

A less invasive system for the direct measurement of ventilation in fish

Junho Eom and Chris M. Wood

Abstract: Most previous systems for quantifying ventilatory flow in fish involve prior anesthesia and difficult surgery to sew or glue membranes to the animal, which are undoubtedly stressful. By modification of the original “van Dam box” design and incorporation of an electromagnetic blood flow probe, we have developed a less invasive system that avoids these problems and provides breath-to-breath measurements of ventilatory flow in real time. The fish can be quickly moved in and out of the apparatus, facilitating repeated measurements on the same animal after different treatments. We have used the system to document the hyperventilatory and hypoventilatory responses to environmental hypoxia and hyperoxia, respectively, in both ~400-g trout (*Oncorhynchus mykiss*) and 10-g goldfish (*Carassius auratus*); the method is easily adaptable to fish of other sizes. Separate experiments on trout have demonstrated that responses to these treatments in buccal pressure amplitude, breathing frequency, and ventilation index are not altered by the attachments used in the apparatus. This less invasive methodology may prove more acceptable to animal ethics committees.

Résumé : La plupart des systèmes existants utilisés pour quantifier le débit ventilatoire de poissons nécessitent une anesthésie préalable et une intervention chirurgicale difficile pour coudre ou coller des membranes au spécimen et entraînent assurément un stress. En modifiant le schéma de « boîte de van Dam » original et en y intégrant une sonde électromagnétique de débit sanguin, nous avons mis au point un système moins effractif qui évite ces écueils et produit des mesures en temps réel du débit ventilatoire entre respirations. Les poissons peuvent être placés dans l'appareil et en être retirés rapidement, ce qui facilite des mesures répétées sur le même spécimen après différents traitements. Nous avons utilisé le système pour documenter les réactions hyperventilatoire et hypoventilatoire à une hypoxie et une hyperoxie ambiantes, respectivement, chez des truites (*Oncorhynchus mykiss*) de ~400 g et des carassins dorés (*Carassius auratus*) de ~10 g; la méthode peut facilement être adaptée à des poissons d'autres tailles. D'autres expériences sur des truites ont démontré que les réponses à ces traitements en ce qui concerne l'amplitude de la pression buccale, la fréquence des respirations et l'indice de ventilation ne sont pas modifiées par les pièces fixées au spécimen qui font partie du dispositif. Des comités d'éthique animale pourraient trouver plus acceptable cette méthode moins effractive. [Traduit par la Rédaction]

Introduction

The measurement of \dot{V}_w , the total ventilatory flow of water (i.e., ventilation volume) through the gills, is fundamental to understanding the respiratory physiology of fish but is not easy to accomplish without stress to both the fish and the investigator. \dot{V}_w represents the total amount of water pumped per unit time by the fish across its respiratory surface and has implications for the workload of breathing, as well as for the efficiency of O_2 extraction from this dense, viscous, O_2 -poor medium. Ideally, \dot{V}_w should be quantified directly and noninvasively. The classic study of Hall (1931) appears to be the first direct measurement, capitalizing on the discrete opercular openings of the pufferfish, attaching them by glass tubes to constant-level overflow chambers so that \dot{V}_w could be directly determined by timed volumetric collection, though opercular closure was undoubtedly impeded. The anatomies of most fish are not amenable to this approach, though in the dragonet (*Callionymus lyra*; Hughes and Umezawa 1968a), rays (Cameron et al. 1971; Graham et al. 1990), and dogfish (*Scyliorhinus* spp., *Squalus* sp.; Lenfant and Johansen 1966; Piiper and Schumann 1967; Hughes and Umezawa 1968b), similar but improved systems were applied by sewing and (or) gluing latex funnels externally around the opercular openings so as to attach them to constant-level overflows without impeding opercular closure. A more widely

used direct measurement system was pioneered by van Dam (1938), who developed a divided chamber fitted with constant-level overflows; a rubber dam fitting tightly around the head of the fish (trout or eel) separated the buccal inflow (anterior chamber) from the opercular outflow so that the water exhaled into the posterior chamber could be quantified by timed collection. Various versions of the “van Dam box” were subsequently customized for fish of different anatomies — for example dogfish (*Squalus suckleyi*; Hanson and Johansen 1970), flatfish (Watters and Smith 1973; Wood et al. 1979), catfish (*Ictalurus punctatus*; Gerald and Cech (1970), mullet (*Mugil cephalus*; Cech and Wohlschlag 1973), suckers (*Catostomus commersonii*; Wilkes et al. 1981), carp (*Cyprinus carpio*; Takeda 1990), and particularly salmonids (e.g., Hughes and Shelton 1957; Cameron and Davis 1970; Davis and Cameron 1971; Wood and Jackson 1980; McKim and Goeden 1982; Smith and Jones 1982; Randall and Cameron 1973; Playle et al. 1990; Kinkead and Perry 1990), for which it was frequently used for about two decades.

As technology improved, electromagnetic flow probes (FPs) originally designed for measuring blood flow were employed for instantaneous ventilatory flow determinations. Initially, funnels fitted with FPs were sealed into the sand so as to overlay the protruding heads of flatfish (Kerstens et al. 1979) and hagfish (*Myxine glutinosa*; Steffensen et al. 1984) that had burrowed into

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sandy substrates. FPs were also substituted for timed overflow collection, thereby providing breath-to-breath rather than time-averaged measurements of ventilatory outflow from the opercular openings of carp (Lomholt and Johansen 1979) and flatfish (Wood et al. 1979). Glass et al. (1990) made an important advance by attaching flow probes to the entrance of a rubber mask sutured around the mouth of the carp, and more recently, very direct measurements of instantaneous inflow were made by attaching the flow-meter to a tube sutured into the single inhalant nostril of hagfish (Perry et al. 2009; Eom and Wood 2019). Except for the “funnel in sand” studies, virtually all of these mask, funnel, tubing, and membrane techniques have required prior anesthesia and surgery (often quite difficult) to suture or glue the device(s) to the fish. An extensive postoperative recovery period in a restraining device is needed, success rates are problematic, and the fish is undoubtedly stressed, which may affect the measurements. It has become increasingly difficult to have such techniques approved by animal ethics committees, which may explain why such direct approaches have been rarely used in recent years.

Less invasive alternatives exist, but they provide only indirect measurement of \dot{V}_w , and all again require prior surgery to implant cannulae into the buccal and (or) opercular cavities for water sampling or pressure measurements. Classic indirect methods include dye dilution approaches (Millen et al. 1966; Jones et al. 1990) and more commonly used Fick principle calculations (e.g., Saunders 1962; Holeton and Randall 1967; Wood and Munger 1994; Kalinin et al. 2000). In the latter, \dot{V}_w is calculated from simultaneous measurements of whole-animal O_2 consumption and partial pressure of oxygen (PO_2) in inspired and expired water. There are errors associated with multiple PO_2 determinations, and expired PO_2 measurements, which are critically important, can be particularly variable (Garey 1967; Davis and Watters 1970). The ventilatory index, calculated as the product of ventilatory pressure amplitude and breathing frequency (measured by pressure transducers attached to buccal or opercular catheters) has also been used as a \dot{V}_w surrogate (e.g., Zhang et al. 2013; Wood et al. 2019; Eom et al. 2020). At best, all of these indirect approaches yield only relative measures of changes in \dot{V}_w .

With this background in mind, our goal was to develop a less invasive system that provided direct \dot{V}_w measurements on a breath-to-breath basis; one that avoided the need for anesthesia, suturing, or gluing; that was manually simple for the investigator; and into which the fish could be quickly introduced and released. A thorough reading of van Dam (1938) suggests that he was able to avoid using anesthesia, sutures, or glue by careful design of the chamber. Here, we have returned to the van Dam approach, combining it with an electromagnetic FP on the inflow (Glass et al. 1990) and a design allowing sealing and simple assembly, so that an unanesthetized fish can be quickly introduced with minimal disturbance. Using our system, we have successfully measured \dot{V}_w in both ~400-g rainbow trout (*Oncorhynchus mykiss*) and ~10-g goldfish (*Carassius auratus*) under moderate hypoxia and hyperoxia, proving that the fish show the expected hyperventilation and hypoventilation, respectively. In a separate series on trout, we have also demonstrated that responses in ventilatory pressure amplitude, breathing frequency, and ventilatory index were unaffected by the system, suggesting that breathing was not constrained. Overall, this system is easy to use for collecting \dot{V}_w data and may prove useful for fish respiration researchers in the future.

Materials and methods

Experimental animals

Experiments were performed under an approved UBC (The University of British Columbia) animal care protocol No. A17-0301 following the guidelines of the Canadian Council on Animal Care. After completion of experiments, all fish were weighed, and the surgically operated fish were euthanized by an overdose (120 mg·L⁻¹) of tricaine methanesulfonate (MS-222, Western Chemicals Inc., Ferndale, Washington, USA; pH was adjusted to 7.0 by titrating with 1 mmol·L⁻¹ NaOH).

Rainbow trout (260–501 g) from Nanaimo River Hatchery (Nanaimo, British Columbia, Canada) and goldfish (9.8–10.1 g) from Noah's Ark (Vancouver, British Columbia, Canada) were held in charcoal-filtered dechlorinated Vancouver City tap water ($[Na^+]$, 0.17 mmol·L⁻¹; $[Cl^-]$, 0.21 mmol·L⁻¹; hardness, 30 mg·L⁻¹ as $CaCO_3$; pH 7.0; temperature, 6.5–9.0 °C for rainbow trout, 15 °C for goldfish) in flow-through systems at UBC. These same temperatures were used in the subsequent experiments. Fish were fed with commercial pellets (EWOS, Surrey, British Columbia, Canada) three times per week and fasted for 1 week prior to experiments.

Rainbow trout operations

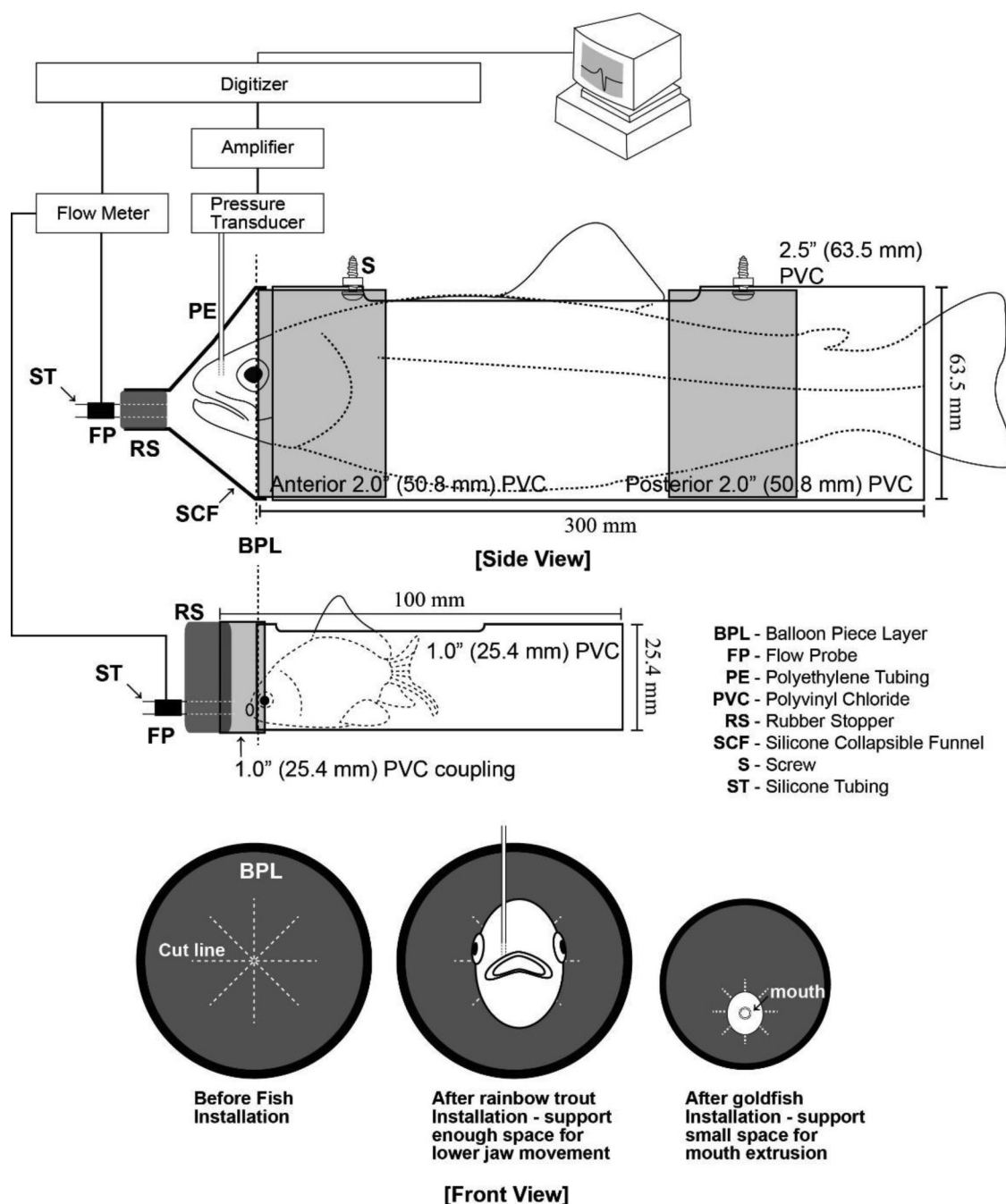
It was not necessary to perform any surgery or cannulation on trout used in the new ventilation measurement system. However, to check whether the system affected the fish's resting ventilation and (or) ventilatory responses to hypoxia and hyperoxia, we performed buccal cannulation on a separate group of trout using the method of Holeton and Randall (1967) to measure buccal pressure amplitude and frequency. Prior to cannulation, the fish were anesthetized in 60 mg·L⁻¹ MS-222 (pH neutralized to ~7.0 as described above) and irrigated via the gills on an operating table. Using an 18-gauge needle, a hole was drilled in the roof of the mouth, and a 3-cm sleeve of polyethylene tubing (PE160, Clay-Adams, Sparks, Maryland, USA; outer diameter (OD) 1.57 mm and inner diameter (ID) 1.14 mm), which had been heat-flared at the mouth end, was inserted. Another flared 30-cm length of PE50 tubing (Clay-Adams, OD 0.97 mm and ID 0.58 mm) was fitted through the PE160 sleeve and cemented with cyanoacrylate glue (Krazy Glue, High Point, North Carolina, USA). The connection between PE160 and PE50 was knotted at the outside with silk sutures. The operated fish were recovered overnight in flowing, aerated fresh water.

System setup for rainbow trout

Figure 1 shows the system setup used for 260- to 501-g trout, and the online Supplementary Media SM1¹ shows a video of how unanesthetized trout are installed in the system. The setup was submerged in an aerated 8-L water bath (reservoir) at the acclimation temperature (6.5–9.0 °C). Prior to fish placement, the centre-cut rubber piece layer, which was cut from a balloon (BPL (balloon piece layer) in Fig. 1; Dollarama, Vancouver, British Columbia, Canada), was layered over the front end of the anterior 2.0" (1 inch = 2.5 cm) small polyvinyl chloride (PVC) piece, which was then plugged into the 2.5" hollow PVC pipe. The fish was placed into the 2.5" PVC, its dorsal fin was positioned so as to fit through the dorsal slit in the 2.5" PVC pipe, and the head was pushed through the centre-cut BPL to a point just posterior to the eyes so as to separate the mouth from the operculae. The free movement of the lower jaw is very important for normal ventilation in rainbow trout, so an additional BPL cut was made if the fish showed any symptoms of irregular ventilation. The position of the posterior 2" PVC pipe was adjusted as necessary to minimize the anterior–posterior movement of the fish, which was also limited by the slit for the protruding dorsal fin. After making these adjustments, a silicone collapsible funnel (SCF, Daiso, Vancouver,

¹Supplementary data are available with the article through the journal Web site at <http://nrcresearchpress.com/doi/suppl/10.1139/cjfas-2020-0177>.

Fig. 1. A diagram of the system setup for rainbow trout and goldfish. For the trout, the system is shown with a buccal cannula in place. The trout is placed in a 2.5" (63.5 mm) diameter PVC pipe. Two 2.0" (50.8 mm) PVC pieces are anteriorly and posteriorly plugged in place and later secured with screws (S) for stabilizing the fish. The centre-cut balloon piece layer (BPL) is stretched over the anterior 2.0" PVC piece and plugged into the 2.5" PVC pipe to separate the buccal and opercular cavities. Once the trout is placed in the 2.5" PVC pipe, the silicone collapsible funnel (SCF) is stretched over anterior 2.0" PVC piece. At this point, it is essential to adjust the BPL so as to allow enough space for lower jaw movement. The anterior opening of the SCF is sealed with a rubber stopper (RS) penetrated with the silicone tubing (ST). The diagram also shows how the overall system is scaled down using a 1" PVC pipe for ventilatory flow measurements in goldfish. In both the trout and goldfish systems, the fish's head is completely sealed in the anterior chamber, so that water inflow during ventilation is only allowed through the ST and is measured by the attached flow-probe (FP).



British Columbia, Canada) was placed over the extended portion of the fish's head by stretching it over the anterior 2.0" PVC piece, completely sealing the anterior part of system. The open anterior end of the SCF was plugged with a rubber stopper (RS) penetrated by silicone tubing (ST, OD 6.35 mm and ID 4.32 mm, length 20 mm). Therefore, the anterior part of system was completely

sealed except for the ST, through which the generated \dot{V}_w flowed and was measured by the attached FP. Using dye, the possibility of leakage was checked after installation of the fish into the system.

When buccal pressure measurements were made, the water-filled PE50 tubing was inserted through a pinhole prepared in the SCF and connected to a pressure transducer (DTP-100, Utah Medi-

cal Products, Midvale, Utah, USA). The analogue buccal pressure signal was amplified (CLA-RTC, Transducer Techniques, Temecula, California, USA) and converted into a digital signal in the digitizer (ADInstruments, Colorado Springs, Colorado, USA), recorded, and analyzed using two points calibration between 0 and 2 cm H₂O in the LabChart software (ADInstruments).

System setup for goldfish

The reservoir temperature was set to 15 °C. The overall system setup was reduced in size by using a 1" PVC pipe rather than a 2.5" pipe. Instead of using anterior and posterior PVC plugs, the cut-lined BPL was directly stretched over the 1" PVC pipe end, and the goldfish was placed into the pipe (Fig. 1). The fish's mouth extended through the cut-lined BPL, with the eyes remaining behind the BPL. Instead of moving the lower jaw, the goldfish opens and closes the mouth by moving the mouth skeletal structure forward and backwards; therefore, less BPL cut line was required. The 1" PVC coupling was fitted over the extended fish's mouth, and the other side of the coupling was sealed with a 1" RS penetrated with the ST (OD 6.35 mm and ID 4.32 mm, length 20 mm). Again, the attached FP measured the \dot{V}_w . The buccal pressure was not measured in goldfish tests. Supplementary Media SM2¹ shows a video of how unanesthetized goldfish are installed in the system.

Flow-meter calibration

In accord with previous reports (Perry et al. 2009; Eom and Wood 2019), the internal machine calibration of the flow-meter (T106 series, Transonic Systems Inc., Ithaca, New York, USA) was not applicable as it was designed for measuring blood flow, so we manually recalibrated with known flow rates of Vancouver water at the experimental temperature. In practice, we first internally calibrated the flow-meter using the two-point machine calibration and then manually recalibrated it. Supplementary Fig. S1¹ shows an example calibration relationship for correcting the measured \dot{V}_w . We also measured the hydraulic resistance of the ST. At a flow rate of 426.6 ± 1.5 mL·min⁻¹ ($N = 83$), comparable to the highest instantaneous \dot{V}_w recorded in our study, the pressure differential across the ST was 0.047 ± 0.009 cm H₂O ($N = 83$), whereas at lower flow rates (means of 64.6, 108.2, and 227.0 mL·min⁻¹), the measured pressure differentials were actually slightly negative (~ -0.04 cm H₂O), perhaps reflecting turbulence and (or) the Venturi effect. Regardless, these values are negligible relative to buccal pressure amplitudes of several cm H₂O (see Figs. 3A, 3D), so the resistance of the ST was insignificant.

Experimental procedures

In Series I, the rainbow trout ($N = 6$) and goldfish ($N = 3$), which had not been operated on, were placed in their respective measurement systems first while under water in a shallow 150-L reservoir (wet table). The systems, with the fish in place, were then transferred to black plexiglass boxes (8-L volume for rainbow trout, 4-L volume for goldfish). This was done without air exposure to the fish by tipping the boxes 90° so that experimenter could slide the system into the box and then return the latter to the upright position. The fish were allowed to settle overnight in the apparatus, and then control ventilatory flow parameters in normoxic water were measured over a 0.5-h period. To change oxygen tension (PO₂), we briefly suspended flow to the box, and pure nitrogen (N₂) gas or oxygen (O₂) gas was bubbled into the reservoir, so as to quickly adjust its PO₂ to the desired value — 50% air saturation (hypoxia) or over 200% air saturation (hyperoxia); this water was then used to rapidly flush the box, and then normal inflow was resumed. The PO₂ of the reservoir was continuously monitored by a dissolved oxygen meter (Model 55, YSI, Yellow Springs, Ohio, USA). The ventilatory parameters were continuously measured over a 1-h treatment period of hypoxia or hyperoxia. At the end of exposure, the water in the reservoir was quickly replaced with normoxic water, the flushing procedure

was performed again, and ventilatory parameters were further recorded over a 0.5-h recovery period.

In Series II, the basic experimental protocol was identical but buccal pressure measurements were made throughout. The operated rainbow trout were placed in the 2.5" PVC pipe system either with ($N = 6$) or without ($N = 6$) the BPL and SCF attachment and then into the black plexiglass boxes. After overnight settling, buccal pressure measurements were made in normoxia (0.5 h), then hypoxia or hyperoxia (1.0 h), and finally during normoxic recovery (0.5 h).

In both Series I and II, the fish were randomly treated first under hypoxia or first under hyperoxia, then allowed to recover overnight, and used again under the alternate treatment.

Data analysis

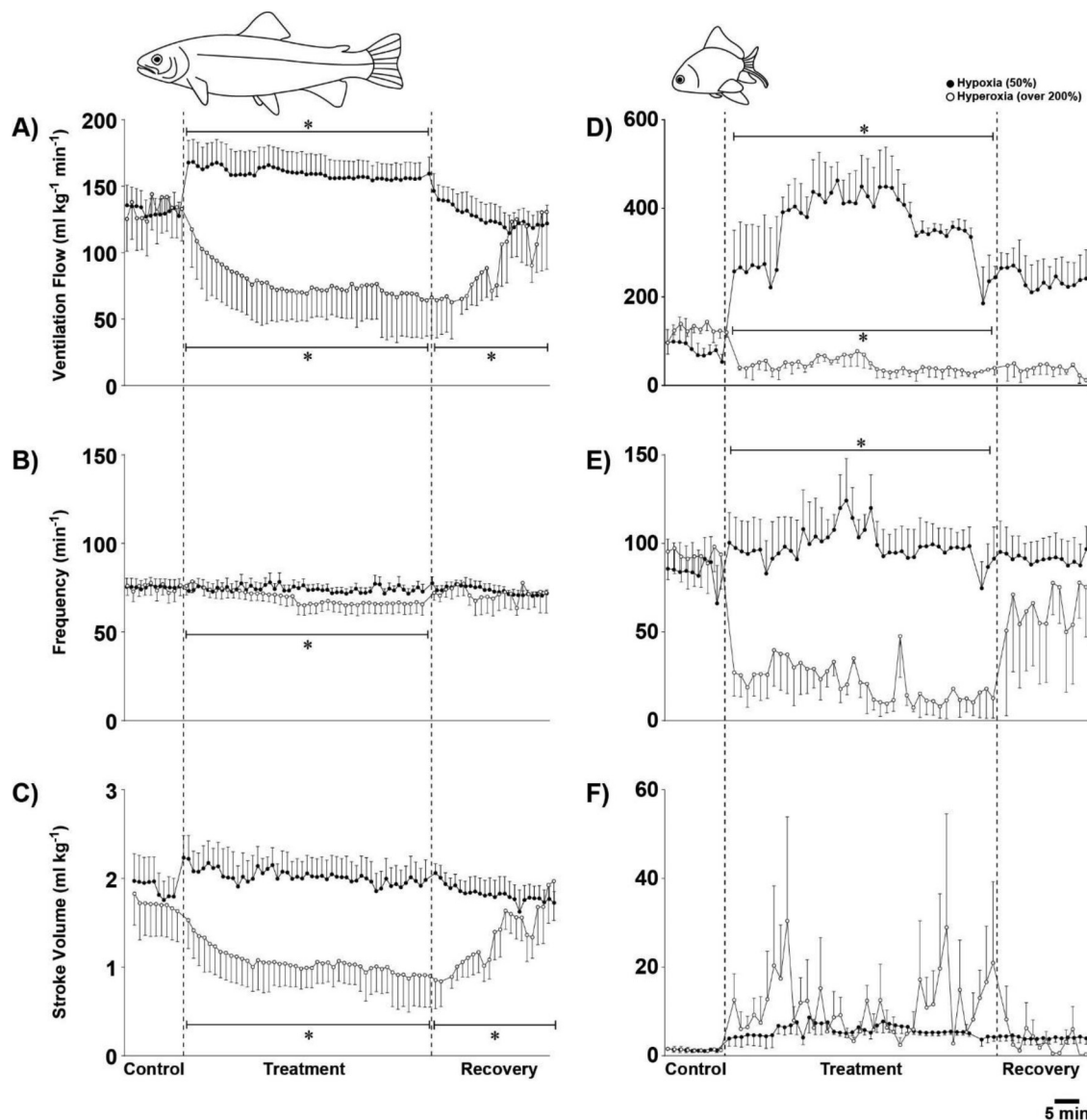
The measured \dot{V}_w and buccal pressure parameters were analyzed using LabChart version 7.0 (ADInstruments). Employing the "Multiple Add to Data Pad" function in LabChart, flow traces were analyzed into ventilation flow (mL·min⁻¹) and breathing frequency (min⁻¹) and pressure traces into buccal pressure amplitude (cm H₂O) and breathing frequency (min⁻¹), respectively. The collected ventilatory parameters were averaged every 3 s in LabChart and exported to Excel for calculation of ventilatory flow per unit body weight (\dot{V}_w , mL·kg⁻¹·min⁻¹), stroke volume (mL·kg⁻¹, using an equation of $\dot{V}_w/\text{frequency}$), and ventilatory index (cm H₂O·min⁻¹, using an equation of buccal pressure amplitude \times frequency). GraphPad Prism 6.0 (La Jolla, California, USA) was used for plotting and statistically analyzing the measured and calculated parameters. Using repeated measures one-way ANOVA in Series I and two-way ANOVA (factors: oxygen, presence-absence of attachments), as well as Student's two tailed t test in Series II, overall significance of averaged ventilatory parameters among control, oxygen (hypoxia or hyperoxia), and recovery period were statistically tested. Also, changed ventilatory parameters in treatments and recovery were compared with averaged control using Dunnett's post hoc test. Throughout, data have been expressed as means \pm 1 SE (N). The threshold for statistical significance was $p < 0.05$.

Results

Using our less invasive system, ventilatory flow measurements were made in Series I on rainbow trout under normoxic control conditions and then in 50% saturation (hypoxia) or in >200% saturation (hyperoxia) (Figs. 2A, 2B, 2C). Representative recordings are shown in Supplementary Figs. S2A, S2B, S2C, and S2D¹. Mean resting \dot{V}_w under normoxia was about 140 mL·kg⁻¹·min⁻¹ with a frequency of about 70 min⁻¹ and a stroke volume of about 2 mL·kg⁻¹ (6.5–9 °C). When challenged with hypoxia (50% saturation), our trout exhibited a significant and sustained 30% increase in \dot{V}_w ($p = 0.0008$; Fig. 2A), achieved almost entirely by an increase in ventilatory stroke volume (Fig. 2C), which was not significant; there was no significant change in frequency (Fig. 2B). Ventilatory parameters returned to normal after about 10 min of normoxia restoration. During hyperoxia, our trout decreased \dot{V}_w by about 40% ($p = 0.0017$; Fig. 2A), achieved by a large decrease in stroke volume ($p = 0.0104$; Fig. 2C) and a very small decrease in frequency ($p = 0.0502$; Fig. 2B). \dot{V}_w returned to normal within 15 min of restoration of normoxia, though there were some ongoing small disturbances of stroke volume and frequency.

We down-sized our system to see whether it could be adapted to much smaller fish, such as 10-g goldfish (Figs. 2D, 2E, 2F; Supplementary Figs. S2E, S2F, S2G, S2H¹). Mean resting \dot{V}_w under normoxia was about 110 mL·kg⁻¹·min⁻¹, with a frequency of 90 min⁻¹ and stroke volume of 1.2 mL·kg⁻¹·h⁻¹ (15 °C). Goldfish also showed hyper- and hypoventilation in hypoxia and hyperoxia, respectively, but the relative changes were larger than those in the

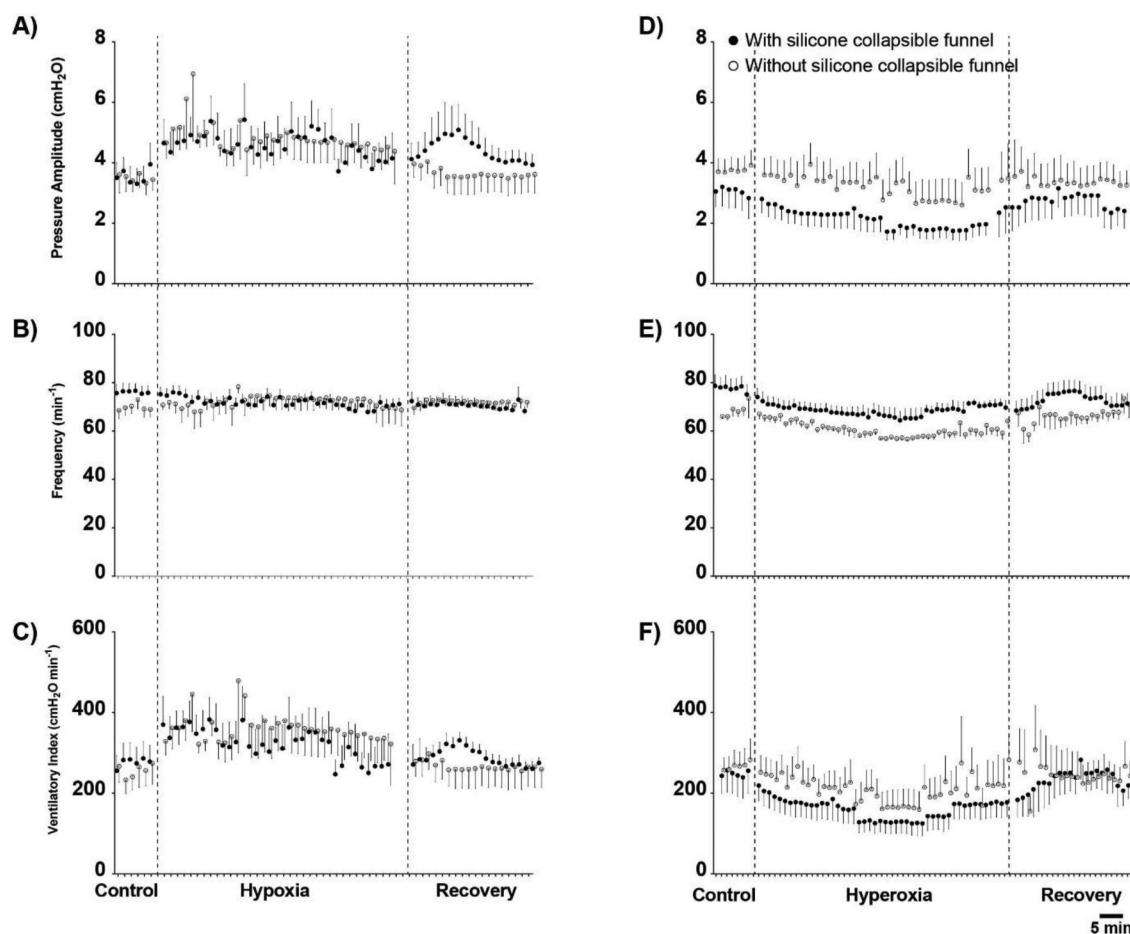
Fig. 2. Ventilatory flow parameters measured during control normoxia, during 1 h in either hypoxia (50% saturation, closed symbols) or hyperoxia (>200% saturation, open symbols) and during recovery in normoxia in rainbow trout ($N = 6$, left-hand panels) and goldfish ($N = 3$, right-hand panels): (A, D) ventilatory flow (\dot{V}_w); (B, E) ventilatory frequency; (C, F) ventilatory stroke volume. Asterisks (on the line: hypoxia, under the line: hyperoxia) indicate periods during which the measured parameters were significantly different from the mean normoxic control value. Measured parameters were averaged every 60 s and plotted. The high variation in the calculated stroke volumes in the goldfish originated from the episodic breathing that occurred during this treatment. Data have been expressed as means ± 1 SE. The threshold for statistical significance was $p < 0.05$.



rainbow trout. Thus, \dot{V}_w increased by 230% during hypoxia ($p = 0.0304$) and recovered only partially during 30 min of normoxia restoration (Fig. 2D). This hyperventilation was achieved by significant increases in frequency (by 25%, $p = 0.0403$; Fig. 2E) and an almost significant increase in stroke volume (by 170%, $p = 0.0522$; Fig. 2F). During hyperoxia, mean \dot{V}_w decreased by 55% ($p = 0.0330$; Fig. 2D). Breathing became intermittent with frequent pauses (Supplementary Figs. S1G, S1H¹), so frequency and calculated stroke volume both became very variable, with the former declining by about 80% (Fig. 2E) and the latter increasing many-fold (Fig. 2F), but these changes were not significant due to large variation and low N number. (Fig. 2). During the 0.5-h period of return to normoxia, ventilatory recovery from hypoxia was incomplete in goldfish, in contrast with trout (Fig. 2).

Using rainbow trout, the experiments of Series II addressed the possibility that our system might restrict the fish's resting ventilation and (or) ventilatory responses to hypoxia and hyperoxia. Buccal pressure amplitude (Figs. 3A, 3D) and ventilatory frequency (Figs. 3B, 3E) were measured in separate trout either with or without the BPL and SCF attachments. There were no significant differences at any time in pressure amplitude (cm H₂O), frequency (min⁻¹), or their product (ventilatory index, cm H₂O·min⁻¹) between the two treatments. In case any differences between the two treatments were missed in the two-way ANOVA, they were also checked by Student's two tailed t test at each time; there were no significant differences. Furthermore, the patterns of ventilatory index changes (35% increase during hypoxia (Fig. 3C), 45% decrease during hyperoxia (Fig. 3F)) were virtually identical to those seen in \dot{V}_w in Series I (30% increase, 40% decrease; Fig. 2A).

Fig. 3. Changes in (A, D) buccal pressure amplitude, (B, E) breathing frequency, and (C, F) ventilatory index in rainbow trout with (solid symbols) or without (open symbols) the silicone collapsible funnel (SCF) and balloon piece layer (BPL) in place. Measurements were made during control normoxia, during 1 h in either hypoxia (50% saturation, left-hand panels) or hyperoxia (>200% saturation, right-hand panels) and during recovery in normoxia. Data have been expressed as means \pm SE ($N = 6$). There were no significant differences between the two treatments (with SCL and BPL versus without SCL and BPL) at any time.



Discussion

Overall, our measured ventilation flow values in our less invasive system were close to those reported by Davis and Cameron (1971) in 210-g trout at similar temperature (8.6 °C) in Vancouver water. Davis and Cameron (1971) used a modified van Dam box with the rubber membrane sewn to the fish's lips. In van Dam's original study (van Dam 1938), where there was no sewing, his single 900-g trout also pumped the same control \dot{V}_w under normoxia at a frequency of 90 min⁻¹ in slightly warmer water (10–12 °C). Our trout exhibited a significant and sustained 30% increase in \dot{V}_w when acutely exposed to hypoxia (50% saturation; Fig. 2A), achieved almost entirely by a nonsignificant increase in ventilatory stroke volume (Fig. 2C). van Dam's trout exhibited an 80–100% increase in \dot{V}_w at the same level of hypoxia (50% saturation), again mainly achieved by increased stroke volume. However, by way of contrast, Davis and Cameron (1971) reported a 600% increase in \dot{V}_w at 40% saturation, and Kinkead and Perry (1990) reported a 200% increase in \dot{V}_w at 46% saturation (10–19 °C), both using sewn-on membranes. Holeton and Randall (1967) reported a 900% increase in \dot{V}_w at 50% saturation (15 °C) calculated by the Fick principle in trout fitted with many catheters. However, in Series II, our trout fitted with only a single buccal catheter (and therefore lightly instrumented) exhibited responses in breathing frequency, pressure amplitude (representative of stroke volume) and ventilatory index (representative of \dot{V}_w) quantitatively similar to those of Series I (Figs. 2, 3). A tentative overall conclusion is

that more invasive techniques tend to amplify the responses to hypoxia.

In response to acute hyperoxia, our trout decreased \dot{V}_w by about 40% (Fig. 2A), mainly due to a large decrease in stroke volume (Fig. 2C). By way of comparison, in trout fitted with sewn-on membranes, reported decreases in \dot{V}_w were about 65% at 10–19 °C (Kinkead and Perry 1990) and 40%–60% at 12–16 °C (Wood and Jackson 1980), again achieved almost entirely by reductions in stroke volume.

During hypoxia, our goldfish at 15 °C increased \dot{V}_w by 230% during hypoxia (Fig. 2D) achieved by 25% increases in frequency (Fig. 2E) and much larger 170% elevations in stroke volume (Fig. 2F). We are not aware of any previous direct measurements of \dot{V}_w in goldfish. However, in the related common carp, Glass et al. (1990) used their stitched mask and flow-meter system (see Introduction) and reported 160% and 240% increases in \dot{V}_w in response to the same hypoxia level (50% saturation) at 10 and 20 °C, respectively. Stroke volume elevations dominated the response at 10 °C, while frequency elevations were more important at 20 °C. Tzaneva and Perry (2010) used impedance techniques to monitor ventilation in goldfish and found that a more severe acute hypoxia (20% saturation) caused 40% increases in ventilatory frequency and 90% increases in amplitude at 7 and 25 °C, respectively. Goldfish are known to normally exploit surface "air gulping" during hypoxia (Burggren 1982), an opportunity that was prevented by the systems used in all three studies, including the present one. This may help explain their large responses to hypoxia. With respect to

hyperoxia, Takeda (1990), using a van Dam box system and a stitched mask, found that hyperoxia decreased \dot{V}_w by about 60% in the related common carp at 25 °C. Our measurements appear to be in general accord with all these studies.

Interestingly, the goldfish that were exposed to hyperoxia showed consistently intermittent ventilation with frequent pauses (Supplementary Figs. S2G, S2H¹), and this pattern was also observed sometimes during normoxia but never in hypoxia (Supplementary Figs. S2E, S2F¹). In two other cyprinids, Glass et al. (1990) and Vulesevic et al. (2006) observed episodic ventilation patterns during normoxia in common carp and zebrafish (*Danio rerio*), respectively, and in the latter, these episodes became more common during hyperoxia and less common during hypoxia, as in the present study. We found that breaths became large, and there was a brief moment where the flow was reversed or in other words became bidirectional (Supplementary Fig. S2H¹). This did not occur in the common carp, where the instantaneous flow trace never became negative (Glass et al. 1990). At present, we are unsure whether the flow reversal in hyperoxic goldfish is real or an artifact, because we noticed that the fish tended to move its mouth forward when taking a large breath. In the closed anterior chamber, this could momentarily reverse the flow through the probe without reversing flow across the gills. Further research is required.

In conclusion, our less invasive system is easy to use, does not involve anesthesia or surgery, and produces reasonable measures of \dot{V}_w on a breath-to-breath basis in both large trout and small goldfish. It will be easily adaptable to fish of different sizes, and with careful design of the BPL and SCF attachments, it could be adapted to other fish with different morphologies. PO₂, PCO₂, and pH sensors can be easily mounted in the inflow and outflow compartments to monitor respiratory gas exchange. As illustrated in Supplementary Media SM1 and SM2¹, the fish can be quickly moved in and out of the system, so it is possible to make repeated measurements on the same animal after different treatments — for example after feeding or a bout of exercise. Success rate is close to 100%, in contrast with about 50% (in our hands) with traditional sewn or glued on rubber membrane techniques. Furthermore, as the procedures are less invasive, the fish is less stressed, so the measurements are more reliable, and the protocol may prove more acceptable to animal ethics committees.

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