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The ventilation mechanism of the Pacific hagfish *Eptatretus stoutii*

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We made anatomical and physiological observations of the breathing mechanisms in Pacific hagfish *Eptatretus stoutii*, with measurements of nostril flow and pressure, mouth and pharyngo-cutaneous duct (PCD) pressure and velum and heart impedance and observations of dye flow patterns. Resting animals frequently exhibit spontaneous apnea. During normal breathing, water flow is continuous at a high rate ($\sim 125 \text{ ml kg}^{-1} \text{ min}^{-1}$ at 12°C) powered by a two-phase unidirectional pumping system with a fast suction pump (the velum, $\sim 22 \text{ min}^{-1}$) for inhalation through the single nostril and a much slower force pump (gill pouches and PCD $\sim 4.4 \text{ min}^{-1}$) for exhalation. The mouth joins the pharynx posterior to the velum and plays no role in ventilation at rest or during swimming. Increases in flow up to $>400 \text{ ml kg}^{-1} \text{ min}^{-1}$ can be achieved by increases in both velum frequency and stroke volume and the ventilatory index (product of frequency \times nostril pressure amplitude) provides a useful proxy for ventilatory flow rate. Two types of coughing (flow reversals) are described. During spontaneous swimming, ventilatory pressure and flow pulsatility becomes synchronised with rhythmic body undulations.

KEYWORDS

coughing, pharyngo-cutaneous duct, swimming, two-phase ventilation, velum, ventilatory stroke volume

1 | INTRODUCTION

The breathing mechanism of hagfishes (Class Myxini), which are arguably the extant vertebrates of the most ancient lineage, is fundamentally different from those in other fishes (Bartels, 1998; Johansen & Strahan, 1963; Malte & Lomholt, 1998). To date, most studies have been on the Family Myxinidae, with only two studies on the Family Eptatretidae (Coxon & Davison, 2011; Perry *et al.*, 2009b). For both families, there is general agreement that the rhythmic up and down movement of the scroll-like velum in the velum chamber of the anterior pharynx serves as the major ventilatory pump, inhaling the ambient water *via* the single anterior nostril duct to the pharynx. This idea that the velum is responsible for the ventilatory water current was first suggested by Cole (1905) based on anatomical studies. Gustafson (1935) and Strahan (1958) added a detailed description of the cartilaginous skeleton and muscles that constitute the velum chamber. Later, Johansen and Hol (1960) applied x-ray analysis with water-soluble contrast media and confirmed that the velum structure moved dorso-ventrally as the major pump for the ventilatory water current as Cole

(1905) had originally suggested. The inhaled water is then expelled through pairs of gill pouches (variable in number both within and between species) and a pharyngo-cutaneous gill duct (PCD). The Eptatretidae differ from the Myxinidae in having separate gill pouch openings to the outside on each side in addition to the exit of the common PCD on the left side *via* an enlarged posterior gill slit.

There have been only two direct measurements of ventilatory water flow in hagfishes, both using blood flow meters in novel configurations. Steffensen *et al.* (1984) measured a resting ventilatory flow rate of about $45 \text{ ml kg}^{-1} \text{ min}^{-1}$ in *Myxine glutinosa* L. 1758 at 15°C by mounting the flow-probe on a cone overlying the partially buried animal. Perry *et al.* (2009b) reported a much higher value of about $235 \text{ ml kg}^{-1} \text{ min}^{-1}$ in *Eptatretus stoutii* (Lockington 1878) at 12°C fitted with a flow-probe attached to a tube tied into the nostril of the non-buried animal. It is unclear whether these very different values reflect differences between the species or the methods used. Furthermore Perry *et al.* (2009b), using hypoxia and hypercapnia as experimental stimuli, concluded that changes in ventilatory flow rate occurred only *via* changes in velum frequency and that stroke volume did not vary.

This conclusion was supported by Coxon and Davison (2011) who recorded the ventilatory frequency response to temperature in *Eptatretus cirrhatus* (Forster 1801) by electromyography of velum contraction, but no direct measurements of ventilatory flow rate were made.

Other elements of the hagfish breathing mechanism also remain incompletely understood. For example, various researchers have questioned the role of gill pouches and their associated ducts as active pumps vs. passive conduits and have even raised the possibility of bidirectional water flow. For example, Goodrich (1930) suggested the gill pouches served as an active water pump in series with the velum chamber for unidirectional water flow and he believed that the gill pouches could even generate a bidirectional water current (inhalation and exhalation) when the nostril was occluded, as might for example occur during feeding. Strahan (1958), however, did not observe the active contraction of gill pouches in live *M. glutinosa* so he concluded that the role of the gill pouches was entirely passive. Subsequently, using x-ray analysis in the same species, Johansen and Hol (1960) observed contraction of the gill pouches during normal unidirectional ventilation. They concluded that water in the pharynx was expelled actively through the gill pouches while valve-like muscular sphincters located in the efferent and afferent gill ducts regulated the amount of expelled water and this was later accepted by Strahan (in Johansen & Strahan, 1963). Another uncertainty is the possible role of the mouth as a conduit, or even a pump, for inhalation; i.e., additional to the well accepted roles of the nostril and velum chamber for these functions. This does not appear to have been directly investigated, though in *M. glutinosa*, Johansen and Hol (1960) observed that x-ray contrast media injected via a tube in the mouth subsequently appeared in the gill pouches and gill ducts, suggesting a functional connection. Various types of flow reversals (coughing and sneezing) either spontaneously, or in response to waterborne particles, have been described in *M. glutinosa* (Johansen & Hol, 1960; Johansen & Strahan, 1963; Steffensen *et al.*, 1984; Strahan, 1958). However, in the Eptatretidae, to our knowledge, the potential roles of the gill pouches and the mouth in ventilation have never been investigated and flow reversals have not been described in members of this family (Coxon & Davison, 2011; Perry *et al.*, 2009b).

With this background in mind, the goal of the present study was to describe the basic breathing mechanisms in *E. stoutii* as a prelude to subsequent studies on its ventilatory responses to temperature (Giacomin *et al.*, 2018) and ammonia (J. Eom, M. Giacomin, A. Clifford, G. Goss and C. M. Wood, January 2019, unpublished data). We started with the simple assumption that any organ connected to the pharynx such as the nostril, the velum chamber, the mouth cavity and the pairs of gill pouches including the PCD exiting through the left posterior gill slit, could serve as ventilatory organs in *E. stoutii*. In order to understand the system, we dissected the animal, we collected water flow data from the nostril, pressure data from the nostril, mouth and PCD, impedance data from the velum chamber and the heart for velum and cardiac rates and we observed dye flow patterns. The focus was on normal ventilatory physiology in resting animals and how this changed during experimental disturbances, during ventilatory reversals (here referred to as nostril coughing and mouth coughing) and during spontaneous bouts of exercise.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

Pacific hagfish *E. stoutii*, body mass 53.9 ± 3.3 g, $n = 149$] were captured in August and September by 22 l sized Korean cone traps that were baited with strips of Pacific hake (*Merluccius productus* (Ayres 1855) and anchored on the bottom at a depth of 100 m in Trevor channel ($48^{\circ} 50.844' N$, $125^{\circ} 08.321' W$) located close to the Bamfield Marine Science Centre (BMSC), off the southwest coast of Vancouver Island, BC, Canada. The captured *E. stoutii* were housed at BMSC in fibreglass tanks (20 m³) served with flow-through seawater and furnished with PVC pipes for shelter. Although hake strips were provided, the animals generally did not feed during the 2 month period of holding. The animals were collected under permits from the Department of Fisheries and Oceans Canada (XR 202 2016 and 194 2017) and the experiments were approved by the University of British Columbia (UBC) (Animal Use Protocol A14-0251) and BMSC Animal Care Committees (AUP RS-17-20) and followed the guidelines of the Canadian Council of Animal Care. Holding and experimental temperature was 11–13°C and salinity was 30–31. After experimentation, animals were euthanised by an overdose of 5 g l⁻¹ neutralised MS-222 followed by evisceration to ensure death.

2.2 | Anatomical studies

In freshly euthanised *E. stoutii*, the head and gill pouch regions were dissected and photographed in order to describe the anatomy of the ventilatory system. In order to understand anatomical features of the mouth cavity, 9 ml of silicone (Silicone I, GE Sealants and Adhesives, Huntersville, NC, USA) dyed with red food colouring were injected into the anterior opening of the nostril cavity of euthanised *E. stoutii* and 3 ml of green or blue silicone into the anterior opening of the mouth cavity respectively. A plastic syringe with attached plastic tube that fit snugly into the respective openings was used for silicone injection. The fish were then immediately dissected to ascertain the distribution of the coloured silicone dyes. In order to further understand functional features of the hagfish mouth cavity, the fish were anaesthetised with MS-222 (see § 2.3), a #18-gauge needle was inserted at an angle of 45° through the ventral side of head into the mouth cavity, a 30 cm length of polyethylene tubing (PE160, OD 1.57 × ID 1.14 mm, Clay-Adams, Sparks, MD, USA) was inserted through the hole and then heat-flared using a butane lighter so it was properly secured inside the mouth cavity.

After the fish recovered from anaesthesia in flowing sea water, a 300 µl volume of red food dye was gently injected into the mouth cavity via the PE160 cannula and the fish was monitored over the following 24 h with a surveillance camera system (Pro-Series HD 720P, Swann Communications; www.swann.com). Food dye was also injected into the water in front of the nostril, in front of the mouth and in front of the gill pouch openings in order to visually observe the direction of ventilatory flow in *E. stoutii*. In order to study the effect of nostril blockage as might occur when the *E. stoutii* immerses its head in its prey, we manually occluded the nostril in a few animals and observed the fate of dye that had been injected into the pharynx via the PCD cannula described in the following section.

2.3 | Physiological recording from various sites in the cardio-respiratory system

Prior to operation for attachment of sensors, the fish were anaesthetised in 0.6 g l⁻¹ MS-222 (neutralised to pH 7.8 with 5 M NaOH) and placed on an operating table. As *E. stoutii* are very hypoxia-tolerant, it was not necessary to maintain gill irrigation. Simple silk sutures (26 mm 1/2C taper, perma-hand silk, Ethicon; www.ethicon.com) fastened to the skin were used to hold recording devices in place. The following recording devices were implanted in various combinations (Figure 1a), but not all devices were installed on all animals: (a) For impedance recording of velum movements, a pair of ~15 cm laminated copper wires (American Wire Gauge #32, Belden; www.belden.com), with ~1 cm stripped off their insulation at the recording ends were inserted under the skin as fish-hook electrodes, knotted externally and secured laterally to the skin around the velum chamber. The entries were made with a #21 gauge needle ~2 cm posterior to the simple eye spots. The other ends of the copper wires, also stripped of insulation, could be connected to an impedance converter (2991, Transmed Scientific, San Luis Obispo, CA, USA). (b) For impedance recording of heart contractions, a similar pair of copper wire electrodes were implanted ventrally under the skin around the heart (~1 cm posterior to the PCD). The *E. stoutii* were very sensitive to attachment of wires in the heart region, frequently showing anti-predation knotting behaviour, so the attached wires as well as other catheters etc. were easily tangled or displaced. Therefore, heart-rate recordings were taken from a separate group of *E. stoutii* fitted only with these heart electrodes. (c) In order to measure ventilatory flow, a 3 cm length of transparent silicone tubing (6.35 mm O.D. and 4.32 mm I.D.) was inserted to fit snugly into the nostril cavity and two stitches were made laterally to the skin to secure the tubing in the nostril cavity. The probe of a flow meter was connected directly to the front of the silicone tubing and used for measuring flow at the nostril associated with ventilation, a technique adapted from that of Perry *et al.* (2009b). (d) In order to measure ventilatory pressures at the nostril, a 3 cm length (non-flared) of PE160 polyethylene tubing was inserted 1 cm deep into the transparent silicone tubing of (c) and secured by two stitches to the silicone tubing. The inserted PE 160 tubing occupied 21.4% of the cross-sectional area of the silicone tubing. It could easily be removed and re-inserted with minimal disturbance to the hagfish, unlike a T-junction tubing tried earlier which was frequently tangled and displaced by the anti-predation knotting behaviour. The secured PE160 tubing in the silicone tubing could then be connected via a #18-gauge needle shaft to another ~30 cm water-filled PE160 tubing that was attached to a medical pressure transducer (DPT-100, Utah Medical Products; www.utahmed.com). (e) In order to monitor pressure events in the mouth cavity, a ~2-cm flared PE-160 catheter was fixed into the mouth, as described in § 2.2. This could then be attached via a #18-gauge needle shaft to a ~30 cm water-filled PE160 catheter connected to a pressure transducer. (f) In order to monitor pressure events in the PCD, a ~2 cm non-flared PE-160 catheter was gently inserted 1 cm deep into the PCD and secured in place by two stitches to the skin. As with the mouth and nostril catheters, this could then be attached via a #18-gauge needle shaft to a ~30 cm water-filled PE160 catheter connected to a pressure transducer.

The copper wire electrodes were connected to impedance converters (Model 2991, Transmed Scientific) to collect the frequency of the velum chamber or heart (beats min⁻¹). The PE160 catheters were connected to medical pressure transducers (DPT-100, Utah Medical Products) to collect ventilatory pressures (cm H₂O) and velum frequencies (min⁻¹) at the nostril, mouth and PCD. A microcirculation ultrasonic flow probe (V-series, Transonic Systems Inc.; www.transonic.com) was connected to a dual channel small animal blood flowmeter (T106 series, Transonic Systems Inc.) to monitor nostril water flow (ml kg⁻¹ min⁻¹) and velum frequency (min⁻¹) as well as the flow direction (inhalation or exhalation). Inhalation was recorded as negative flow and exhalation as positive flow. Ventilatory stroke volume (ml kg⁻¹ velum stroke⁻¹) was calculated as nostril water inflow divided by velum frequency. The measured analogue signals were amplified (Load Cell Amplifier signal conditional with Removable Terminals Connectors, Transducer Techniques; www.transducertechniques.com), converted to digital signals in a PowerLab data integrity system (ADInstruments; www.adinstruments.com) and were visualised and analysed in LabChart 7.0 software (ADInstruments). Two surveillance camera systems (Pro-Series HD 720P, Swann Communications) simultaneously recorded the *E. stoutii* (for monitoring of behaviour) and the computer screen (for monitoring physiological parameters) for later correlation of behaviour with interpretation of the simultaneously collected physiological data (Figure 1a).

Following implantation of recording devices, most of the *E. stoutii* ($n = 119$) were allowed to recover overnight in flowing anaesthetic-free seawater before measurements of nostril ventilatory variables. In order to collect simultaneous ventilatory signals in the nostril duct, mouth cavity, velum chamber and PCD at rest and during spontaneous swimming activity, another group of hagfish ($n = 3$) were allowed to recover for only 30 min before physiological recordings commenced. By this time, the *E. stoutii* had resumed their normal coiled posture and ventilatory flows and pressures were at normal resting levels. This much shorter recovery period was necessary to minimise tangling and extrusion of the multiple recording devices. The three animals from which the simultaneous multiple recordings were made successfully represent a small subset of the total number (> 10) that were attempted. In general, *E. stoutii* showed higher activity levels at night-time so the operations were performed in late afternoon and the recordings were made mostly at night. The recording area was screened from the general laboratory by black plastic sheeting but was next to a window and thus exposed to natural photoperiod.

2.4 | Calibration of the recording systems

The flow probe detected both the magnitude and direction of flow, so correct orientation was essential. In our recordings, negative values (*i.e.*, below zero flow) represent inhalation through the nostril and positive values represent exhalation, as occurs during coughing, for example. As noted by Perry *et al.* (2009b), the intrinsic calibration of the flow probe proved to be altered in seawater, so the probe was recalibrated by flowing salinity 30 seawater at 12°C through the probe at known rates (determined gravimetrically), using a peristaltic pump. Voltage outputs were converted into flow units (ml min⁻¹) by the

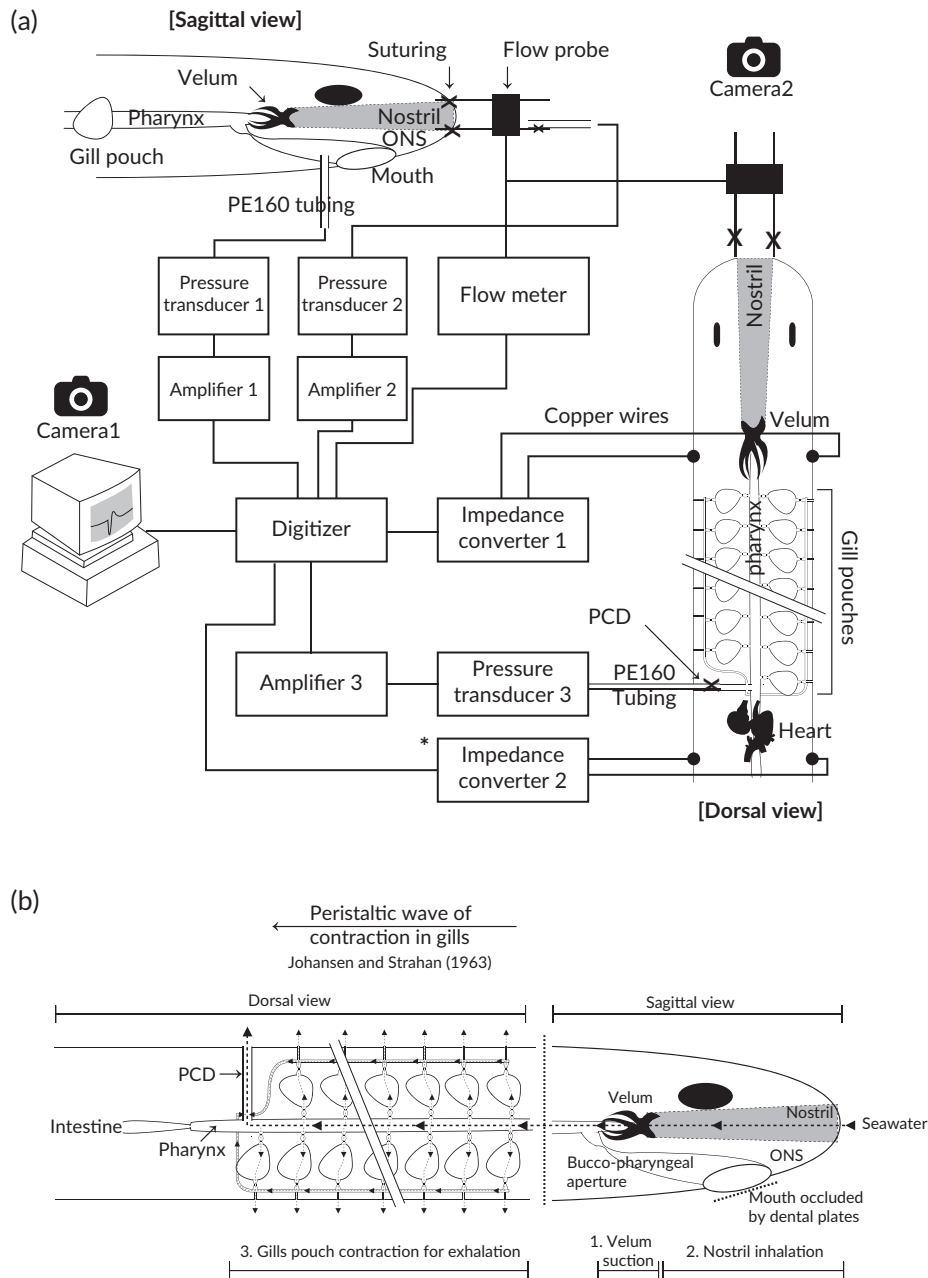


FIGURE 1 Schematic diagram of (a) recording attachments to nostril, mouth cavity, velum chamber, and pharyngo-cutaneous duct (PCD) and (b) a simple model summarizing our current understanding of the unidirectional ventilatory water flow mechanism in *Eptatretus stoutii* at rest and during swimming

LabChart software. The pressure transducers were zeroed to the water surface and calibrated with a column of water in the range of 0–4 cm H_2O . These units were used for parallelism with previous studies on ventilatory pressures in fish. Note that 1 cm H_2O is equivalent to 0.09801 kPa. The impedance measurements gave faithful recordings of frequency of velum and heart contractions but could not be used as indices of contraction strength because amplitude varied with the precise placement of the electrodes.

2.5 | Experimental procedures

Measurements of cardiorespiratory parameters in resting, undisturbed *E. stoutii* under control conditions (normoxic water, 12°C) were

available from 122 animals (99 for various ventilatory variables, 23 for heart rate). Many of these animals ($n = 119$) were subsequently subjected to treatments that altered ventilation such as exposure to various levels of ammonium bicarbonate (NH_4HCO_3) and ammonium chloride (NH_4Cl) in the water or injections of the ammonium salts in isotonic saline. Many also exhibited coughing and periods of spontaneous swimming. Recordings from these periods of altered ventilation were analysed to better understand the overall working of the ventilatory system. In addition, simultaneous measurements were obtained from the three animals fitted with nostril duct, mouth cavity, velum chamber and PCD recording devices for parallel recording at all these sites at rest, during coughing and during spontaneous exercise.

2.6 | Statistical analyses

Data have been reported as mean \pm SE. Nostril and mouth coughing were repeatedly generated in three animals by dye injection into the respective cavities and these same three animals were used for detailed analysis of the swimming responses. One-way repeated-measures ANOVA followed by Dunnett's test were applied to compare the changed respiratory variables against respective control values in GraphPad Prism 6.0 (www.graphpad.com). Relationships (with 95% confidence intervals and 95% prediction intervals) among various ventilatory variables were assessed by simple linear regression. The threshold for statistical significance was $P < 0.05$.

3 | RESULTS

3.1 | Anatomical observations

The mouth of the *E. stoutii* is a hollow sac-like structure located ventral to the single anterior nostril and separated horizontally from it by a thin sheet of muscle, which has been termed the oronasohypophyseal septum (ONS) in *Eptatretus burgeri* (Girard 1855) by Oisi *et al.* (2013). The entrance of the mouth can be sealed by bilaterally paired dental plates that are covered with the lingual teeth used for predation. When the plates are closed, inhalation of water through the mouth would appear to be impossible. The velum chamber, which consists of a complex cartilaginous skeleton and muscular structure that can be expanded and contracted, lies posterior to the nostril and is directly connected to it at the front end and to the anterior pharynx at the back end. Injections of red-dyed silicone into freshly-euthanised animals revealed that the nostril cavity leads directly to the velum chamber and then to the pharynx and gill pouches (Figure 2). Silicone injected into the nostril never appeared in the mouth. In contrast, injections of green or blue-dyed silicone into the mouth revealed that the mouth is not connected to the velum chamber but rather leads directly to the anterior pharynx, just posterior to the velum chamber. The injected dye in the pharynx exited mostly via the opening of the PCD posterior and to the left of the gill pouches and partially via the paired gill pouches. Thus, the nostril-velum-pharynx route and the mouth-pharynx route lie in parallel.

Injections of dye into the water close to the nostril of live hagfish indicated a steady inhalation powered by the contractions of the velum chamber and indeed the rhythmic velum contractions could sometimes be seen at the body surface. Dye inhaled through the nostril subsequently exited through the posterior gill slits and the PCD in a pulsatile fashion (Figure 3a). In some cases, rhythmic slow pulsatile contractions of the PCD could be observed visually, whereas movement of the paired gill pouches was never detected visually. However, in instances where the dye inhaled into the nostril induced nostril coughing, it was ejected both through the nostril and then through the PCD opening (Figure 3b). Dye placed in the water close to the mouth or directly injected into the mouth was not inhaled into the pharynx, therefore ejection of this dye never occurred through either the paired gill pouches, PCD opening, or nostril duct during normal ventilation. However, in instances where direct dye injection to the mouth cavity stimulated mouth coughing, the injected dye was

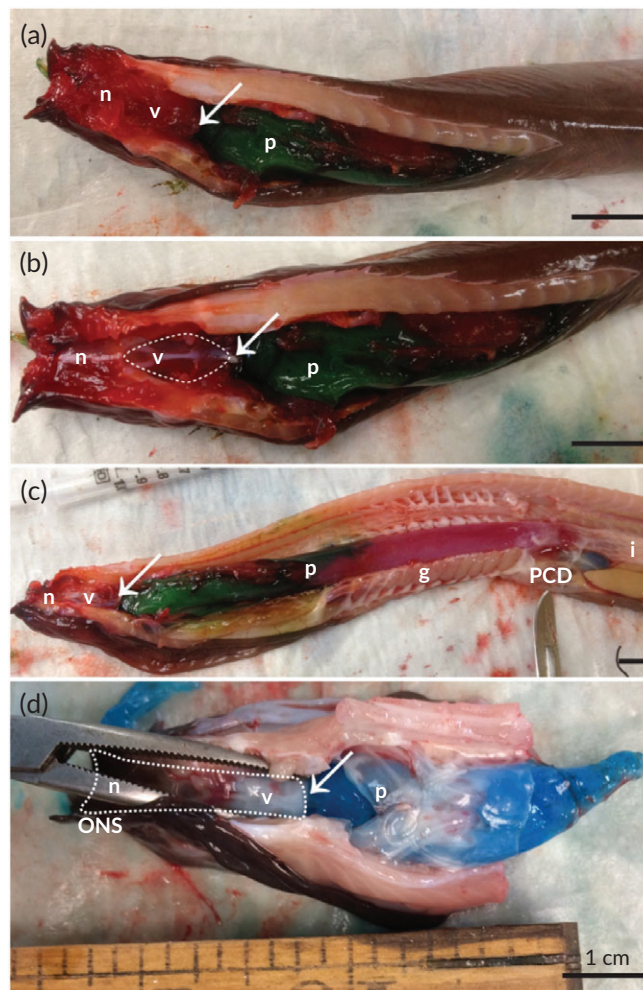


FIGURE 2 Dissection of a freshly euthanized *Eptatretus stoutii* injected with dyed silicone. (a) Red silicone injected into the nostril was subsequently found between the nostril (n) and velum chamber (v), as well as in the pharynx (p), part of the gill pouches (g) and the pharyngo-cutaneous duct (PCD). (b) The nostril cavity and velum chamber were exposed after removal of part of the red silicone. (c) Green silicone and (d) blue silicone injected into the mouth cavity was subsequently found in the anterior part of pharynx (p) but did not move forward to the velum chamber or nostril. The oronasohypophyseal septum (ONS) is depicted within the white dotted area. Scale bar 1 cm

immediately ejected to the ambient environment through the mouth (Figure 3c). When the injection did not induce mouth coughing, the dye was forced back up the injection tubing and stayed there more than 24 h (Figure 4), indicating that it was never inhaled in resting *E. stoutii*. Similarly, dye placed just outside the openings of the paired gill pouches and PCD was never inhaled. In addition, we briefly occluded the nostril in some *E. stoutii* (Figure 3d). These animals immediately showed muscular contraction (possibly by the pharyngeal constrictor muscle) in the ventral area, but this did not cause the injected dye in the pharynx (which had been previously injected via the PCD cannula) to exit through the gill pouches or PCD (Figure 3e). Eventually the fish violently coughed the dye out through the mouth (Figure 3f). After release of the nostril occlusion, the *E. stoutii* immediately initiated unidirectional ventilatory inflow through the nostril so the remaining dye in the pharynx was ejected through the gill pouches

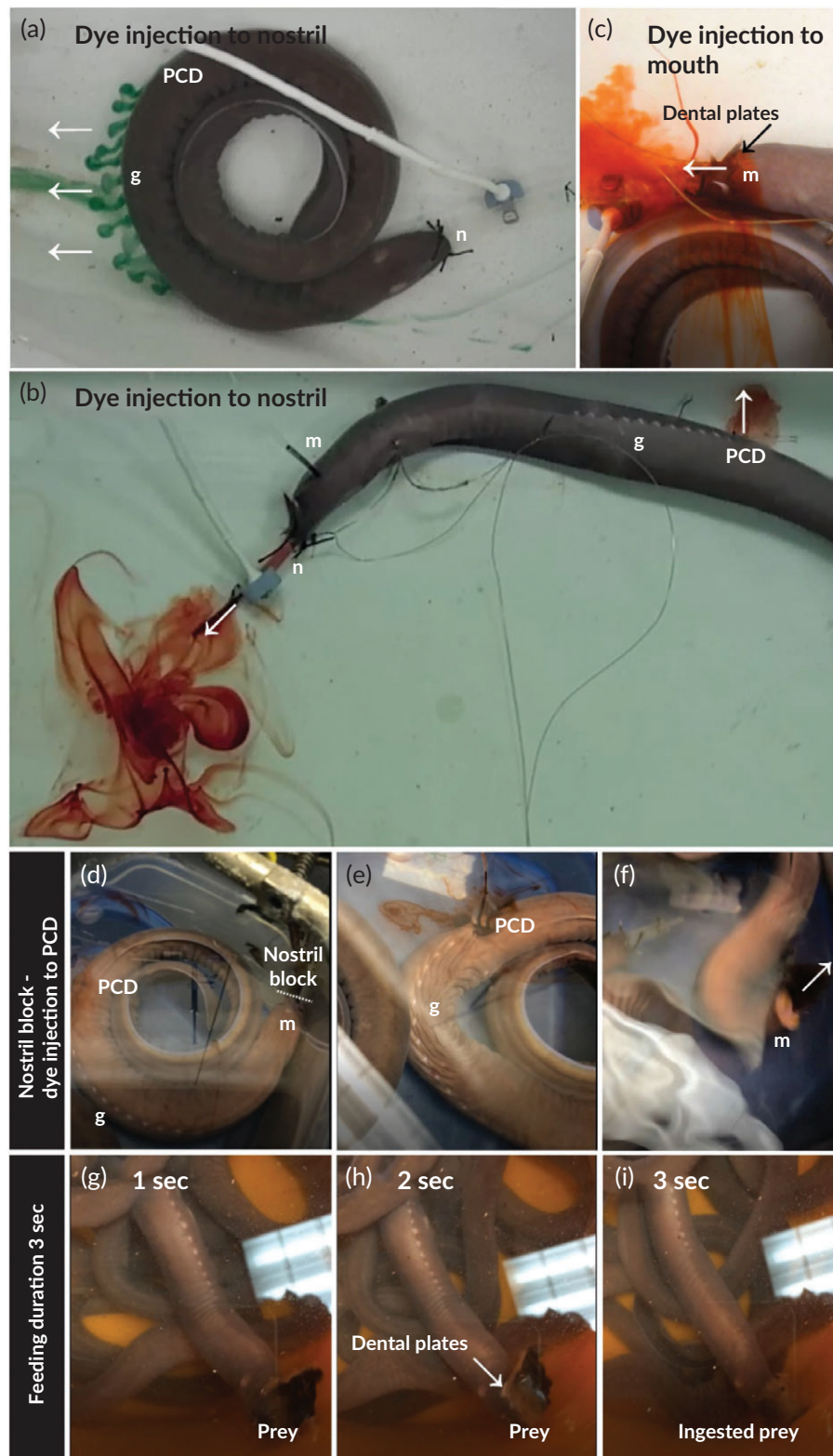


FIGURE 3 (a) After dye injection to the nostril (n) of live *Eptatretus stoutii*, (b) it induced nostril coughing, with serial dye ejection from the nostril (n), 12 pairs of gill pouches (g) and then the pharyngo-cutaneous duct (PCD). In (b), the ventilation measurement sensors can be seen, attached to the nostril (n; flow meter), mouth cavity (m; pressure measuring catheter) and pharyngo-cutaneous duct (PCD; pressure measuring catheter), and skin around the velum chamber (v; wires for impedance measurement). (c) In contrast, in an example where dye injection into the mouth cavity induced mouth coughing, the dye was ejected only back out through the mouth. (d) After occluding the nostril, (e) the animals immediately showed muscular contraction in the ventral area but this did not cause the injected dye in the pharynx to exit through the gill pouches or PCD. (f) Eventually the fish violently coughed the dye out through the mouth. Panels (g), (h), and (i) depict the time course of feeding by engulfment



FIGURE 4 Dye injection to the mouth cavity of a live *Eptatretus stoutii*: the location of the injected dye was monitored at (a) 0 h, (b) 2 h, and (c) 24 h. In this example, the fish immediately flushed the dye back up the tubing (▼) and it remained stored in the tubing over 24 h. There was no evidence that it was ever inhaled

and PCD. Thus, there was no evidence of bi-directional flow in *E. stoutii* gill pouches and the PCD.

Using chunks of anchovy, feeding behaviour was also observed in a few animals. The *E. stoutii* first oriented its head towards the prey item (Figure 3g), then exposed and opened its paired dental plates in front of the prey item (Figure 3h) and engulfed the chunk of anchovy immediately (Figure 3i). Overall, this sequence of feeding events was completed within 3 s.

3.2 | Ventilation of *E. stoutii* under control resting conditions

Key parameters are summarised in Table 1. After operation, most hagfish ($n = 99$) were allowed to recover overnight in flowing seawater and their ventilatory movements were recorded via the inserted pressure-measuring tubing, flow probes and impedance wires at the respective sites for 5 min, sometimes longer. These recordings are defined as control resting ventilation in this study. Of the healthy 99 animals in which ventilatory measurements were made under control resting conditions, 35 *E. stoutii* (35.4%) exhibited no detectable breathing during the observation period. As most of our recordings were of short duration, the mean duration of apnea is not known, but some animals did not breathe for several hours. In non-breathing animals, ventilation could usually be initiated by stressors such as

pinching or prodding. Some of these *E. stoutii* immediately stopped breathing again while others continued to breathe. These 35 non-breathing animals were not included in the averages of Table 1, but this observation indicates that prolonged periods of ventilatory arrest are common in *E. stoutii*.

During simultaneous recordings, the frequencies of the nostril duct (pressure and flow), mouth cavity (pressure), velum region (impedance) and PCD (pressure) showed similar values, reflecting the pulsatile movement of the velum chamber. Therefore, averaged frequencies from the nostril duct, mouth cavity and velum chamber have been reported as velum frequency in Table 1, averaging about 22 min^{-1} (range $4.7\text{--}77.5 \text{ min}^{-1}$). This rhythmic velum contraction and relaxation resulted in a negative absolute pressure in the nostril of about $-0.6 \text{ cm H}_2\text{O}$ (range = -0.89 to $-0.35 \text{ cm H}_2\text{O}$) with a pressure pulse amplitude of approximately $0.05 \text{ cm H}_2\text{O}$ (range = $0.01\text{--}0.10 \text{ cm H}_2\text{O}$). Ventilatory index (see below), the product of pressure pulse amplitude times velar frequency, averaged about $1.3 \text{ cm H}_2\text{O min}^{-1}$ (range = $0.11\text{--}6.22 \text{ cm H}_2\text{O min}^{-1}$). The frequency of spontaneous coughing events under control resting conditions was about 0.65 min^{-1} (range = $0.48\text{--}0.82 \text{ min}^{-1}$) and heart rate averaged about 16 min^{-1} (range = $3.2\text{--}20.7 \text{ min}^{-1}$) and was therefore similar to mean velum frequency in *E. stoutii*.

As a result of the continuous but fluctuating negative pressure created in the nostril by the velum chamber contraction cycle, water was inhaled into the nostril cavity at a mean flow rate of about $-125 \text{ ml kg}^{-1} \text{ min}^{-1}$ (range = -267 to $-22 \text{ ml kg}^{-1} \text{ min}^{-1}$) in resting animals (Table 1). Water flow was continuous, fluctuating with the velum cycle, but never dropping to zero between velum beats. The mean ventilatory stroke volume for each velum contraction cycle was about 7 ml kg^{-1} (range = $2.35\text{--}10.96 \text{ ml kg}^{-1}$). In simultaneous recordings, the trough (most negative point) in nostril pressure corresponded to the greatest nostril inflow (Figure 5). Water was exhaled through 12 pairs of branchial pouches and the PCD.

The three *E. stoutii* set up to characterise coughing and spontaneous swimming events were also employed to make detailed pressure measurements in the mouth and PCD, together with recordings of nostril flow (Table 2). The absolute mean pressure (about $+0.005 \text{ cm H}_2\text{O}$, range = $0.002\text{--}0.008 \text{ cm H}_2\text{O}$) in the PCD was very low and variable but slightly above ambient pressure on average. Two types of

TABLE 1 Ventilation variables (mean \pm SE) collected under resting control conditions from 64 *Eptatretus stoutii*. During control conditions, 35 out of 99 fish (35.4%) did not breathe spontaneously and are not included here. Heart rates were recorded from an additional 23 fish. Sample size (n) is given in parenthesis

| Variable | Value |
|--|-------------------------|
| Nostril flow ($\text{ml kg}^{-1} \text{ min}^{-1}$) | -124.6 ± 25.81 (16) |
| Stroke volume (ml kg^{-1}) | 7.11 ± 1.15 (16) |
| Velum frequency (min^{-1}) | 21.84 ± 1.86 (64) |
| Nostril absolute pressure ($\text{cm H}_2\text{O}$) | -0.62 ± 0.27 (11) |
| Nostril pressure amplitude ($\text{cm H}_2\text{O}$) | 0.05 ± 0.01 (64) |
| Ventilatory index ($\text{cm H}_2\text{O min}^{-1}$) | 1.27 ± 0.28 (64) |
| Coughing frequency (min^{-1}) | 0.65 ± 0.17 (30) |
| Heart rate (min^{-1}) | 16.40 ± 0.49 (23) |

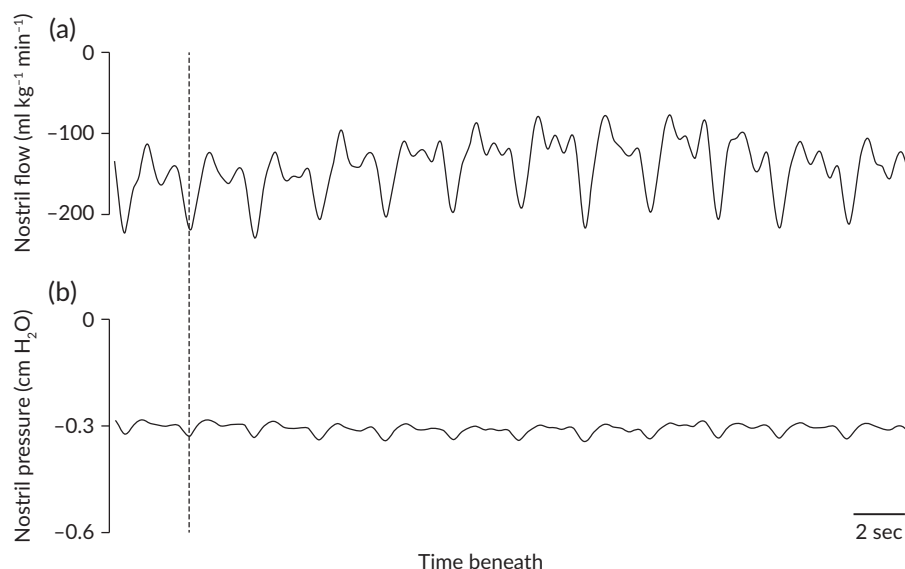


FIGURE 5 Simultaneous recording of (a) flow and (b) pressure in the nostril duct of *Eptatretus stoutii*. -----, Line highlighting patterns of greatest flow [most negative value in (a)] and lowest pressure [most negative value in (b)] were well matched in the nostril duct, as indicated by the dotted vertical line

frequencies were detected in the PCD, one (fast mode; Figure 6d) which was matched to the velum frequency with a mean pressure amplitude of about 0.008 cm H₂O (range = 0.007–0.009 cm H₂O) and the other [slow-mode (SM); Figure 6d] with a frequency of about 4.4 min⁻¹ (range = 4.13–4.73 min⁻¹) and a mean pressure amplitude of about 0.05 cm H₂O (range = 0.045–0.055 cm H₂O) which was probably generated by the contraction–relaxation of the gill pouches. The interpretation of this SM ventilation frequency is explored further in § 4; note that this phenomenon was recorded not only in these three fish, but also in an earlier group ($n = 6$; data not shown) where recordings were made from the PCD catheter only. Note also that contraction of the velum chamber (Figure 6c) also created a pulsatile negative absolute pressure in the mouth cavity (Figure 6b; mean = -0.44 cm H₂O, range = -0.10 to -1.24 cm H₂O) with a pressure amplitude of about 0.2 cm H₂O (range = 0.06–0.23 cm H₂O). The trough in mouth cavity pressure (*i.e.*, least negative mouth pressure value) corresponded to the peak in nostril inflow, exactly opposite the pattern in nostril pressure.

TABLE 2 Simultaneous recordings of ventilation variables (mean \pm SE) from the nostril duct, velum chamber, mouth cavity, and PCD of three *Eptatretus stoutii* under control resting conditions. Velum frequency represents averaged frequency among nostril duct, mouth cavity, velum chamber, and PCD fast mode

| Variable | Value |
|--|---------------------|
| Velum frequency (min ⁻¹) | 15.07 \pm 1.58 |
| Nostril flow (ml kg ⁻¹ min ⁻¹) | -154.70 \pm 17.83 |
| Mouth Cavity absolute pressure (cm H ₂ O) | -0.44 \pm 0.20 |
| Mouth Cavity pressure amplitude (cm H ₂ O) | 0.16 \pm 0.05 |
| PCD absolute pressure (cm H ₂ O) | 0.005 \pm 0.003 |
| PCD fast-mode pressure amplitude (cm H ₂ O) | 0.008 \pm 0.001 |
| PCD slow-mode pressure amplitude (cm H ₂ O) | 0.050 \pm 0.005 |
| PCD slow-mode frequency (min ⁻¹) | 4.43 \pm 0.30 |

3.3 | Analysis of ventilatory flow and pressure relationships

Over 3000 data points from 42 animals were available where nostril flow and nostril pressures were recorded simultaneously under a variety of conditions [rest, spontaneous activity, ammonia injections, exposure to high environmental ammonia (HEA)] in which ventilatory flow varied greatly, from close to 0 ml kg⁻¹ min⁻¹ to > 400 ml kg⁻¹ min⁻¹ (Figure 7). Clearly, changes in both velum frequency (Figure 7a) and stroke volume (Figure 7b) contributed to changes in flow, with the stroke volume explaining a slightly greater percentage of the variance (34%, based on R^2) than did frequency (26%), though both relationships were highly significant ($P < 0.001$). Flow was strongly related to pressure amplitude ($P < 0.001$) which explained 52% of the variance in flow (Figure 7c). Stroke volume was also significantly related to pressure amplitude ($P < 0.001$), which accounted for about 25% of the variance (Figure 7e). Therefore, pressure amplitude and stroke volume are not independent variables, explaining why the contributions of frequency (Figure 7a), stroke volume (Figure 7b) and pressure amplitude (Figure 7c) appear to explain more than 100% of the variance in flow. The ventilatory index (Figure 7d) was strongly correlated ($r = 0.64$, $P < 0.001$) with ventilatory flow and can be employed as a useful proxy, accounting for 42% of the variance in the latter.

3.4 | Simultaneous recordings from nostril duct, mouth cavity, velum chamber, and pharyngo-cutaneous duct (PCD)

Ventilatory parameters from different respiratory organs were measured simultaneously in a subset of three fish to better understand the relationships between them at rest, as well as during coughing and spontaneous exercise (Figures 8–11). Nostril coughing is defined as coughing stimulated by inhalation of dye into the nostril, while mouth coughing is defined as coughing initiated by injection of dye into the mouth. Under control resting conditions (Table 2), the absolute values

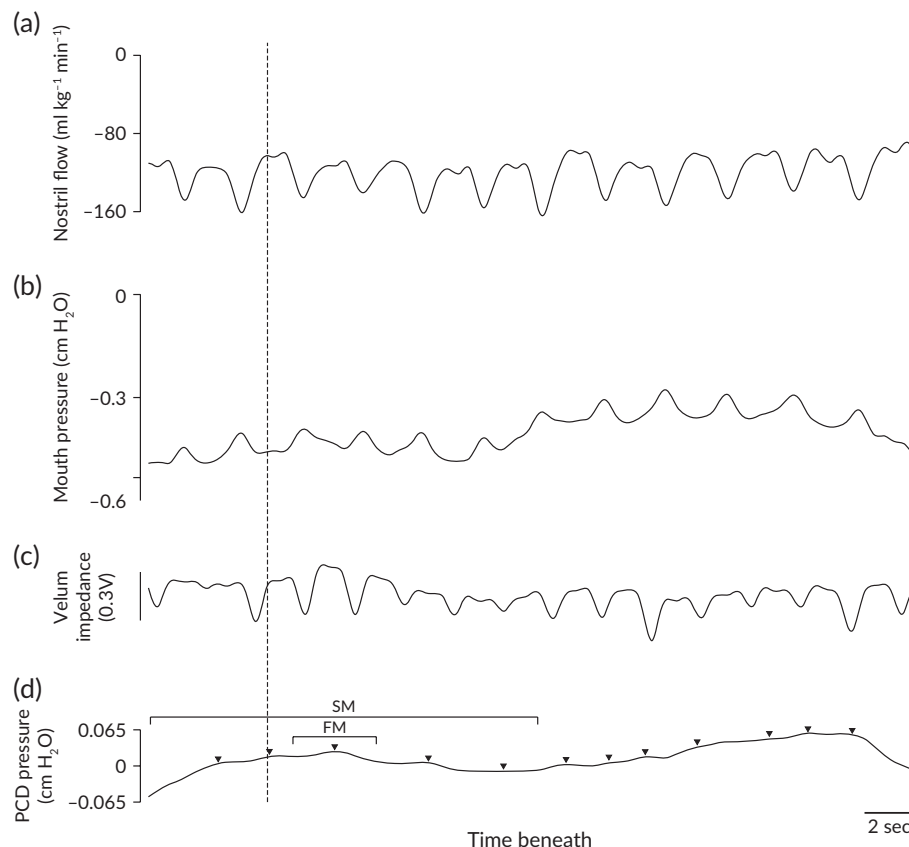


FIGURE 6 Simultaneous ventilation recordings of (a) nostril flow (b) mouth cavity pressure, (c) velum chamber impedance and (d) pharyngo-cutaneous duct (PCD) pressure in a resting *Eptatretus stoutii* under control conditions. (d) Two types of ventilatory pressure cycles were detected in the PCD, slow mode (SM) which appeared to be generated by the gill pouches and fast mode (FM) which correlated with the velum movement. ▼, Ventilatory rhythms in fast mode (FM), while the larger brackets indicate the slow pressure wave of slow mode (SM). -----, Line highlighting patterns of simultaneous ventilatory movements: lowest nostril flow [least negative value in (a)], lowest mouth pressure [most negative value in (b)], velum chamber movement and highest PCD pressure. The lowest nostril flow and lowest mouth pressure were exactly 180° out of phase with nostril pressure seen in Figure 5

were similar to those for the larger group of fish in Table 1. The changes seen during nostril coughing, mouth coughing and spontaneous swimming are summarised in Figure 8. Spontaneous nostril coughing occurred far more often than spontaneous mouth coughing.

During nostril coughing *E. stoutii* showed greatly different patterns of ventilatory variables compared with control resting conditions. For example, they changed the direction of nostril flow from unidirectional inhalation averaging about $155 \text{ ml kg}^{-1} \text{ min}^{-1}$ in these three fish to unidirectional exhalation, averaging about $100 \text{ ml kg}^{-1} \text{ min}^{-1}$, a highly significant change (Figure 8a). Although nostril pressure was not measured in these three specimens, based on others in which nostril coughing occurred, the absolute pressure (and pressure amplitude) increased greatly to highly positive values in the nostril during the flow reversal. Absolute pressures and pressure amplitudes also increased greatly to highly positive values in both the mouth cavity (Figure 8d,e) and PCD (fast mode; Figure 8f,g); all of these changes were significant. The SM pressure waves could not be diagnosed because of the short duration of the coughing event. However, the velum frequency (measured by impedance; Figure 8b) and the PCD fast-mode frequency (Figure 8c) did not change. After dye administration to the nostril (Figure 9) and prior to the actual nostril cough, the apparent frequency slowed and the pressure became more positive and then more negative in the mouth cavity (Figure 9b). Then the actual

cough (Figure 9) occurred with simultaneous pressure increases in the nostril duct (with flow reversal; Figure 9a), the mouth cavity (Figure 9b) and the PCD (Figure 9d) and an indication of velum chamber contraction in the impedance trace (Figure 9c). Dye was immediately ejected anteriorly through the nostril opening and then posteriorly mainly through the PCD and to a lesser extent through the gill pouches (Figure 3a, c).

During mouth coughing, the fish essentially stopped nostril flow (Figure 8a), but there were significantly greater pressure amplitudes in both the mouth cavity (Figure 8d) and PCD (fast mode; Figure 8f) as well as significantly greater absolute pressures in both locations (Figure 8e,g). To nostril coughing, velar frequency (Figure 8b) and PCD fast-mode frequency (Figure 8c) were not altered during mouth coughing. Usually (but not always) the fish showed a large reduction (Figure 10d) of the PCD pressure to negative values after dye administration and prior to mouth coughing. Just before the cough, *E. stoutii* exhibited contraction of the nostril (Figure 10a), the velum chamber (Figure 10c) and the PCD (Figure 10d) while the mouth cavity was expanded (more negative pressure; Figure 10b). The dye in the mouth cavity was never ejected to the environment via the paired gill pouches or PCD but rather via the mouth opening, with the brief opening of the dental plates at this time. This never occurred during nostril coughing.

During spontaneous swimming, mean nostril flow did not change (Figure 8a) despite significant increases of PCD fast-mode frequency

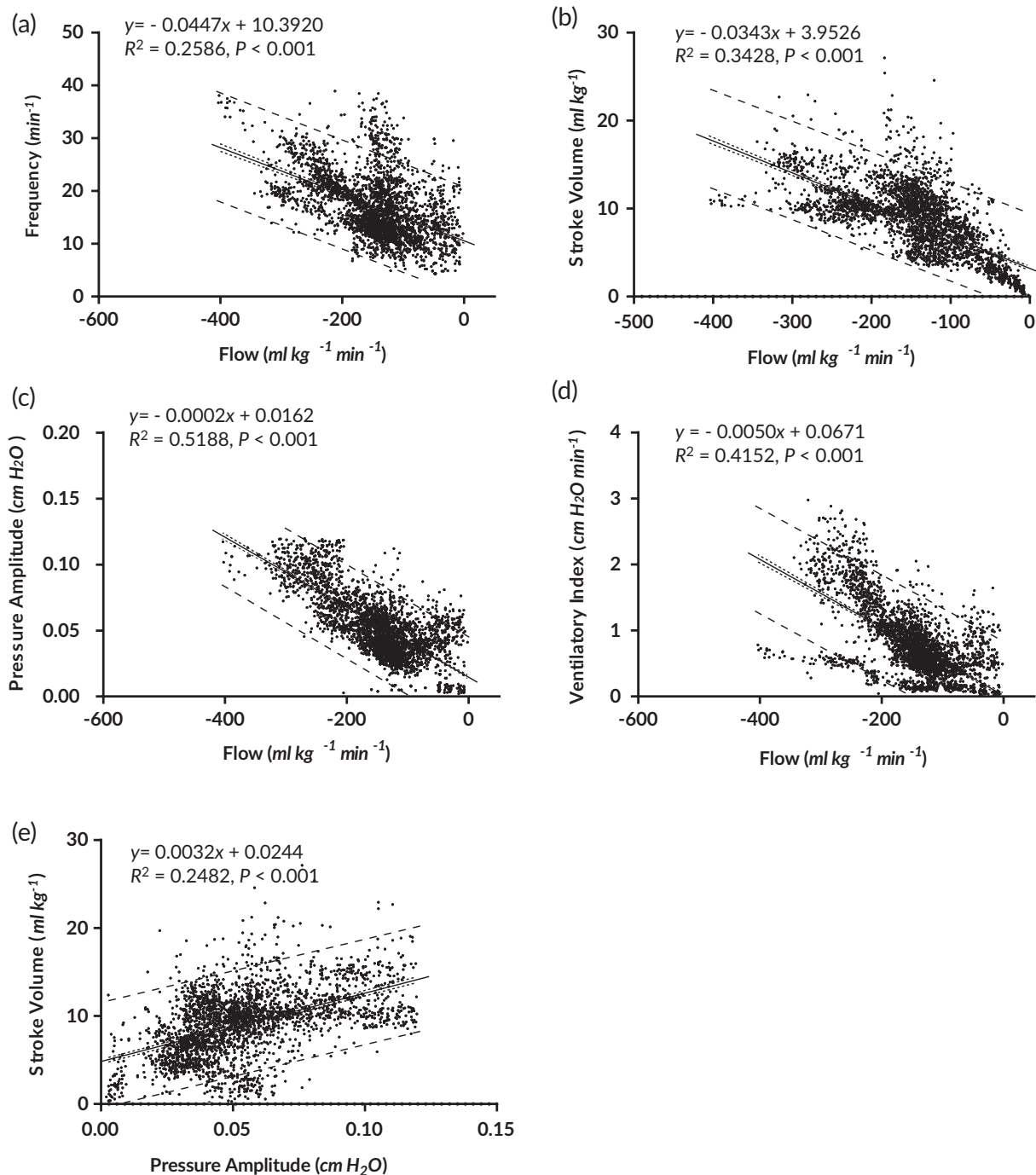


FIGURE 7 Linear regression relationships (—) for 3134 data points from 42 *Eptatretus stoutii*. Nostril flow was plotted against (a) frequency, (b) stroke volume, (c) pressure amplitude and (d) ventilatory index. (e) Stroke volume in relation to pressure amplitude. ———, 95% CI for the regression; ·····, 95% prediction interval or population CI

(Figure 8c). The PCD fast-mode pressure amplitude (Figure 8f) and absolute pressure (Figure 8g) were significantly elevated, but mouth cavity pressure amplitude (Figure 8d) and mouth cavity absolute pressure (Figure 8e) were not changed. During the transition period, pressure amplitudes gradually increased. Eventually the overall frequencies from nostril duct, mouth cavity and PCD (fast mode) were synchronised at about 34 min^{-1} (range = $32.0\text{--}35.5 \text{ min}^{-1}$). These values were matched to the frequency of body undulation in swimming performed by the animal (average 34.2 min^{-1}). The impedance trace became noisy during swimming events and could not be reliably interpreted, so it is

unclear whether velum frequency also changed or not. Our visual observations confirmed that the dental plates were not exposed and remained closed during spontaneous swimming.

4 | DISCUSSION

A simple model of our understanding of the ventilatory mechanism in *Eptatretus stoutii* based on observations made in the present study is shown in Figure 1b.

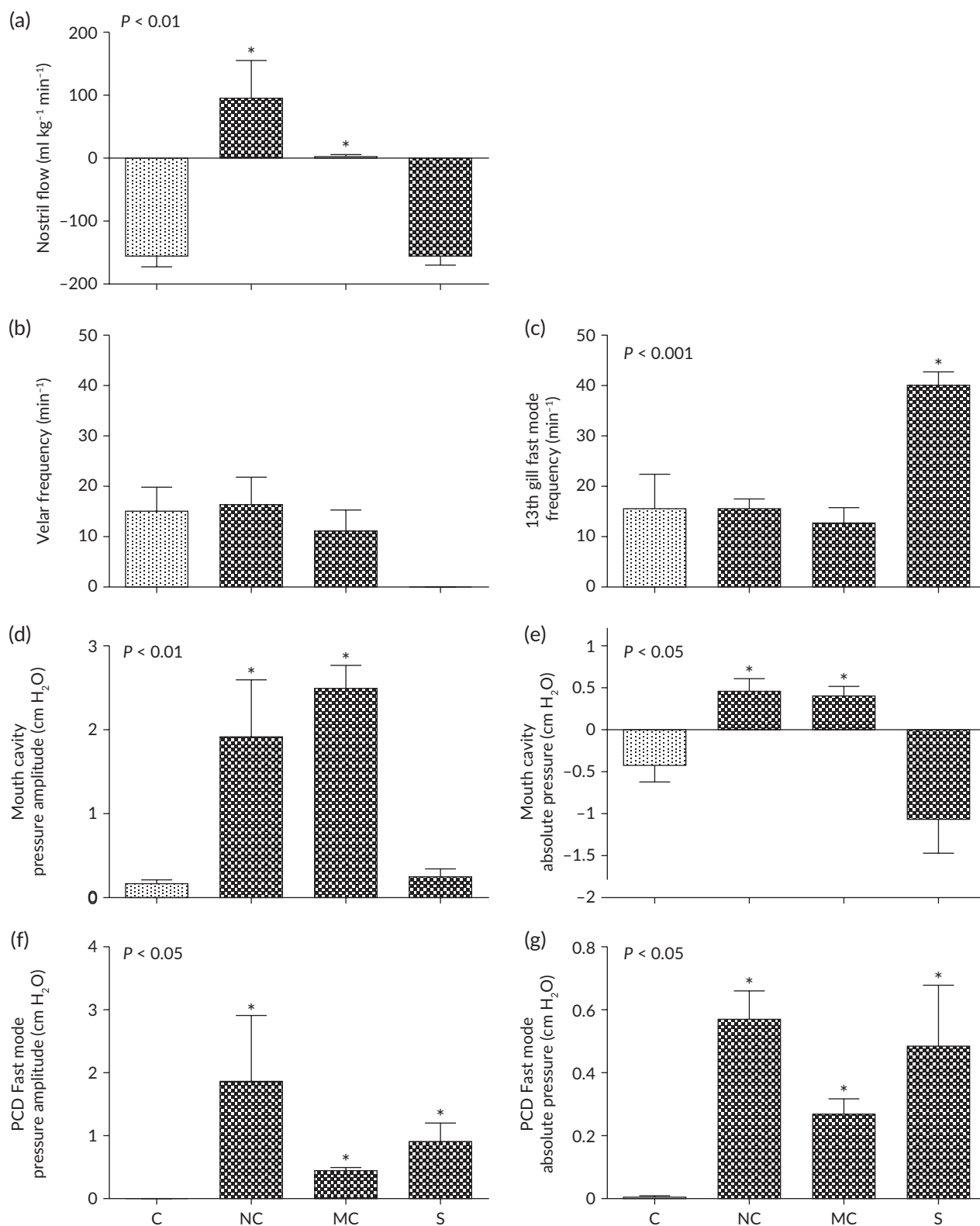


FIGURE 8 Ventilatory variables recorded simultaneously under control conditions (C), during nostril coughing (NC), during mouth coughing (MC), and during spontaneous swimming (S) in the same three *Eptatretus stoutii*. (a) Nostril flow rate, (b) velum frequency (there is no velar frequency for spontaneous swimming in panel as the velum impedance recording became noisy), (c) pharyngo-cutaneous duct (PCD) fast mode (FM) frequency (the pulsatility of flow and pressure, *i.e.*, fast mode frequency, became equal to that of body undulations), (d) mouth-cavity pressure amplitude, (e) mouth-cavity absolute pressure, (f) PCD FM pressure amplitude and (g) PCD FM absolute pressure. *P* values indicate overall significance of the one-way ANOVA, and asterisks indicate significant differences from the Control Value (c)

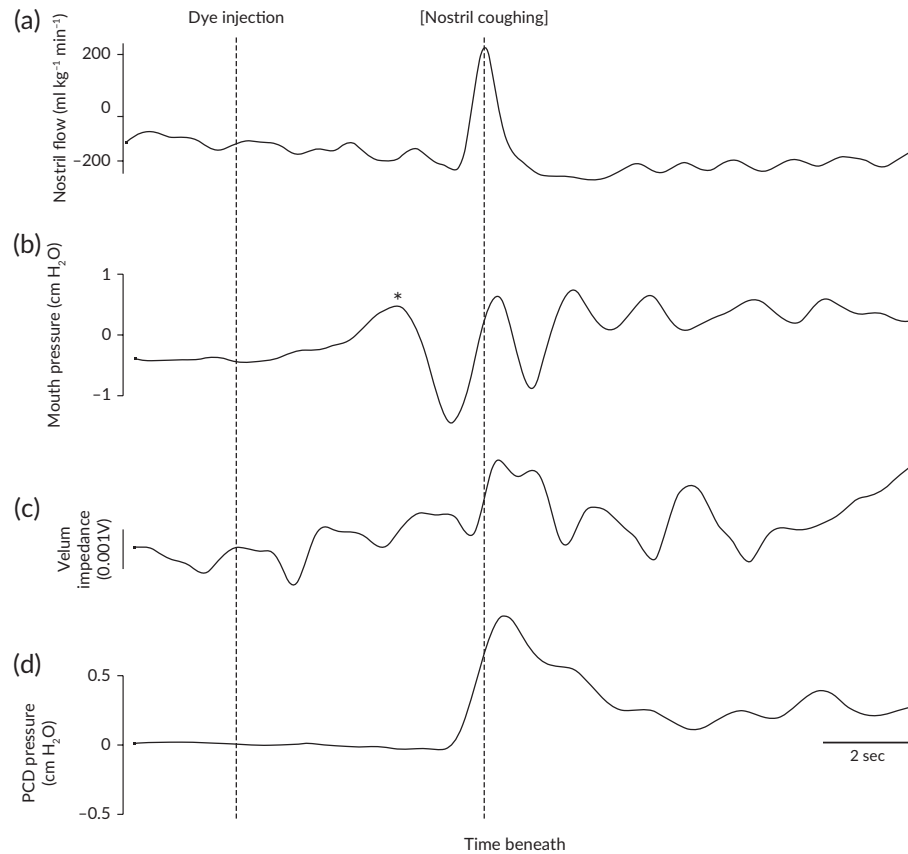


FIGURE 9 Simultaneous recordings of (a) nostril flow (b) mouth cavity pressure, (c) velum chamber impedance and (d) pharyngo-cutaneous duct (PCD) pressure in a *Eptatretus stoutii* where nostril coughing was induced by dye inhalation into the nostril (left-hand —). Following dye administration and prior to the actual nostril cough, the apparent frequency slowed and the pressure became more positive and then more negative in the mouth cavity (*). Then the actual cough occurred (right-hand -----) with simultaneous pressure pulses in the nostril duct [with flow reversal; (a)], the mouth cavity (b), and the PCD (d), and a rise in the impedance trace from the velar chamber (c)

4.1 | Anatomical observations

Our anatomical studies indicate both similarities and differences in *E. stoutii* from previous observations in the Myxinidae. As in the latter, the nostril duct lies dorsal to the mouth cavity and is horizontally separated from it by the oronasohypophyseal septum (ONS), a simple muscular membrane-like structure. The nostril duct leads directly to the anterior velum chamber but in contrast to *M. glutinosa* which has a short ONS (Dawson, 1963; Johansen & Strahan, 1963; Strahan, 1958), *E. stoutii* has a relatively longer ONS, so the mouth cavity is connected not to the velum chamber but to the anterior pharynx by a bucco-pharyngeal aperture that lies posterior to the velum (Figure 1b); this was confirmed by the silicone injection experiment (Figure 2). Simultaneous recordings of pressure in the mouth cavity and pressure and flow in the nostril revealed reciprocal patterns of fluctuations at the frequency of velum contraction (dotted line in Figure 6a,b), presumably due to passive distension and relaxation of the ONS by the action of the velum pump. Owing to the ONS barrier and the post-velum connection point of the bucco-pharyngeal aperture, water exchange between the velum chamber and mouth cavity probably does not normally occur (Figure 1b). Furthermore, the mouth cavity is normally completely sealed (Figures 1b and 4) by the paired dental plates and probably also by contraction of the bucco-pharyngeal aperture when the animal is not feeding. If the short ONS

model of *M. glutinosa* were present, with the anterior connection of the velum chamber to both the nostril duct and mouth chamber, then the rhythmic movement of the velum would generate qualitatively similar pressure patterns in the two chambers, in contrast to the reciprocal patterns seen in the present study.

4.2 | Patterns of ventilation

In accord with many previous studies on Myxinidae and two on Eptatretidae (Coxon & Davison, 2011; Perry *et al.*, 2009b), our observations indicate that ventilation in the *E. stoutii* is primarily driven by the rhythmic contraction of the velum apparatus, which creates a fluctuating but continuous negative pressure in the nostril (Figure 1b). The resulting inflow through the nostril is pulsatile but continuous, never dropping to zero between velum contractions, in agreement with Steffensen *et al.* (1984) and Perry *et al.* (2009b), so water flow through the gills is also probably continuous. The pressure pulses in the nostril caused by the velum-contraction cycle could also be detected in the PCD and in the mouth cavity, where the frequencies (fast-mode frequency only for PCD) were the same as those of the impedance recordings from the velum.

Indeed, two types of ventilatory movements were detected in the PCD under resting conditions: a SM frequency of 4–5 positive pressure fluctuations per minute onto which was superimposed very small amplitude pressure fluctuations representing the fast-mode (FM) frequency of

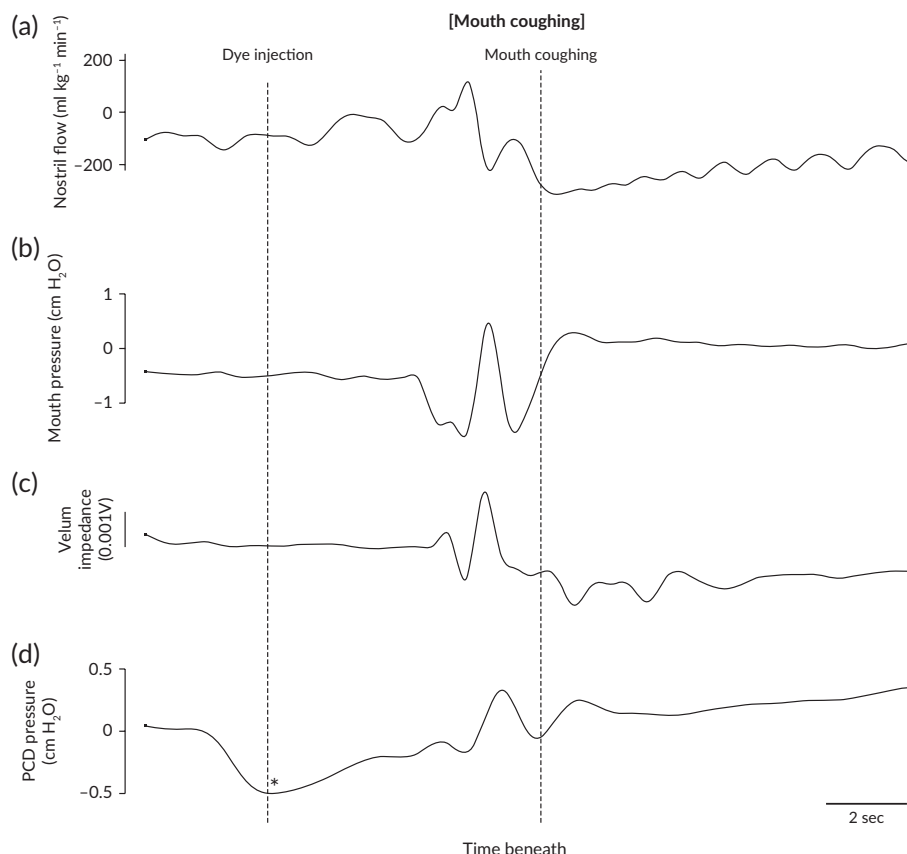


FIGURE 10 Simultaneous recordings of (a) nostril flow (b) mouth cavity pressure, (c) velum chamber impedance and (d) pharyngo-cutaneous duct (PCD) pressure in *Eptatretus stoutii* where mouth coughing was induced by dye injection into the mouth cavity. Usually (but not always) before mouth coughing, fish showed a large reduction (*) of the PCD pressure to negative values

velum contractions (Figure 6d and Table 2). Anatomically, the velum chamber is connected to the paired gill pouches and the PCD via the long pharynx (Figure 1b), so the pressure wave from the rhythmic movement of the velum chamber could be transmitted to the measurement site at the PCD. We interpret the larger SM pressure pulses as evidence of active contraction of the gill pouches or PCD and their associated ducts, which thereby help power exhalation (Figure 1b). While this possibility was initially denied in *M. glutinosa* (Strahan, 1958), it was later proven to occur in this species (Johansen & Hol, 1960; Johansen & Strahan, 1963). These authors described a “peristaltic wave from the proximal end of the afferent (gill) duct to the proximal end of the efferent duct,” which is probably the same as our SM contractions. However, it is not clear from these studies on Myxinidae whether the gill pouch contractions occur at a lower rate than the velum chamber contractions. *Eptatretus stoutii* appears to operate a two-phase ventilatory system with a faster suction pump for inhalation and a much slower force pump for exhalation (Figure 1b). We saw no evidence of inhalation through the gill pouches or PCD.

Under control conditions, *E. stoutii* showed an all-or-nothing pattern of breathing, either ventilating rhythmically or not at all; 35% of the animals examined exhibited no spontaneous ventilation. Regular rhythmic ventilation seemed to be more common after sunset, perhaps reflecting the nocturnal nature of these animals. During periods of apnea, mild stressors such as pinching or gently touching the skin would usually cause ventilation to start up, though in some animals it would soon stop again. These periods of ventilatory arrest are in accord with the ability of this species to tolerate anoxia and to

suppress metabolic rate for long periods (Cox *et al.*, 2011). As heart rate was not recorded in the same animals, we do not know whether the heart slowed and kept beating regularly with increased cardiac stroke volume as it does during experimental anoxia (Cox *et al.*, 2010); this is an important topic for future investigation.

When breathing rhythmically, *E. stoutii* exhibited ventilatory flow rates of -125 to -155 $\text{ml kg}^{-1} \text{min}^{-1}$ at rest (Tables 1 and 2). These values are midway between the -45 $\text{ml kg}^{-1} \text{min}^{-1}$ reported for *M. glutinosa* by Steffensen *et al.* (1984) and the -235 $\text{ml kg}^{-1} \text{min}^{-1}$ reported by Perry *et al.* (2009b) for *E. stoutii*, the latter through a method almost identical to that of the current study. However, Perry *et al.* (2009b) did not report episodes of apnea or coughing, so it is possible that their animals were less settled than those of the present study. Regardless, these resting ventilatory flow rates are very comparable with those of teleosts (Perry *et al.*, 2009a; Wood *et al.*, 1979), which is surprising considering that resting $\dot{M}O_2$ is exceptionally low in *E. stoutii* (Cox *et al.*, 2011; Giacomini *et al.*, 2018; Munz & Morris, 1965; Perry *et al.*, 2009b), only about one third of typical rates in comparably sized teleosts (Clarke & Johnston, 1999). This anomaly was previously noted by Perry *et al.* (2009b) and suggests that O_2 extraction efficiency at the gills is very low, which is surprising as it is generally believed that a counter-current exchange system operates in the gills of hagfish. However, the fact that resting *E. stoutii* spends substantial periods in apnea suggests that it may hold previously inhaled water in the gill pouches during this time to extract the maximum possible amount of O_2 from that water. Possibly, time-averaged O_2 extraction efficiency could be much higher than during the periods of

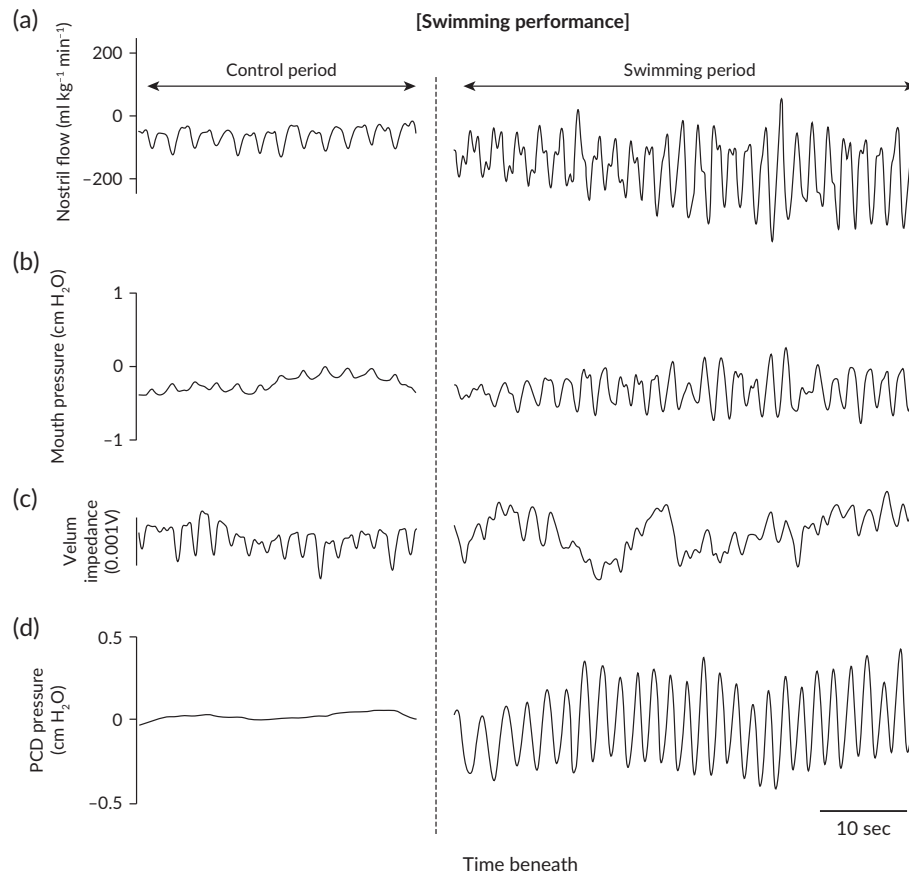


FIGURE 11 Simultaneous recordings of (a) nostril flow, (b) mouth cavity pressure, (c) velum chamber impedance and (d) pharyngo-cutaneous duct (PCD) pressure prior to and during a period of spontaneous swimming in *Eptatretus stoutii*

active ventilation alone. A study of O_2 extraction dynamics at the gills relative to the breathing pattern is required to clarify the situation.

Eptatretus stoutii in the present study were able to increase their ventilatory flow rate to at least $400 \text{ ml kg}^{-1} \text{ min}^{-1}$ during experimental disturbances (Figure 7), comparable with the elevations reported by Perry *et al.* (2009b) in response to hypoxia or hypercapnia. Our data clearly show that *E. stoutii* are capable of large variations of ventilatory flow by changing not only velum frequency (Figure 7a) but also stroke volume (Figure 7b) which differs from the conclusion of Perry *et al.* (2009b) that only changes in frequency occur. Furthermore, increases in stroke volume were correlated with increases in nostril pressure amplitude (Figure 7e), indicating that the velum chamber contraction can become more powerful. Interestingly, most *E. stoutii* in this study showed increasing frequency or increasing stroke volume separately, apparently dependent on the nature of the treatment or types of stressors. For example, *E. stoutii* mostly increased velum frequency in response to tactile disturbance, similar to the responses reported by Perry *et al.* (2009b) during hypoxia and hypercapnia, but mostly increased stroke volume first and then velum frequency later under circumstances of HEA treatment or ammonium salt injection. The ventilatory index proved useful in capturing this variability and was strongly correlated ($r = 0.64$, $P < 0.001$) with ventilatory flow rate, providing a useful proxy for the latter (Figure 7d). In this regard, despite the very different breathing mechanism, *E. stoutii* is similar to many teleosts, where the ventilatory index is also a useful proxy for ventilatory flow (Perry *et al.*, 2009a).

4.3 | Coughing

In the present study, two types of ventilatory flow reversals were seen, which we have termed nostril coughing and mouth coughing. These were often induced by the presence of dye in the respective cavities. Nostril coughing occurred spontaneously far more often than mouth coughing and a related study found that spontaneous coughing frequency was temperature-dependent (Giacomin *et al.*, 2018), suggesting that coughing is a normal part of ventilation. Obviously coughing events will serve to clear irritant particles and noxious dissolved substances, but in future studies it will be of interest to investigate whether they play any direct role in respiratory gas exchange. Hagfishes are known to have not only an olfactory-like chemosensory nasal epithelium in the nostril duct but also Schreiner organs scattered on the body surface. These are thought to be sensory cells due to numerous sensory buds composed of microvilli on the apical cell surface (von Düring & Andres, 1988). Therefore, dye in the nostril was probably sensed by the nasal epithelium or Schreiner organs while dye in the mouth was probably sensed by the Schreiner organs. Interestingly, the two previous investigations on ventilation in the Eptatretidae made no mention of flow reversals (Coxon & Davison, 2011; Perry *et al.*, 2009b). However, as discussed below, if only velum frequency is monitored, as in the study of Coxon and Davison (2011), coughing events could well be missed.

Nostril coughing appears to be the same phenomenon as sneezing, described previously in the Myxinidae (Johansen & Hol, 1960;

Johansen & Strahan, 1963; Steffensen *et al.*, 1984; Strahan, 1958). Strahan (1958) hypothesised that this was largely due to contraction of the pharyngeal constrictor muscle that encircled the posterior part of the velum chamber and this was confirmed by Johansen and Hol (1960) by X-ray cinematography of radio-opaque contrast medium. Their study also implicated contraction of the velum chamber itself.

In *E. stoutii*, after dye administration to the nostril and prior to the actual nostril cough, pressure recordings in the mouth cavity indicated that the apparent velum frequency in the mouth cavity slowed (even though the true velum frequency did not change) and the mouth pressure first increased then decreased greatly. During the cough itself which ensued within 5 s, the fish increased absolute pressures and pressure amplitudes in the nostril duct, mouth cavity and PCD simultaneously, reversal of flow in the nostril occurred to outflow values comparable with normal inflow values and the impedance trace suggested contraction of the velum chamber itself (Figures 8 and 9). This resulted in ejection of the dye first through the nostril duct, then *via* the PCD (major route) and paired gill pouches (minor route), after which the fish immediately recovered the unidirectional ventilation. The slowed frequency and the biphasic pressure cycle in the mouth cavity (Figure 9b) appeared to be a key event and may have reflected tight closure of the bucco-pharyngeal aperture, so that noxious material did not enter the mouth during the nostril cough. The contractile force for the overall event probably originated from contraction of the pharyngeal constrictor muscle and the posterior part of the velum chamber, as deduced by Strahan (1958) and Johansen and Hol (1960).

In mouth coughing, dye was ejected only through the mouth. Johansen and Hol (1960) described an event in *M. glutinosa* that may have been the same, where contrast media was ejected through the mouth from the gill area, powered by contraction of the gill pouches and associated ducts, as well as the musculature of the pharynx and body wall. *Eptatretus stoutii* often (but not always) exhibited a large relaxation of the PCD producing very negative pressures (Figure 10d) before the surge of positive pressure in all compartments (Figure 8d–g) that ejected dye anteriorly through the mouth. None exited posteriorly through the gill pouches, PCD, or nostril. The negative gill pressure perhaps served to drain water from the pharynx into the gill pouches, preparatory to generating back pressure and flow to eject the dye out through the mouth. The bilaterally paired dental plates were opened during ejection, probably by protractor muscles and retractor muscles located ventral to the mouth cavity. The velum chamber must have been shut off from the pharynx at this time, perhaps by the pharyngeal constrictor muscle that encircles the posterior margin of the velum chamber (Johansen & Hol, 1960; Strahan, 1958). This would explain why there was no ejection through the nostril and why nostril flow dropped to zero (Figure 8a) while velum frequency continued unchanged (Figure 8b). As the mouth is not used in normal breathing, it may be that mouth coughing is mainly used to clear irritant particles ingested during feeding.

4.4 | Ventilation during swimming

To the best of our knowledge, the current observations are the first data on hagfish ventilation during swimming. Hagfishes are known as anguilliform locomotors; *i.e.*, eel-like swimmers that use lateral

oscillations of the posterior 2/3 of the body while the head remains oriented in a straight and forward direction (Long *et al.*, 2002). In our study, the relatively heavy flow-probe attached to the silicone tubing in the nostril duct restricted movement of the fish's head. During spontaneous swimming, frequencies of nostril duct and mouth cavity were synchronised with the frequency of the body undulations. The absolute pressure and pressure amplitude increased in the PCD and the SM frequency could no longer be seen; only the fast-mode frequency could be detected at this site (Figure 11d), while there were no changes in mouth pressures (Figure 11b). The velar impedance trace usually became noisy and undecipherable during swimming (Figure 11c), so it is unclear whether velar frequency increased or not (Figure 8b). It seems likely that the increased fast-mode frequency was generated by body undulation not by velum movement during spontaneous swimming performance, as mean ventilatory flow did not change (Figures 8a and 11a). Overall, these observations suggest that *E. stoutii* may transfer some of the work of breathing to the swimming muscles by using ram ventilation. This would occur only *via* the nostril as the dental plates remained closed. Otherwise, the ventilatory mechanism appeared to be basically the same as at rest (Figure 1b). However, in future, measurements in truly free-swimming hagfishes will be required to confirm these ideas.

Overall, our observations indicate that *E. stoutii* has a complex, unique ventilatory system (Figure 1b) similar but not identical to that of the Myxiniidae. Key features include a two-phase unidirectional pumping system with a fast suction pump (the velum) which inhales water through the nostril and a much slower force pump (the gill pouches, PCD and associated structures) for exhalation. Water flow is continuous. Two types of coughing (flow reversals) occur: nostril coughing (sneezing) where water and irritant materials are expelled forcefully through both the nostril and gill pouches and mouth coughing, where they are expelled only through the mouth. The mouth, which joins the pharynx posterior to the velum chamber, plays no role in ventilation of resting, swimming and nostril-coughing *E. stoutii* as the dental plates remain closed, but is probably an important route for inhalant water and food particles in feeding *E. stoutii*. Increases in ventilatory flow can be achieved by both increases in velum frequency and increases in stroke volume, the latter reflected in increases in nostril pressure amplitude. Ventilatory index and is strongly correlated with ventilatory flow and therefore provides a useful index for the latter. When the fish is actively breathing, ventilatory flow rates are relatively high, in the range of teleosts, despite very low O₂ consumption rates, but long periods of spontaneous apnea are common. Ram ventilation powered by the swimming muscles may occur during anguilliform swimming, but this observation remains to be confirmed. In future, it will be of great interest to understand how this complex system is controlled and co-ordinated at a central (Central Nervous System) and peripheral level (chemoreceptors) and to study what happens to breathing during feeding, when the hagfish opens its mouth and engulfs or immerses its head its prey.

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Author contributions

J. E. generated data. C. M. W. and J. E. analyzed data and prepared the manuscript together.

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