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Cite this article: Wood CM, Eom J. 2019
The internal CO₂ threat to fish: high PCO₂ in
the digestive tract. *Proc. R. Soc. B* **286**:
20190832.
<http://dx.doi.org/10.1098/rspb.2019.0832>

Received: 15 April 2019

Accepted: 25 June 2019

Subject Category:

Development and physiology

Subject Areas:

physiology

Keywords:

trout, goldfish, feeding, bicarbonate (HCO₃⁻),
blood gases, climate change

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Electronic supplementary material is available
online at [https://dx.doi.org/10.6084/m9.
figshare.c.4561118](https://dx.doi.org/10.6084/m9.figshare.c.4561118).

The internal CO₂ threat to fish: high PCO₂ in the digestive tract

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Our goal was to use novel fibreoptic sensors to make the first direct PCO₂ measurements in the digestive tracts of live freshwater fish (anaesthetized, artificially ventilated, 12°C). PCO₂ levels in gastrointestinal fluids were substantially higher than in blood, and were elevated after feeding. In the carnivorous, gastric rainbow trout, the mean PCO₂ in various parts of the tract increased from 7–13 torr (1 torr = 0.1333 kPa) during fasting to 20–41 torr after feeding, relative to arterial levels of 3.5–4 torr. In the agastric, omnivorous goldfish, the mean gut levels varied from 10–13 torr in fasted animals to 14–18 torr in fed animals, relative to arterial levels of 5–7 torr. These elevated PCO₂ values were associated with surprisingly high HCO₃⁻ concentrations (greater than 40 mmol l⁻¹) in the intestinal chyme. Incubations of food pellets with acid or water revealed endogenous PCO₂ generation sufficient to explain gastric PCO₂ in fed trout and anterior intestine PCO₂ in fed goldfish. The impacts of possible equilibration with venous blood draining the tract are assessed. We conclude that fish are already coping with PCO₂ levels in the internal gastrointestinal environment many-fold greater than those of current concern in the external environment for climate change and aquacultural scenarios.

1. Introduction

PCO₂ levels in the blood of water-breathing fish are very low because the solubility of O₂ in water is very low relative to the solubility of CO₂ [1]. As a result, the relatively high ventilation required to acquire sufficient O₂ from the water washes out PCO₂ from the bloodstream to very low levels, typically just a few torr (1 torr = 0.1333 kPa)—i.e. less than about 0.7 kPa, 0.7% CO₂). This is very different from air-breathers, where the effective solubility of the two gases in the respiratory medium is the same, and as a result, blood PCO₂ levels are much higher (typically 35–50 torr, or about 5–7 kPa, 5–7% CO₂). The low PCO₂ in fish blood is critical in setting the correct pH in the body fluids, and only small elevations in PCO₂ will have large effects (respiratory acidosis) on acid–base status. In turn, this will interfere with blood O₂ transport through Bohr and Root effects [2–4]. Fish are efficient in compensating respiratory acidosis by accumulating HCO₃⁻ through transbranchial Na⁺/H⁺ or Cl⁻/HCO₃⁻ exchanges, but neurosensory, behavioural, mitochondrial, and metabolic disturbances accompany this compensation [5,6]. As a result, there is now growing concern about how fish will deal with the elevated environmental PCO₂ levels predicted to occur with climate change—for example +0.75 torr, or about +0.1 kPa, +0.1% by the end of the century [7,8].

However, in mammals, there is evidence that the PCO₂ in the digestive tract is much higher than in the blood, reflecting CO₂ production by the interactions of gastric acid (HCl) with food and HCO₃⁻ secreted into the lower tract via hepatic bile and pancreatic fluid, as well as CO₂ produced in bacterial fermentation [9–11]. For example, the mean PCO₂ levels in human flatus were 1.5–8-fold greater than normal blood PCO₂ levels, depending on diet [12–14]. If the same is true in fish, then physiological mechanisms may already be in place to prevent this internal PCO₂ threat from equilibrating with the blood and disrupting acid–base regulation and O₂ transport. However, if the same is not true in fish, then digestive processes must be fundamentally different than in mammals.

Our objective was to use novel fiberoptic PCO_2 sensors to make the first direct measurements of PCO_2 in the gastrointestinal tracts of live fish. These sensors were first employed by Lee *et al.* [15] to measure PCO_2 in the haemolymph of dragonflies. In order to evaluate fish with very different feeding habits and gut morphology, the carnivorous rainbow trout (*Oncorhynchus mykiss*) that has an acid-secreting stomach was compared with the omnivorous goldfish (*Carassius auratus*) that lacks a stomach and therefore lacks gastric acid secretion. The goldfish also exhibits higher blood PCO_2 levels due to the presence of the inter-lamellar cell mass in the gills [16] at the low acclimation temperature used here (12°C). In order to understand the impact of feeding, both fed and fasted fish were examined, and the same commercial pellets were fed to both species.

2. Material and methods

(a) Experimental animals

Rainbow trout *O. mykiss* ($N = 23$; 130–426 g) and goldfish *C. auratus* ($N = 15$; 41–143 g) were obtained from Miracle Springs Trout Hatchery (Fraser Valley, British Columbia, Canada) and The Little Fish Company (Surrey, British Columbia, Canada), respectively. Fish were held at 12°C in flowing dechlorinated Vancouver tap water ($Na^+ = 0.09$, $Cl^- = 0.10$, $Ca^{2+} = 0.10$, $Mg^{2+} = 0.011$, $K^+ = 0.004$ mmol l⁻¹, hardness = 3.3 mg l⁻¹ CaCO₃, pH = 7.0) for several months prior to experimentation. Both species were fed to satiation every third day with commercial pellet food (45% protein, 24% lipid; BioTrout 4.0 mm, Bio-Oregon™, Longview, WA, USA). Fish for the fed treatment were taken from the feeding tanks at 12–72 h after feeding, while fish for the fasted treatments had not been fed for at least 7 days to ensure that the digestive tract was empty. All experiments were approved by the University of British Columbia Animal Care Committee (AUP A18-0271) under the guidelines of the Canadian Council on Animal Care.

The fish were anaesthetized with an initial knockdown in 0.1–0.2 g l⁻¹ MS-222 (NaOH-neutralized, Syndel Laboratories, Parksville, British Columbia, Canada), placed on an operating table, and continuously irrigated via the gills with temperature-controlled water (12°C). The water contained neutralized MS-222 at a concentration appropriate for each species (trout ~ 0.03 g l⁻¹, goldfish ~ 0.09 g l⁻¹), so as to maintain stage 5 anaesthesia—‘loss of reflex activity, total loss of reactivity, shallow opercular movements’ [17]. The dorsal aorta was cannulated [18] to facilitate blood sampling, and then PCO_2 and pH measurements commenced. Typical experimental periods were 3–5 h, and the fish continued to ventilate throughout. At the end of the experiment, the fish was euthanized by an overdose of neutralized MS-222 and weighed. The digestive tract was removed, and spread out. In some individuals, the total length of the intestine together with the fork length of the body were measured. In most fish, the stomach (trout only), and the anterior, mid, and posterior intestinal compartments were ligated, and the contents (fluids or chyme) were collected into sealable 2 ml plastic centrifuge tubes for terminal measurements. After immediate measurement of the pH, the samples were centrifuged (2 min, 5000g), and the supernatant was flash-frozen in liquid N₂ for later assay of total CO₂ content. Blood samples were similarly centrifuged, and the plasma supernatant was flash-frozen for the later measurement of lactate concentrations.

(b) PCO_2 , pH, and total CO₂ measurements

PCO_2 and pH were measured in all samples in live animals, as well as in incubated food pellets, and pH was additionally

measured in terminal chyme/fluid samples immediately after collection. Total CO₂ was measured only in freshly thawed supernatants from terminal samples, using a model 965 total CO₂ analyzer (Corning Instruments, Corning, NY, USA) calibrated with NaHCO₃ standards. Validation tests demonstrated that values did not change as long as the samples were flash-frozen, and assayed shortly after thawing on ice. Plasma lactate concentration was measured on freshly thawed samples using a Lactate Pro Meter (Arkray Inc., Kyoto, Japan) which has been validated for fish plasma [19,20].

The PCO_2 was measured using novel optical PCO_2 microsensors mounted inside #23 hypodermic needles mounted on 1 ml plastic syringes. The syringe was secured in a model M33301R three-axis micro-manipulator (World Precision Instruments, Sarasota, FL, USA), so that the probe could be appropriately positioned. These microsensors were prototype devices (200001368) manufactured by PreSens Precision Sensing GmbH (Regensburg, Germany). Each sensor was connected to a prototype single-channel electronic meter (200001367), with the output displayed on a personal computer running prototype software (200001488). Prior to each experiment, the PCO_2 microsensor was calibrated at the experimental temperature (12°C) using 0.9% (154 mmol l⁻¹) NaCl solutions equilibrated with CO₂-in-air mixes (typically 0.04, 0.1, 0.5, 1, 3, and 5%, and occasionally 10%) created by a 301aF precision gas-mixing pump (Wösthoff Messtechnik GmbH, Bochum, Germany). Calibration typically took about 1.5 h. The various calibration solutions were kept at 12°C in sealed Erlenmeyer flasks to allow calibration checks during and after the experiment. PCO_2 measurements were made by inserting the needle-mounted microsensor into the desired solution or section of the digestive tract, and then advancing the syringe plunger so that approximately 1 mm of the sensor tip was exposed. Recordings were made at 2 min intervals, and final stable values were typically attained after 10–20 min.

The pH was measured using a flexible oesophageal pH micro-electrode (MI-508; 1.4–1.6 mm OD) together with a flexible micro-reference electrode (MI-402; Microelectrodes Inc., Bedford, NH, USA) connected to a model 220 pH meter (Corning Instruments). The electrodes were calibrated at 12°C using precision buffers (Fisher Scientific, Toronto, Canada). The pH electrode was inserted into the target fluid or through the hole made in the digestive tract by the #23 hypodermic needle, and was advanced several millimetres forward in the latter. The micro-reference electrode was placed in the solution or into the body cavity of the fish.

(c) Experimental procedures

It was not possible to make all measurements in all fish due to optode breakage, loss of calibration, and unforeseen events, and the procedure varied from fish to fish. In general, arterial blood PCO_2 and pH were measured at both the start and the end of the experiment, by allowing blood from the catheter to fill a 0.5 ml centrifuge tube (thermostatted to 12°C) under its own pressure, with the catheter discharging at the bottom of the tube. The PCO_2 and pH electrodes were advanced to the bottom of the tube. Next, in some fish, measurements were made in the peritoneal cavity by blind puncture. The peritoneal cavity was surgically opened to expose the gastrointestinal tract which was kept moist throughout with physiological saline. In goldfish, measurements were made in the anterior, mid, and posterior intestines, and in trout, in the stomach, pyloric caecae (fed fish only), anterior, mid, and posterior intestines in random order. Additionally, in some fed trout, it was possible to insert the PCO_2 optode into the subintestinal vein. This was done immediately after measuring PCO_2 in the compartment from which the vein was collecting blood (e.g. posterior intestine), and before measuring PCO_2 in the compartment at the site of measurement (e.g. mid-intestine). The experiment usually ended with final arterial blood recordings, followed by euthanasia, and the terminal measurements described earlier.

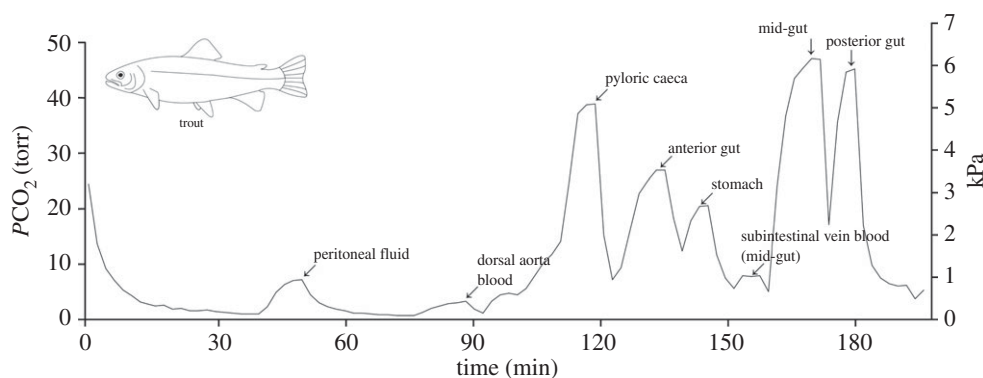


Figure 1. Original recording of PCO_2 with a PreSens fibreoptic microsensor in various sections of the digestive tract and blood of a fed rainbow trout.

(d) PCO_2 and pH measurements in incubated food pellets

To investigate whether PCO_2 elevations could be endogenously generated in the food pellets, they were incubated in sealed glass bottles with no air-space (2 ml septum HPLC vials, Waters Corporation, Milford, MA, USA) at 12°C with either distilled water or 100 mmol l⁻¹ HCl (to mimic gastric acid) for 2.5 or 24 h. PCO_2 and pH measurements were made by penetrating the septum. The added fluid to pellet ratio was 0.57 ml g⁻¹. Considering that the original moisture content of the food was 0.22 ml g⁻¹, the overall % water was about 50%, similar to that measured in chyme sampled from the stomach at 2–24 h post-feeding [21].

(e) Calculations and statistical analyses

In cases where measured PCO_2 and HCO_3^- concentrations were compared with calculated values, the Henderson–Hasselbach equation was used for the latter, with values for pK' and CO_2 solubility for trout plasma [22].

Data have been expressed as means \pm 1 s.e.m. (N = number of fish). Two-way ANOVA was used to analyse the measurements in the intestine within each species (factors: section, feeding) and in incubated food pellets (factors: fluid type, time), followed by Tukey's multiple comparison test to identify specific differences. For the arterial blood and peritoneal cavity, comparisons between fed and fasted values were made using Student's two-tailed unpaired t -test. Student's two-tailed paired t -test was used to evaluate differences between *in vivo* and post-euthanasia values measured or calculated in the same animals. Where necessary, data were appropriately transformed to ensure normality and homogeneity of variances. A significance level of $p < 0.05$ was used throughout.

3. Results

(a) Rainbow trout

The ratio of intestinal length, relative to body fork length, averaged 0.92 ± 0.02 (5). Figure 1 illustrates a representative experiment on a fed rainbow trout. It can be seen that PCO_2 values varied greatly among recording sites, but that all measured values in the gastrointestinal tract were markedly higher than in the blood. Directly measured mean PCO_2 values in arterial blood from the dorsal aorta were about 3.5–3.8 torr, close to resting values (approx. 3.2 torr) recorded by direct measurement in rainbow trout [23]. These did not differ significantly between the start and the end of the 3–5 h experiments, so average values have been reported in figure 2a. Arterial PCO_2 was not affected by feeding status.

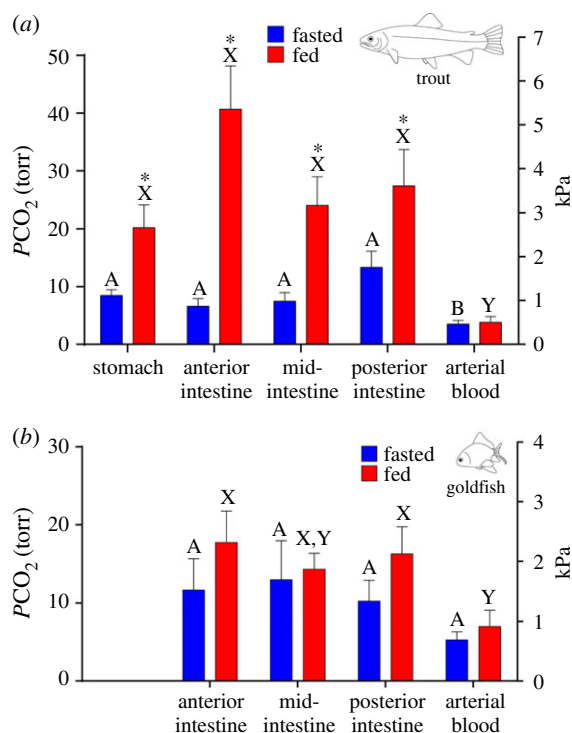


Figure 2. (a) Direct measurements of PCO_2 in the stomach, anterior, mid, posterior intestine, and arterial blood of fasted ($N = 6-7$) and fed ($N = 6-9$) rainbow trout. (b) Direct measurements of PCO_2 in the anterior, mid, posterior intestine, and arterial blood of fasted ($N = 6$) and fed ($N = 5-6$) goldfish. Means \pm 1 s.e.m. Asterisks indicate a significant effect of feeding ($p < 0.05$) within a section. Within a feeding treatment, means not sharing the same letter are significantly different ($p < 0.05$). (Online version in colour.)

However, in the gastrointestinal tract, by two-way ANOVA, there was a significant overall effect of feeding but not of section or interaction. In fasted trout, the mean PCO_2 in the tract was two- to fourfold greater than blood levels, ranging from about 7 torr in the anterior intestine to 13 torr in the posterior intestine, with no significant differences among the sections. In fed trout, this difference increased to 5–10-fold blood levels, with the mean PCO_2 values ranging from about 20 torr in the stomach to 41 torr in the anterior intestine. Again, there were no significant differences among the sections. The pyloric caecae in fed trout exhibited very similar PCO_2 values [35.6 ± 2.4 (8) torr] to those in the anterior intestine [40.7 ± 7.5 (8) torr] to which they are directly connected. We were concerned that opening the peritoneal cavity might disturb the values in the gut. However, this seems unlikely as

Table 1. A comparison of PCO_2 values (torr) directly measured in the subintestinal vein of fed trout with measurements in the preceding intestinal compartment from which the vein was collecting blood, and in the intestinal compartment at the point of measurement.

| | subintestinal vein | preceding intestinal compartment | intestinal compartment at point of measurement |
|---------|--------------------|----------------------------------|------------------------------------------------|
| trout A | 1.9 anterior | 7.7 mid | 25.9 anterior |
| trout B | 1.9 mid | 10.4 posterior | 15.5 mid |
| trout C | 34.0 mid | 37.2 posterior | 29.4 mid |
| trout D | 9.1 anterior | 46.0 mid | 27.1 anterior |
| trout E | 3.4 mid | 33.4 posterior | 23.4 mid |

PCO_2 values measured in the peritoneal cavity *before* opening it were close to blood levels, much lower than gastrointestinal levels, and did not differ with feeding status [4.5 ± 0.4 (8) torr in fasted trout versus 5.8 ± 1.5 (8) torr in fed trout]. Plasma lactate concentrations were also not significantly different in fasted [8.0 ± 0.9 (6) $mmol\ l^{-1}$] versus fed trout [6.9 ± 0.8 (9) $mmol\ l^{-1}$].

By visual observation, intestinal blood flow was greatly increased in fed animals, and in five fed trout, it was possible to insert the PCO_2 optode into the engorged subintestinal vein without impeding blood flow. Table 1 summarizes the measurements and figure 1 illustrates one example. In four of the five, the PCO_2 in the vein (2–9 torr) was low, far below that in the preceding intestinal compartment from which the vein was collecting blood, and also far below that in the intestinal compartment at the point of measurement from which it was also possibly collecting blood. Thus, PCO_2 equilibration did not occur. However, in one fish, subintestinal vein PCO_2 was high (34 torr), only slightly below that in the preceding intestinal compartment (37 torr).

For gut pH, there were significant overall effects for feeding, gut section, and their interaction, though the latter two became non-significant when the stomach was removed from the analysis. In fasted trout, the mean pH in the stomach was about 2.4, and this rose to 7.2–7.4 throughout the intestinal tract (figure 3a). Although pH tended to rise moving down the tract, there were no significant differences among the three intestinal sections, and the blood pH was in the same range in these anaesthetized fish. Feeding resulted in significant increases in all intestinal sections (by 0.3–0.5 pH units), as well as a non-significant rise in the arterial blood (by 0.15 pH units), whereas the increase in the mean stomach pH to 4.3, an elevation of 1.9 units, was much greater. As with PCO_2 , the pH in the pyloric caecae of fed trout [7.50 ± 0.14 (8)] was very similar to that of the anterior intestine [7.46 ± 0.14 (8)]. In the unopened peritoneal cavity, pH values were close to blood levels and did not differ with feeding status [7.34 ± 0.11 (6) in fasted trout versus 7.38 ± 0.13 (8) in fed trout].

The direct PCO_2 and pH measurements allowed calculation of HCO_3^- concentrations by the Henderson–Hasselbalch equation (figure 4a). There were significant overall effects of feeding, section, and interaction, though the interaction became non-significant when the stomach was removed from

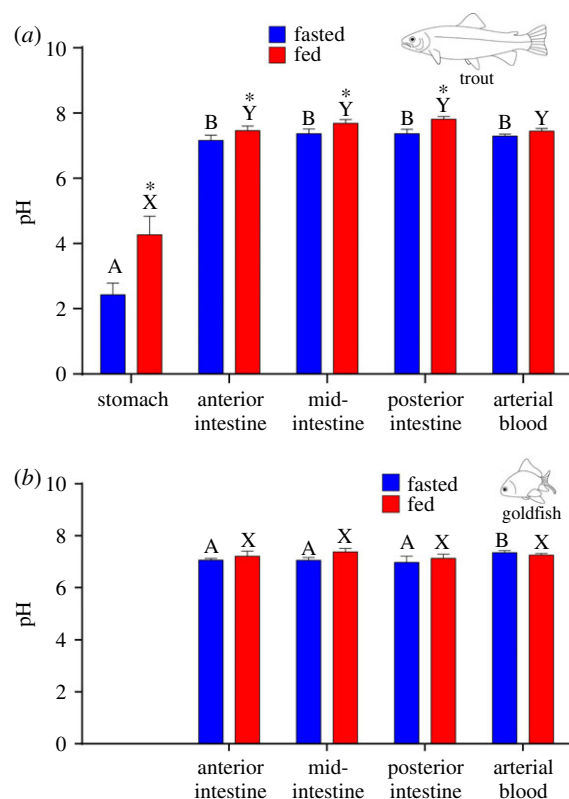


Figure 3. (a) Direct measurements of pH in the stomach, anterior, mid, posterior intestine, and arterial blood of fasted ($N = 6-7$) and fed ($N = 6-9$) rainbow trout. (b) Direct measurements of pH in the anterior, mid, posterior intestine, and arterial blood of fasted ($N = 6$) and fed ($N = 5-6$) goldfish. Other details as in the legend of figure 2. (Online version in colour.)

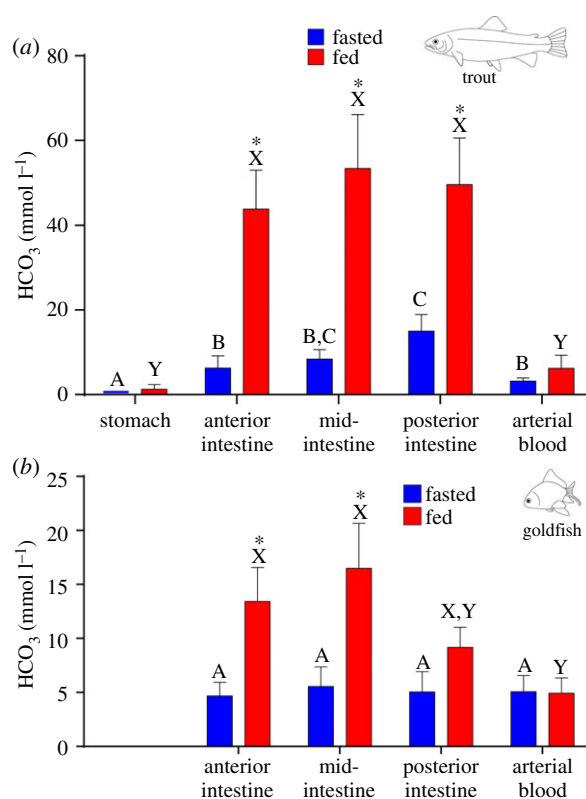


Figure 4. (a) Calculated HCO_3^- concentrations, in the stomach, anterior, mid, posterior intestine, and arterial blood of fasted ($N = 6-7$) and fed ($N = 6-9$) rainbow trout. (b) Calculated HCO_3^- concentrations in the anterior, mid, posterior intestine, and arterial blood of fasted ($N = 6$) and fed ($N = 5-6$) goldfish. The calculations based on the Henderson–Hasselbalch equation used direct measurements of PCO_2 and pH. Other details as in the legend of figure 2. (Online version in colour.)

the analysis. In fasted trout, the mean calculated $[\text{HCO}_3^-]$ rose from almost zero in the stomach to 6–8 mmol l^{-1} in the anterior and mid-intestine, and about 15 mmol l^{-1} in the posterior intestine, relative to 3 mmol l^{-1} in the arterial blood plasma of these anaesthetized fish. With feeding, the calculated $[\text{HCO}_3^-]$ remained at zero in the stomach, but rose significantly by four- to fivefold in all sections, reaching about 50 mmol l^{-1} in the mid and posterior intestine, relative to 6 mmol l^{-1} in the arterial blood. The mean calculated $[\text{HCO}_3^-]$ in the pyloric caecae $[42.9 \pm 9.8 \text{ mmol l}^{-1} (7)]$ was virtually identical to that in the anterior intestine $[43.8 \pm 9.2 \text{ mmol l}^{-1} (7)]$. The calculated $[\text{HCO}_3^-]$ values in the peritoneal cavity [fasted: $5.0 \pm 1.9 (6)$, fed: $8.5 \pm 3.1 (6) \text{ mmol l}^{-1}$] were non-significantly higher (by 30–50%) than the calculated blood plasma levels [fasted: $3.2 \pm 0.7 (6)$, fed: $6.3 \pm 3.0 (6) \text{ mmol l}^{-1}$].

Terminal measurements of pH in the chyme/fluid collected post-euthanasia showed very similar patterns to the direct *in vivo* measurements, and there were no significant differences on a paired basis (electronic supplementary material, table S1).

Terminal measurements of total CO_2 facilitated an almost direct determination of $[\text{HCO}_3^-]$ in the chyme/fluid (electronic supplementary material, table S1). These measurements did not differ significantly from calculated *in vivo* values and confirmed that $[\text{HCO}_3^-]$ was very high in the intestine, though a clear separation between fed and fasted trout was not apparent. These also allowed calculation of PCO_2 in the terminal samples (electronic supplementary material, table S1). Relative to the *in vivo* measurements, these calculated post-euthanasia PCO_2 values tended to be somewhat higher in fasted trout (significant only in the anterior intestine on a paired basis), while values in fed trout were similar to *in vivo* measurements and reinforced the elevation that occurred with feeding.

(b) Goldfish

The ratio of intestinal length, relative to body fork length, was 3.6-fold greater than in trout, averaging $3.30 \pm 0.18 (12)$. The mean PCO_2 in arterial blood from the dorsal aorta was about 5.0–7.0 torr, which is in the normal range for goldfish at low temperature (see Introduction), and did not differ between fasted and fed animals (figure 2*b*). Within the digestive tract, the mean PCO_2 values were two- to threefold greater than those in the blood. Two-way ANOVA revealed no significant effect of feeding, section, or interaction, though the mean PCO_2 levels tended to be higher in fed animals (14–18 torr) than in fasted animals (10–13 torr) (figure 2*b*). PCO_2 values in the peritoneal cavity *before* opening it were close to blood levels and did not differ with feeding status [$6.2 \pm 0.9 (9)$ torr in fasted goldfish versus $10.8 \pm 2.6 (4)$ torr in fed goldfish]. There was no significant difference in plasma lactate levels between fasted [$4.0 \pm 0.3 (6) \text{ mmol l}^{-1}$] and fed goldfish [$5.5 \pm 0.7 (6) \text{ mmol l}^{-1}$].

The mean pH values in the intestine tended to be slightly higher in fed goldfish (7.13–7.38) than fasted goldfish (6.97–7.06), relative to the mean blood values of 7.25 and 7.36, respectively, but again there was no significant effect of feeding, section, or interaction (figure 3*b*). In the peritoneal cavity, pH values were in the same range [$7.19 \pm 0.15 (4)$ torr in fed fish versus $7.31 \pm 0.05 (9)$ torr in fasted animals].

HCO_3^- concentrations (figure 4*b*) calculated from the *in vivo* pH and PCO_2 measurements revealed a significant overall

Table 2. PCO_2 and pH measured in food pellets incubated in sealed vials with distilled water or with 100 mmol l^{-1} HCl. Means ± 1 s.e.m. (*N*). Different superscript letters indicate significant differences over time within a fluid incubation.

| | | 2.5 h | 24 h |
|------------------------------|-----------------------|------------------------|------------------------|
| distilled water | PCO_2 (torr) | $8.4 \pm 0.5^a (5)$ | $13.9 \pm 2.6^b (4)$ |
| 100 mmol l^{-1} HCl | PCO_2 (torr) | $21.7 \pm 1.2^x *(5)$ | $28.5 \pm 1.8^y *(4)$ |
| distilled water | pH | $5.72 \pm 0.06^a (6)$ | $5.66 \pm 0.02^a (4)$ |
| 100 mmol l^{-1} HCl | pH | $5.12 \pm 0.06^x *(4)$ | $5.32 \pm 0.06^x *(4)$ |

*Significant difference ($p < 0.05$) between distilled water and 100 mmol l^{-1} HCl values.

influence of feeding, but no significant influence of section or interaction. In fasting goldfish, the mean calculated $[\text{HCO}_3^-]$ was low (about 5 mmol l^{-1}) in all sections. Feeding elevated the mean calculated $[\text{HCO}_3^-]$ by two- to threefold, differences which were significant in the anterior (13 mmol l^{-1}) and mid-intestine (16 mmol l^{-1}) only. The mean arterial blood levels were about 5 mmol l^{-1} and unaffected by feeding, and those in the peritoneal cavity were essentially identical (data not shown).

Terminal measurements of pH in the chyme/fluid collected post-euthanasia tended to be slightly higher relative to values measured *in vivo*, but none of the differences were significant on a paired basis except in the mid-intestine of fasted goldfish (electronic supplementary material, table S2).

HCO_3^- concentrations derived from post-euthanasia measurements of total CO_2 in chyme/fluid confirmed a significant overall effect of feeding, with no significant influence of section or interaction (electronic supplementary material, table S2). However, there were consistent marked differences in absolute values between these 'direct' measurements and the calculated *in vivo* values. The 'direct' measurements were rather variable, but fasting values averaged about 20 mmol l^{-1} (versus 5 mmol l^{-1} calculated *in vivo*), and fed values averaged 30–48 mmol l^{-1} (versus 9–16 mmol l^{-1} calculated *in vivo*). Most of these differences were significant on a paired basis. PCO_2 values calculated from these terminal samples were also generally higher than *in vivo* measurements, and showed a greater elevation in fed relative to fasting animals which was significant overall, with no significant effect of section or interaction (electronic supplementary material, table S2). On a paired basis, the differences between calculated post-euthanasia PCO_2 and measured *in vivo* PCO_2 were about twofold overall, and significant in the mid and posterior intestine of fed goldfish.

(c) PCO_2 and pH measurements in incubated food pellets

In food pellets incubated with fluids in sealed vials (table 2), the pH was about 5.7 with distilled water, and significantly lower (about 5.2) with 100 mmol l^{-1} HCl, actually higher than in trout stomach chyme *in vivo* (figure 3*a*), so the concentration of HCl used was not too high. Notably, PCO_2 increased significantly over time, reaching about 14 torr with distilled water, and 28 torr with 100 mmol l^{-1} HCl by 24 h.

4. Discussion

(a) High PCO_2 in the fish gut and potential consequences for the blood

These novel fiberoptic sensors provided the first direct PCO_2 measurements in the digestive tracts of fish, and revealed that PCO_2 in chyme/fluids of two freshwater species are clearly elevated above blood levels (figure 2*a,b*). Furthermore, this difference becomes much more pronounced after feeding, especially in the rainbow trout (5–10-fold greater than arterial PCO_2). To put these levels in perspective, these elevated PCO_2 levels in the gut environment are 10–50-fold higher than those that are of current concern in the external environment in climate change scenarios (see Introduction), and several fold higher than those now raising concern in intensive aquaculture [6,24,25]. Given the negative impacts of high blood PCO_2 in fish, the highly vascularized nature of the digestive tract [26,27], and its design for efficient absorption of other substances, do high gut PCO_2 levels have an impact on blood PCO_2 ?

Our few direct measurements in the subintestinal vein of trout (table 1 and figure 1) are not conclusive, but do demonstrate that in four of the five fed trout, the venous blood PCO_2 was far out of equilibrium with the PCO_2 in the intestine, and indeed much lower than it. To our knowledge, there are only two prior reports of PCO_2 in venous blood draining from the tract. Both are low. Eliason *et al.* [28] examined seawater-acclimated Atlantic salmon (9°C) that had been fasted 24–48 h, so were likely still in the post-prandial state. PCO_2 levels were 2.8–4.8 torr in blood sampled from the hepatic portal vein. However, the measurements were made with an ‘i-stat’ portable clinical analyser which calculates PCO_2 from an on-board script, and the validity of this analyser for PCO_2 measurements in fish blood has been challenged [29]. Cooper *et al.* [30] sampled from the subintestinal vein of seawater-acclimated rainbow trout (12°C) that had been fasted more than 72 h. Subintestinal vein PCO_2 was higher (4.7 torr) than in either arterial or mixed venous blood (2–3 torr). However, PCO_2 was not directly measured but rather calculated from pH and total CO_2 measurements by the Henderson–Hasselbalch equation.

The extent of blood equilibration with high gut PCO_2 should be investigated in future by more extensive direct measurements to address the following questions. Does this high PCO_2 play a role in O_2 delivery to the enterocytes by the Bohr and Root effects? Does some type of recycling mechanism maintain high PCO_2 levels in the tract, and prevent equilibration with the effluent blood flow? If the high CO_2 levels in the chyme are not taken away by the bloodstream, what is their fate? We are aware of no reports of flatulence in water-breathing fish, but in humans, CO_2 is the most abundant gas in expelled flatus [14].

The fact that all measurements were made on anaesthetized, artificially ventilated fish is a limitation of the current approach. The measured blood pH and calculated plasma [HCO_3^-] values indicate that these animals were exhibiting metabolic acidosis, a conclusion reinforced by measured lactate levels (7–8 mmol l^{-1} in trout, 4–5 mmol l^{-1} in goldfish) that were well above resting values but about half of those reported in exhaustively exercised trout [31] and goldfish [32]. Directly measured arterial blood PCO_2 values were representative of those recorded in resting trout [23] and

goldfish [16], so there appeared to be no limitation on CO_2 excretion (i.e. no respiratory acidosis). We are aware of no evidence that acidosis itself impacts gut blood flow, but exercise, hypoxia, and general disturbance are all known to do so [27,33]. By visual observation, we are confident that gastrointestinal blood flow was greatly increased in our fed preparations, and our results clearly show elevated gut PCO_2 levels with feeding. Nevertheless, caution is needed in extrapolating the present data to true *in vivo* values in unanaesthetized, undisturbed fish.

(b) Origin of high PCO_2 in the fish gut

The high gut PCO_2 levels clearly originate within the gastrointestinal tract itself, as PCO_2 measured in the unopened peritoneal cavity was close to blood levels. In this regard, fish are like mammals, which also exhibit high PCO_2 in the chyme/intestinal fluid [12–14] and lower PCO_2 in the peritoneal cavity [34]. In mammals, the two major sources of CO_2 in the tract are metabolic production by the microbial community, and the reaction of gastric HCl with carbonates in foods and HCO_3^- secreted in the bile and pancreatic fluids [9–11]. In fish, a third source (discussed below) may be H^+ and HCO_3^- secreted by the intestinal epithelium. Our simple measurements on incubated food pellets illustrated that pH becomes acidic and PCO_2 rises rapidly to greater than blood levels when just distilled water is present, and these effects are greatly augmented when 100 mM HCl is used (table 2). Therefore, direct production by gastric HCl acting on food pellets can explain the high PCO_2 in the stomach of fed trout (figure 2*a*), and the action of circum-neutral secreted fluid on pellets can explain the somewhat lower levels in the intestine of the agastric goldfish (figure 2*b*). Yet, PCO_2 levels in the tract are elevated above blood levels even in fasted fish, so presumably, these result from the residual metabolism of microbes and of the tract itself.

Recently, Brijis *et al.* [35] reported that in fasted trout, the whole digestive system consumes 11–25% of the whole animal’s O_2 uptake. Considering that blood flow to the fish digestive tract represents 10–40% of cardiac output in fasted fish and increases 1.35–2.1-fold after feeding [27,36–42], O_2 consumption and CO_2 production of the tract probably increase to a comparable extent. Indeed, the O_2 consumption of isolated intestinal epithelia from marine toadfish, measured *in vitro*, was elevated twofold at 6 h after feeding [43]. Presumably, some of the CO_2 produced may enter the gastrointestinal fluids.

We were surprised to find high HCO_3^- concentrations, which increased after feeding, in the chyme/intestinal fluids of both of these freshwater fish (figure 4*a,b*). While these values were calculated from the Henderson–Hasselbalch equation, rather than directly measured, they were qualitatively confirmed by measurements in chyme/intestinal fluid supernatants from post-euthanasia sampling (electronic supplementary material, tables S1 and S2). The latter were actually total CO_2 determinations from which HCO_3^- concentrations were calculated, but they were essentially direct HCO_3^- measurements, because [HCO_3^-] comprises more than 90% of the total CO_2 at typical intestinal pH values. High rates of intestinal HCO_3^- secretion were first reported in seawater-acclimated rainbow trout [44]. They are now known to play a key role in virtually all marine teleosts, serving to precipitate Ca^{2+} and Mg^{2+} ions from ingested seawater so as to

reduce the osmotic gradient opposing water absorption across the intestinal epithelium (reviewed by Grosell [45]). The presence of high HCO_3^- in the intestinal chyme/fluid of two freshwater teleosts, especially in the goldfish that never encounters seawater and has no need to drink, is a novel finding, and raises questions as to its function. In seawater teleosts, intestinal H^+ secretion runs in parallel to HCO_3^- secretion, but at a lower rate, titrating off a significant proportion of the latter [44–47]. Furthermore, HCO_3^- secretion increases after feeding in marine fish [43,48]. If the same phenomena occur in these freshwater species, then they could make a significant contribution to the high gut PCO_2 values recorded in the present study.

(c) Is there evidence of high PCO_2 in the digestive tract of other fish?

There are no direct PCO_2 measurements in other species. However, post-euthanasia terminal samples of pH and total CO_2 in chyme/intestinal fluid are available for some species, from which PCO_2 can be calculated. Wood *et al.* [46] calculated that such intestinal PCO_2 values were generally low (less than 5 torr) in fasted fish, but rose as high as 85 torr in fed killifish [46], 25 torr in fed toadfish [49], and 8 torr in fed flounder [50]. These values should be interpreted with caution. The present comparisons with direct *in vivo* measurements show that this approach works reasonably well in trout (electronic supplementary material, table S1) but only qualitatively in goldfish, with large quantitative overestimations for this species (electronic supplementary material, table S2). While post-euthanasia changes may be one confounding factor, uncertainties in pK' and CO_2 solubility coefficients are probably more important. These have never been determined for chyme/gastrointestinal fluids, so plasma values [22] were used. Furthermore, unless substantial carbonic anhydrase is present in chyme, disequilibrium conditions will very likely exist, making Henderson–Hasselbalch calculations even more problematic.

(d) Implications

The potential impact of high intestinal PCO_2 on blood O_2 delivery to the enterocytes should now be investigated with direct measurements of O_2 and CO_2 parameters in the tract, and in the inflowing arterial and outflowing venous blood. There is a particular need for these measurements in marine fish, because their high intestinal secretion rates of HCO_3^- suggest that gut PCO_2 may also be very high. Potentially, high gut PCO_2 levels could either promote or inhibit digestive and absorptive efficiencies, and this would be a useful topic to investigate from the viewpoint of aquacultural productivity. A commercial diet was used in the present study, but its composition could be manipulated experimentally to create higher or lower PCO_2 levels in the gut. Natural diets high in carbonate-rich bone, carapace, and shells might create even higher gut PCO_2 . There is also a need to understand how PCO_2 levels in the tract interact with other acid–base events associated with feeding, such as the post-prandial ‘alkaline tide’ in carnivores [51,52] and the ‘acidic tide’ [46] in agastric omnivores, as well as with the elevated environmental PCO_2 levels that occur in present-day aquaculture and future climate change scenarios [6].

Data accessibility. All the original raw data are in electronic supplementary material, table S3.

Authors' contributions. The study was conceived by C.M.W. and jointly performed by C.M.W. and J.E. C.M.W. wrote the first draft and J.E. edited it. Both authors gave final approval for publication and agree to be held accountable for the work performed herein.

Competing interests. We declare we have no competing interests.

Funding. Supported by an NSERC (Canada) Discovery grant no. (RGPIN-2017-03843) to C.M.W., and made possible by the generous gift of prototype PCO_2 fibreoptic PCO_2 electrodes, electronic meter and software by PreSens Precision Sensing GmbH.

Acknowledgements. We thank Sunita Nadella for statistical analyses, Matt Regan and Jeff Richards for the gift of the goldfish, and Fernando Martinez Ferreras, Miriam Ubach Granados, and Laura Perez Medina of PreSens Precision Sensing GmbH for advice and support.

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