverse cross-sectional area (A), wetted perimeter (P) and hydraulic diameter (D_h, where $D_h = 4A/P$; White, 2003, p. 376) were calculated from the segmented images that had led to Model 2. Calculations were performed at 32.5 μm intervals. The uncertainty associated with the segmentation that resulted in Model 2 was estimated to be ± one or two voxels. In the geometrically complex regions of the nasal passageway, this uncertainty led to an error of 10 - 15 % in A and D_h; in the less complex regions, the error in these parameters was on the order of 5 %. The perimeter was little affected by the uncertainty in segmentation (~ 0.5 % error). Variation in the transverse cross-sectional area and the perimeter of the nasal passageway was independently confirmed by an analysis of the raw MRI data using ImageJ (http://rsb.info.nih.gov/ij).

Internal volumes were calculated for the nasal duct, junction region and nasal chamber by the methodology of Craven et al., (2007, pp. 1329-1330), and confirmed using ScanIP (Simpleware Ltd, Exeter, UK). The surface area of the olfactory epithelium was estimated using Rhinoceros (Robert McNeel and Associates, Version 3.0) as follows. The outline of the olfactory epithelium in transverse MR images at 32.5 μm intervals was traced with the Free-Form command (Curve menu: Free-Form → Control Points). The resultant curves for each image were extruded by 32.5 μm with the Extrude Curve command (Surface menu: Extrude Curve → Straight) and the cumulative area of the resultant surfaces calculated with the Mass Properties

command (Analyze menu: Mass Properties → Area). The total surface area of the olfactory epithelium was the sum of the cumulative areas. This estimate for the surface area of the olfactory epithelium includes the sides of the olfactory lamellae, together with the lateral walls of the nasal chamber opposing the two outermost lamellae. The estimate does not include the free edges of the lamellae. Nor does it include the surface associated with the protrusion of the central lamella, because this surface is covered with Schreiner organs (Braun and Northcutt, 1998, Fig. 32.4B). The estimate was supported by similar estimates made using the methodology of Craven et al. (2007, pp. 1329-1330) and ScanIP (Simpleware Ltd, Exeter, UK).

Variation in Reynolds and Womersley numbers along the nasal passageway

Whether flow is laminar or turbulent is determined by the Reynolds number, *Re* (Vogel, 1994, pp. 84-88; Craven et al., 2007, p. 1330):

$$Re = \frac{D_h u}{\nu}$$

where u is the average flow speed and ν is the kinematic viscosity of the fluid, in this case seawater (salinity 35 ‰) at 10 °C ($\nu = 1.35 \times 10^{-6}$ m^2 s^{-1}; Denny, 1993, p. 63). This equation can, however, be rewritten as:

$$Re = \frac{4Q}{P\nu}$$

by substituting $D_h = 4A/P$ and $u = Q/A$ (Vogel, 1994, pp. 32-33), where Q is the volumetric flow rate through the nasal passageway. The modified equation shows that the Reynolds number is inversely proportional to the wetted perimeter (P) of the nasal passageway. We used this modified equation to calculate the Reynolds number at 32.5 µm intervals along the nasal passageway. The figure used for the volumetric flow rate was based on reported ventilation rates of hagfish, which vary according to the mass of the fish and the temperature of their surroundings (Steffensen et al., 1984, Table 1; Perry et al., 2009, p. 231). The mass of our specimen taken directly from its preservative is 45 g. Steffensen et al., (1984, Table 1) give a flow rate of approximately 1 ml min^{-1} at 7 °C for resting *Myxine glutinosa* of a similar mass. From Perry et al., (2009, p. 231), who give a value of 235 ± 33 ml min^{-1} kg^{-1} at 13 °C for resting *Eptatretus stoutii*, the flow rate in the nasal passageway of our specimen may be calculated as 10 ml min^{-1}, although this may be an overestimate, for reasons discussed by these authors. Therefore 5 ml min^{-1} at 10 °C was used as a reasonable estimate of the flow rate in the nasal passageway of the resting hagfish.

The Womersley number (Wo; Loudon and Tordesillas, 1998) characterises the degree to which a flow is unsteady. The Womersley number was calculated at 32.5 µm intervals along the nasal passageway using the equation (Loudon and Tordesillas, 1998, p. 64; Craven et al., 2007, p. 1330):

$$Wo = \frac{D_h}{2} \sqrt{\frac{2\pi f}{\nu}}$$

where *f* in this case is the breathing frequency of the hagfish. The maximum breathing frequency of *Eptatretus stoutii* at rest at 13 °C is 0.36 Hz, similar to other species of hagfish (Perry et al., 2009, p. 232), and this was the value used to calculate the Womersley number along the nasal passageway.

Endoscopy

Endoscopy was performed with a Nikon Coolpix 995 camera attached to a Hopkins 30° solid rod endoscope. Illumination was provided by a xenon light source (Baxter, model AR5500).

Results

Classification of specimen

The label on the vessel in which the hagfish was originally stored did not classify it precisely - it stated only that the specimen belonged to 'Myxinoidea', a synonym for the order Myxiniformes (Adam and Strahan, 1963, p. 2). However, based on the number of external gill openings (12), the number of dental cusps (approximately 40), the length of the dorsal nasal barbel (3 mm), the length of the pre-branchial region (65 - 66 mm) and the prominent ventral fin, the specimen is likely to be *Eptatretus stoutii* (Nelson, 1994, p. 28; Wisner and McMillan, 1990, p. 796, p. 798, Tables 1 and 6), a species which is relatively easy to acquire.

General comments on the MRI scan

The MRI scan encompasses the first 3 cm of the specimen's head, from the barbels to the velar chamber, but it does not include the gill regions (Fig. 2, main image, and Fig. 3). Individual features such as the dental cusps, inner ears, primitive eyes, optic nerves and velum may be readily distinguished, indicating good internal preservation of the specimen (see also Supplemental Material for an animation demonstrating most of these features). Large dark circular patches in some images (Figs. 2B and 2F) probably signify the presence of metallic fragments on the surface of or within the

specimen (a supposition supported by an X-ray micro-computed tomography scan of the specimen; RLA and JPLC, unpublished).

Initially, we were concerned that the hydrophobic fluid (Fomblin) in which the specimen was immersed during the MRI scan might cause the hydrophilic olfactory lamellae to aggregate. However, the lamellae are not aggregated and, apart from a small V-shaped gap, they appear to be regularly spaced (Figs. 2G and 2H). Indeed there is little difference in the spacing between the lamellae in this MRI scan and a preliminary MRI scan acquired with the specimen immersed in aqueous preservative (WMH and JPLC, unpublished). We assume that the regular spacing of the lamellae in Fomblin is due to two factors. First, the lamellae are partially constrained by their attachment to the roof and posterior wall of the nasal chamber. Second, the lamellae are supported and segregated by the static pressure exerted by Fomblin, which is almost twice as dense as water (Sianesi et al., 1994, Table 2). The V-shaped gap in the lamellae may be a postmortem change. It is not an air bubble, which would give a readily identifiable dumb-bell shape (Callaghan, 1991, Fig. 4.16).

The MRI scans were acquired with a T_1 weighting, i.e. the image intensity was weighted by the longitudinal relaxation time (T_1) of the tissue (Haacke et al., 1999, p. 356). Different tissues have different T_1 values depending on the concentration of macromolecules (Fullerton and Cameron, 1988, pp. 149-150), water binding and water content (Young, 1988, p. 85). In a T_1-weighted MRI scan, tissue with a short T_1 gives a high signal (brighter image), whereas tissue with a long T_1 gives a lower signal (darker image). Thus, it is possible to distinguish different tissue types. For example, in Figures 2G, 2K and 2L it can be seen that the olfactory epithelium has a

lower signal (longer T_1) than the lamellar tissue on which it is mounted, possibly indicating greater water content. The ability to discriminate the olfactory epithelium in individual MR images allowed us to make a reasonable estimate of its surface area ('Morphometric analysis', Materials and Methods).

Anatomy and morphometry of the nasal passageway

The reconstructed nasal passageway is shown in Figure 3. The nasal duct is 9 mm long and 3 - 4 mm wide, and is oval in transverse section (Figs. 2A and 2B). A longitudinal ridge and conical protrusion are present at the entrance to the nasal duct (LR1 and Co, Fig. 2, main image, and Figs. 2A and 4). A photograph of a living specimen of *Eptatretus burgeri* shows what appears to be a similar conical protrusion (Martini, 1998, Fig. 5.4c). Ridges arising from the cartilaginous rings that support the nasal duct are evident (CR, Fig. 2, main image, and Figs. 2L, 3A and 4A), as are the numerous Schreiner organs lining this duct (SO, Fig. 4A).

The junction between the nasal duct and the nasal chamber is characterised by several notable features (Fig. 5; see Supplemental Material for a virtual model of the junction region). The first of these features is a spur in the roof of the nasal duct that marks the beginning of the junction region (DS, Fig. 2, main image, and Figs. 2C, 3D, 4 and 5A; see also arrow, Figs. 1A and 1C). This spur can be seen in previous studies (Theisen, 1973, Fig. 1; Theisen, 1976, Fig. 1; Zeiske et al., 1992, Fig. 2.2; Braun, 1998, Fig. 8A), but it has not been commented on before.

The second notable feature in the junction region is a short fold that encircles the nasal passageway (JF, Figs. 2D-F, 2K, 2L, 3D, 4 and 5A). The fold stems from the dorsal spur as two separate sections (see also Braun, 1998, Fig. 8A) that descend sharply, at an angle of approximately 45° to the horizontal, meeting on the floor of the nasal passageway (Fig. 3D). The free edge of the fold creates an oblique pentagonal aperture with an elongated apex (PA, Fig. 5). The posterior edge of the fold overlaps the protrusion on the central lamella (described below), leaving a narrow gap between the two (Gp, Fig. 2F; white square, Fig. 3D). The posterior surface of the fold forms two lateral channels that meet underneath the protrusion (PC, Figs. 2E, 2F, 3A, 3B and 5B; white disks, Fig. 3D). One of these posterior channels is visible in Figure 1A of Theisen (1976; see also Fig. 1C in this report), but again was not mentioned.

Theisen (1976, p. 168) described the junctional fold as a 'valve', which suggests that it controls the overall direction of the flow, and that it has a degree of mobility. However, the unidirectional, albeit rhythmic, nature of flow in the nasal passageway arises from the pumping action of the velum, which drives flow continuously through the nasal passageway (Gustafson, 1935, p. 5) - there is no need for a valve. Furthermore, it is difficult to envisage any significant mobility in the junctional fold given its limited extent. Therefore we do not believe that the junctional fold acts as a valve.

The third notable feature in the junction region is a second longitudinal ridge on the floor of the nasal passageway (LR2, Figs. 2E and 2F). This ridge creates two broad shallow channels anterior to the protrusion of the central lamella (BC, Figs. 2E and

2F). In fact this ridge may be the slender structure to the right of the vertical line labelled 'v' in Figure 1A of Theisen (1976; redrawn as Fig. 1C in the present report).

The final notable feature in the junction region is a constriction created by the junctional fold and the inwardly inclined walls and floor of the nasal passageway. The constriction is evident in the shape of the nasal passageway (Cs, Figs. 2K, 2L, 3A and 3B) and the decrease in transverse cross-sectional area in this region (Figs. 2D and 6A).

The volumes of the nasal duct and junction region are 72 and 11 mm^3, respectively. Therefore, at a volumetric flow rate of 5 ml min^{-1} (see 'Variation in Reynolds and Womersley numbers along the nasal passageway', Materials and Methods), fluid entering the nasal passageway will take about 1 s to reach the nasal chamber.

The nasal chamber comprises the seven expected olfactory lamellae, and the incipient nasopharyngeal duct (Figs. 2G and 2H; see Supplemental Material for a virtual model of the nasal chamber). The nasopharyngeal duct forms a broad channel ventral to the lamellar array; the same duct in *Myxine* species, on the other hand, is exceedingly narrow (Fig. 1B; Theisen, 1973 and 1976, Figs. 2 and 1B, respectively). Apart from the V-shaped gap (see 'General comments on the MRI scan', above), the lamellae are closely packed and uniformly spaced (Fig. 2G). The outer lamellae are inclined towards the centre of the array in both transverse (Fig. 2G) and horizontal sections (Figs. 2K and 2L), in a similar fashion to *Myxine glutinosa* (Fig. 1B; Theisen 1973, Fig. 2; Fänge, 1998, Fig. I.1). Where the lamellae are attached to the roof and posterior wall of the nasal chamber, the sensory channels are slightly enlarged (En,

Fig. 2K); this enlargement is also evident in Figure 8 of Cole (1913), which shows a transverse section through the nasal chamber of a specimen of *Myxine*. We were unable to detect the interlamellar ridges which Theisen (1973, Fig. 2) observed in the posterior part of the nasal chamber of *Myxine glutinosa* (R, Fig. 1B).

The seven olfactory lamellae are mainly responsible for the striking increase in the perimeter of the nasal passageway evident in the nasal chamber region (Fig. 6B). The estimated surface area of the olfactory epithelium, including that present on the lateral walls of the nasal chamber (Figs. 1B and 2L), is approximately 115 mm^2. The volume of the nasal chamber is 37 mm^3. The estimated depth of the sensory channels (Fig. 1B, inset) is 65 - 130 μm. Transverse cross-sectional areas increase markedly in the nasal chamber (Fig. 6A); the increase correlates with an anterior-to-posterior expansion in the width of the nasal chamber (Figs. 3A and 3B). The maximum transverse cross-sectional area of the nasal passageway is found towards the rear of the nasal chamber (Fig. 6A).

As noted by Theisen (1976, p. 168; see also Braun and Northcutt, 1998, Fig. 32.4B), the anterior edge of the central lamella projects from the lamellar array (Fig. 2K). Furthermore, the anterodorsal edge of this lamella extends into the junction between the nasal duct and the nasal chamber (ADE, Figs. 2D-F and Fig. 3D), terminating just anterior to the dorsal spur. But the most prominent feature of the central lamella, visible even to the naked eye, is a large protrusion situated on its anteroventral edge (P, Figs. 2F, 2L, 3D, 4 and 5; see also Fig. 1C). The protrusion, which has a triangular frontal aspect (Fig. 5A) and appears spade-like in transverse and horizontal cross-sections (Figs. 2F and 2L), spans the three central lamellae (Fig. 5A) and partially

obstructs the incipient nasopharyngeal duct (Fig. 2, main image, and Fig. 3D). Although Theisen (1976, p. 168) refers to the protrusion as two ventral 'extensions' of the central lamella, in the present specimen this feature is a single, discrete protrusion from the anterior edge of the central lamella. The protrusion is missing in *Myxine* (Theisen, 1973, Fig. 1), or much reduced (Theisen, 1976, p. 168).

Variation in Reynolds and Womersley numbers along the nasal passageway

Reynolds numbers throughout the nasal passageway are low (< 30; Fig. 6C), indicating laminar flow (Vogel, 1994, pp. 84-85). The profiles of Reynolds numbers and perimeters are inverted versions of each other (compare Figs. 6B and 6C), because the Reynolds number is inversely proportional to the perimeter (see 'Variation in Reynolds and Womersley numbers along the nasal passageway', Materials and Methods). Reynolds numbers in the nasal chamber ($Re \sim 5$) are significantly lower than in the remainder of the nasal passageway (Re typically 20 - 28), and are comparable with the upper limit of Reynolds numbers estimated for flow within the sensory channels of other fish ($Re \sim 4$; Cox, 2008, Table 2).

Womersley numbers in the nasal chamber are less than one (Fig. 6D), as would be expected from a periodic flow of low frequency in narrow channels (Loudon and Tordesillas, 1998, p. 73). Thus flow in the nasal chamber is likely to be 'quasi-steady', i.e. the oscillating velocity profile is parabolic, with the greatest amplitude in the centre of the sensory channels (Loudon and Tordesillas, 1998, Fig. 2). Womersley numbers in the remainder of the nasal passageway are, on the other hand, greater than

unity. Therefore, in the regions anterior and posterior to the nasal chamber, velocity profiles are not parabolic (Loudon and Tordesillas, 1998, Fig. 2) and flow is inherently unsteady.

Discussion

Transport of odorant in the nasal passageway

We begin by noting that the velar pump, which is responsible for driving flow through the nasal passageway of a hagfish, generates oscillating flow with a frequency range of 0.3 - 0.4 Hz (Perry et al., 2009, p. 232), similar to the frequency range associated with other biological positive displacement pumps (~ 1 Hz, Loudon and Tordesillas, 1998, p. 73).

Our results show that the nasal duct is long and broad, consistent with its function as a conduit for the bulk movement of fluid from the external environment to the nasal chamber (LaBarbera and Vogel, 1982, p. 57). The dimensions of the nasal duct will, however, delay the arrival of odorant in the nasal chamber by about 1 s, increasing the response time to an olfactory stimulus and therefore adversely affecting the hagfish's olfactory ability. The need for the nasal duct at all may be connected with the high density of Schreiner organs lining it (SO, Fig. 4A; Braun, 1998, Fig. 9). Transport of whatever chemical stimulus these sensory organs respond to will be affected by the unsteady (but laminar) flow (*Wo* ~ 2) within this duct (Loudon and Tordesillas, 1998, Fig. 2).

Figures 3 and 6 show that the nasal chamber is shorter (4 mm) than the nasal duct (9 mm), as would be expected of a biological fluid transport system (LaBarbera and Vogel, 1982, p. 57). The sensory channels are narrow (65 - 130 μm), favouring the

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flux of odorant molecules to the olfactory epithelium, which is likely to be inversely proportional to some power of the depth of each channel (Levich, 1962, p. 116). It is notable that the depth of the sensory channels in *Myxine glutinosa* (approximately 40 μm; Theisen, 1973, Fig. 2), and in other fish (10 - 70 μm; Cox, 2008, Table 2; 10 - 150 μm; Abel et al., 2010, Table A1) is similar to that of the specimen described here. Transport of odorant from the circulating fluid to the olfactory epithelium will also be governed by the laminar (Re ~ 5), quasi-steady flow ($Wo < 1$) in these channels (Loudon and Tordesillas, 1998, p. 73). Loudon and Tordesillas (1998, Fig. 7) suggest that the magnitude of the maximum velocity gradient 'at the wall' (in this case at the surface of the olfactory epithelium) is greatest for $Wo < 1$, which would favour odorant transport (Vogel, 1994, p. 197). The transport process should also be favoured by the increase in the transverse cross-sectional area seen in the nasal chamber (Fig. 6A) which, according to the principal of continuity (Vogel, 1994, pp. 32-34), will reduce the average flow speed in the nasal chamber, and therefore increase the time available for diffusion to the olfactory epithelium (LaBarbera and Vogel, 1982, p. 58). That said, fluid spends more time (~ 1 s) in transit to the nasal chamber than it does within it (0.4 s), the opposite of what might be expected of an efficient fluid transport system (LaBarbera and Vogel, 1982, p. 56).

The estimated surface area of the olfactory epithelium of the hagfish specimen is 115 mm^2, similar to estimates that can be made from drawings of the nasal chamber of *Myxine glutinosa* (90 mm^2, Theisen, 1973, Fig. 1), *Eptatretus stoutii* (220 mm^2, Theisen, 1976, Fig. 1A) and *Myxine circifrons* (180 mm^2, Theisen, 1976, Fig. 1B). The estimate of the olfactory sensory surface area of the hagfish specimen is also similar to the total olfactory sensory surface area of a minnow, *Phoxinus phoxinus* (68

mm^2; Teichmann, 1954, Table 1), a fish with a well-developed olfactory system (Teichmann, 1954, pp. 177-183) and very good olfactory sensitivity (Kleerekoper, 1969, Table 5). On the other hand, the estimate is one to two orders of magnitude less than the total olfactory sensory surface area of eels and sharks (727 mm^2, Teichmann, 1954, Table 12; ~ 100 cm^2, Kajiura et al., 2005, Fig. 6), two groups of fish with very well developed olfactory systems (Liermann, 1933, pp. 5-7; Theisen et al., 1986; Zeiske et al., 1987) and excellent olfactory sensitivity (Teichmann, 1959, Table 12; Tricas et al., 2009, Table 1). In any case, these sensory surface area comparisons alone suggest that hagfish have good olfactory sensitivity.

How flow is distributed over the olfactory lamellae in the hagfish is a question that has been previously addressed by Theisen (1976, p. 168). She suggested that the junctional fold acts in association with the protrusion of the central lamella (P and JF, respectively, Figs. 3D and 5A) 'to direct water into the spaces between the olfactory laminae [lamellae]'. In the absence of experimental and computational fluid dynamics, we can only make limited remarks on this issue: 1) the constriction in the junction region (Cs, Figs. 2D, 2K, 2L, 3A and 3B) will increase the average flow speed prior to the nasal chamber; 2) the protrusion of the central lamella will block flow entering the nasopharyngeal duct to some extent (P, Fig. 2, main image, and Figs. 3D and 5A); 3) the inclination of the outer lamellae (in the horizontal plane) towards the centre of the lamellar array (Figs. 2K and 2L) may help direct flow into the sensory channels. The enlarged sections of the sensory channels (En, Fig. 2K) may further influence flow in the nasal chamber. Enlarged sensory channels have also been observed in the olfactory lamellar arrays of other fish (Abel et al., 2010, p. 471).

In the above discussion, we have assumed that the hagfish is at rest. We have not taken into account the effect of swimming, or that of a current. We have also not taken into account the effect a mucus layer would have on transport of odorant to the olfactory epithelium.

Anatomical features that may assist location of an odour source

Although the single olfactory organ will limit the hagfish's ability to identify the source of an odour, there are several anatomical features within the nasal passageway that may offset this limitation by splitting inspired flow into two streams. These include the longitudinal ridge and conical protrusion at the entrance to the nasal duct (LR1 and Co, Fig. 2, main image, and Figs. 2A and 4), the dorsal spur and the two pairs of channels in the junction region (DS, BC and PC, Figs. 1-5), and the leading edge and protrusion of the central lamella (e.g. Fig. 5A). The presence of a dorsal longitudinal fold in the nasal passageway of *Myxine glutinosa* (Theisen, 1973, p. 272 and Fig. 1), and what appears to be a similar conical protrusion at the entrance to the nasal duct of *Eptatretus burgeri* (Martini, 1998, Fig. 5.4c) suggests that in these species too the limitation of the single olfactory organ may be offset. Curiously, the European flounder, *Platichthys flesus*, whose two olfactory organs are asymmetric in both structure and location, also has a cone-like feature in each incurrent nostril (Liermann, 1933, p. 15 and Fig. 8). Braun (1996, p. 263) has previously noted that 'lampreys and hagfish show hints of a paired olfactory system in their development and in the gross morphology of their olfactory organs and [nasal] ducts'. The hagfish study he was referring to, however, described a paired region in the posterior part of

the nasal duct of an embryonic specimen of *Myxine glutinosa* (Janvier, 1974, p. 195).

The features we describe relate to an adult hagfish.

Acknowledgements

Supplemental Material

The following are available as electronic supplemental material:

1. **Hagfish animation.mov** Animation showing the locations of the dental cusps (orange), inner ears (yellow), brain (pink), primitive eyes and optic nerves (both turquoise) within the head of the hagfish specimen.

2. **Junction.stl** Virtual model of the junction between the nasal duct and the nasal chamber, including the anterior part of nasal chamber. This file may be opened with ParaView (http://www.paraview.org/).

3. **Nasal chamber.stl** Virtual model of the nasal chamber. This file may be opened with ParaView (http://www.paraview.org/).

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Footnotes

[1]Note that there are alternative names for the various regions, e.g. the nasal duct is designated the prenasal sinus in some work (Braun, 1998).

[2]Although Zeiske et al., (1992, p. 14) state that there 'about' seven olfactory lamellae, all published diagrams of the nasal chambers of extant hagfish show exactly seven.

Figure Legends

Fig. 1. Schematic sections through the hagfish head. (**A**) Vertical section through the head of *Myxine glutinosa*. The dashed line marks the position of the transverse section shown in (**B**). Redrawn from Figure 2.2 of Zeiske et al., (1992). The vertical white band is a discontinuity that appears in the original figure. (**B**) Transverse section through nasal chamber of *Myxine glutinosa*. The location of this section within the head is shown by the dashed line in (**A**). The nasal passageway is red, the olfactory lamellae are white and the olfactory epithelium is dark grey. Inset: boxed region in main picture, indicating the depth of the sensory channel (d). Redrawn from Figure 2 of Theisen (1973). (**C**) Vertical section through the nasal passageway of *Eptatretus stoutii*. The entrance to the nasal duct is not shown. Redrawn from Figure 1A of Theisen (1976). Arrows: dorsal spur. d: depth of the sensory channel; Br: brain; CL: central lamella; DC: dental cusps; JF: junctional fold; Jn: junction region; La: lamella; LR2: possible location of the second longitudinal ridge described in the Results section; M: mouth; NB: nasal barbel; NC: nasal chamber; ND: nasal duct; NPD: nasopharyngeal duct; OB: oral barbel; OE: olfactory epithelium; P: protrusion of the central lamella; PC: peripheral channel; R: ridge; SC: sensory channel; SN: single nostril; Sp: spinal cord; V: velum; VC: velar chamber.

Fig. 2. MR images of the head of the hagfish specimen. Main image: vertical section midway through the hagfish head. (**A-J**) Transverse images. The right side of each transverse image corresponds to the right flank of the hagfish. (**K, L**) Horizontal images. The top of each horizontal image corresponds to the right flank of the hagfish.

Labels A to L in the main image indicate the location of the transverse and horizontal images. The location of the main image is marked on the top of images (**A**) to (**J**) and on the left side of images (**K**) and (**L**). (**A-B**) Nasal duct, anterior and mid-regions, respectively; (**C-E**) junction between the nasal duct and nasal chamber, anterior, mid- and posterior regions, respectively; (**F-H**) nasal chamber, anterior, mid- and posterior regions, respectively; (**I**) nasopharyngeal duct; (**J**) velar chamber. Arrowhead in (**G**): V-shaped gap in lamellar array. ADE: anterodorsal extension of central lamella; Ar: artefact arising from zero-filling of MR data (see 'Magnetic resonance imaging', Materials and Methods); BC: broad ventral channel in junction region; Br: brain; Co: conical protrusion at entrance to nasal duct; CL: central lamella; CR: cartilaginous ring; Cs: constriction in nasal passageway; DC: dental cusp; DS: dorsal spur; En: enlarged region of sensory channel; Ey: eye; Gp: narrow gap between junctional fold and protrusion of central lamella; JF: junctional fold; La: lamella; LR2: longitudinal ridge in junction region; M: mouth; MF: location of possible metallic fragment; ND: nasal duct; NP: nasal passageway; NPD: nasopharyngeal duct; OB: oral barbel; OE: olfactory epithelium; P: protrusion of central lamella; PB: palatine bar; PC: posterior channel in junction region; SC: sensory channel; SN: single nostril; VC: velar chamber; VS: velar scroll. The scale bar in (**A**) also applies to MR images (**B**-**L**).

Fig. 3. Reconstruction of the hagfish nasal passageway *in situ*. (**A**) Dorsal view. Grey: head region; blue: nasal passageway. The scale bar also applies to (**B**) and (**C**). (**B**) Ventral view. (**C**) Vertical section. The mouth region is not shown. (**D**) Boxed region in (**C**). Dotted line: dorsal extent of protrusion of central lamella. White square: gap between junctional fold and protrusion of central lamella. White disks: dorsal and ventral sections of a posterior channel in the junction region. ADE: anterodorsal

extension of central lamella; Co: conical protrusion at entrance to nasal duct; CL: central lamella; CR: ridge arising from cartilaginous ring; Cs: constriction in junction region; DS: dorsal spur; JF: junctional fold; Jn: junction region; NB: nasal barbels; NC: nasal chamber; ND: nasal duct; NPD: nasopharyngeal duct; OB: oral barbel; P: protrusion of central lamella; PC: peripheral channel; SC: sensory channel; SN: single nostril.

Fig. 4. Anterior views of the nasal passageway of the hagfish specimen. (**A**) Photograph from endoscopy. (**B**) Model of head. Co: conical protrusion at entrance to nasal duct; CR: ridge arising from cartilaginous ring; DS: dorsal spur; JF: junctional fold; Lp: dorsal lip of single nostril; LR1: longitudinal ridge at entrance to nasal duct; NB: nasal barbels; OB: oral barbel; P: protrusion of central lamella; SO: Schreiner organs. Scale bars are based on the width of the nasal duct at its entrance.

Fig. 5. Models of the junction between the nasal duct and the nasal chamber of the hagfish specimen. (**A**) Anterior and (**B**) posterior views. CL: central lamella; DS: dorsal spur; JF: junctional fold (lying between dashed line and white outline); La: lamella; P: protrusion of central lamella; PA: pentagonal aperture of junctional fold (white outlines); PC: posterior channel. Scale bars are based on the width of the protrusion of the central lamella (P).

Fig. 6. Variation in transverse cross-sectional area (**A**), perimeter (**B**), Reynolds number (**C**) and Womersley number (**D**) along the nasal passageway of the hagfish specimen. Jn: Junction region; NC: nasal chamber; ND: nasal duct; NPD: nasopharyngeal duct.