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The gaseous gastrointestinal tract of a seawater teleost, the English sole (*Parophrys vetulus*)

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ABSTRACT

There has been considerable recent progress in understanding the respiratory physiology of the gastrointestinal tract (GIT) in teleosts, but the respiratory conditions inside the GIT remain largely unknown, particularly the luminal PCO_2 and PO_2 levels. The GIT of seawater teleosts is of special interest due to its additional function of water absorption linked to HCO_3^- secretion, a process that may raise luminal PCO_2 levels. Direct measurements of GIT PCO_2 and PO_2 using micro-optodes in the English sole (*Parophrys vetulus*; anaesthetized, artificially ventilated, 10–12 °C) revealed extreme luminal gas levels. Luminal PCO_2 was 14–17 mmHg in the stomach and intestinal segments of fasted sole, considerably higher than arterial blood levels of 5 mmHg. Moreover, feeding, which raised intestinal HCO_3^- concentration, also raised luminal PCO_2 to 34–50 mmHg. All these values were higher than comparable measurements in freshwater teleosts, and also greater than environmental CO_2 levels of concern in aquaculture or global change scenarios. The PCO_2 values in subintestinal vein blood draining the GIT of fed fish (28 mmHg) suggested some degree of equilibration with high luminal PCO_2, whereas subintestinal vein PO_2 levels were relatively low (9 mmHg). All luminal sections of the GIT were virtually anoxic ($PO_2 \leq 0.3$ mmHg), in both fasted and fed animals, a novel finding in teleosts.

1. Introduction

The respiratory physiology of the gastrointestinal tract (GIT) in fish has been relatively well-studied with respect to O_2 supply by the blood (reviewed by Seth et al., 2011) but relatively little is known about the respiratory conditions inside the lumen of the tract, or about how the other two respiratory gases (CO_2 and ammonia) are handled by the GIT. For example, to our knowledge there have been no measurements of PO_2 in the GIT lumen of any fish species, while a few recent investigations have reported ammonia levels that are many fold greater than blood levels (Bucking et al., 2013; Pelster et al., 2015; Rubino et al., 2014; Wood et al., 2019). A very recent study (Wood and Eom, 2019) was the first to directly record PCO_2 levels *in vivo* using a needle-type fibre-optic PCO_2 sensor ("micro-optode"). Surprisingly, in two freshwater (FW) teleosts, the carnivorous gastric rainbow trout (*Oncorynchus mykiss*) and the omnivorous agastric goldfish (*Carassius auratus*), PCO_2 levels in the gastrointestinal fluids/chyme of various parts of the GIT were much higher than in arterial blood. Thus in fasted fish of both species, GIT PCO_2 values were approximately 10 mmHg (1 mmHg = 0.1333 kPa), 2–3 fold higher than arterial blood values. With feeding, luminal PCO_2 increased moderately in the goldfish but dramatically in the trout to 20–41 mmHg.

As in mammals (Altman, 1986; Kurbel et al., 2006; Steggerda, 1968; Suarez et al., 1997; Tomlin et al., 1991), these high PCO_2 values were speculatively attributed to a combination of endogenous metabolism of the GIT tissues, metabolism of the gut microbiome, and to the reaction of gastric HCl with endogenous carbonates in the food, as well as with biliary, pancreatic, and/or intestinal HCO_3^- secretion. Additionally, simultaneous H^+ and HCO_3^- secretion by the intestinal epithelium can occur in fish (Grosell, 2011; Guffey et al., 2011; Wood et al., 2010) and could also contribute to elevations in luminal PCO_2.

A subsequent survey of indirectly measured PCO_2 values (calculated *via* the Henderson –Hasselbalch equation from terminal measurements of pH and total CO_2 concentration in the GIT fluids, as reported in the literature) in a wide variety of teleost and elasmobranch species yielded comparably high values, with a tendency for greater values in fed

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animals (Wood, 2019). As discussed subsequently, there are multiple uncertainties in terminal values obtained in this manner, but they nevertheless support the patterns seen in the direct measurements on FW trout and goldfish. Overall, these findings raise questions about how this high PCO_2 is generated, whether it equilibrates with the venous blood draining the GIT, and how it might impact blood O_2 transport *via* Bohr and Root effects (Nikinmaa, 2006).

The primary goal of the present study was to use this same technology (PCO_2 micro-optodes) to make the first direct measurements of luminal PCO_2 values in the GIT of a seawater (SW) teleost, under fasted and fed conditions. We selected the carnivorous, gastric English sole (*Parophrys vetulus*, also often known as lemon sole) because it is abundant, has suitable anatomy, readily eats in captivity, and has a relatively well studied intestinal physiology (Grosell et al., 1999; Grosell et al., 2001; Grosell and Wood, 2001). A parallel objective of our experiments was to use PO_2 micro-optodes to make the first ever direct measurements of PO_2 in the GIT of any teleost.

We hypothesized that luminal PCO_2 values in the English sole would be much higher than arterial blood levels, and even higher than in the carnivorous FW rainbow trout since the metabolic rate and blood flow requirements of the GIT appear to be higher in SW than FW teleosts (Brijs et al., 2015), likely due to the major role of intestinal HCO_3^- and H^+ secretion in the osmoregulation of SW teleosts (Grosell, 2011; Guffey et al., 2011; Wood et al., 2010). Following feeding, we hypothesized that stomach pH would decrease due to digestive HCl secretion, and intestinal pH would increase due to HCO_3^- secretion. We hypothesized that these acid-base movements would also result in a higher luminal PCO_2 value in fed relative to fasting fish. Based on limited reports on venous blood draining from the GIT (Eliason et al., 2007; Cooper et al., 2014; Wood and Eom, 2019), we speculated that the subintestinal venous blood would not equilibrate with the luminal PCO_2, but would instead maintain a low PCO_2. Despite quite extensive studies on the cardiovascular system of the GIT (Seth et al., 2011), there has been no investigation on the PO_2 profile of the lumen in fish, to our knowledge. Based on the mammalian GIT that is known to be virtually anoxic (Espey, 2013; Kurbel et al., 2006), we hypothesized that the teleost lumen would exhibit similarly low luminal PO_2.

2. Material and methods

2.1. Experimental animals

Experiments were performed at Bamfield Marine Sciences Centre (BMSC; Bamfield, BC, Canada) in August–September 2018 and 2019. English sole (436.9 ± 27.7 g; $N = 5$ in 2018 and $N = 14$ in 2019) were caught from the wild (Barkley Sound, BC) by line fishing under Department of Fisheries and Oceans Canada collection permits XR-204.18 and XR-212.19, and transferred to outdoor seawater flow-through tanks filled with 4 cm of sand to allow the animals to burrow. Fish were held at 10–12 °C and 32 ppt salinity under natural photoperiod. All fish were allowed to recover from capture for at least 1 week before experimentation. The fasted group was not fed for 7 days prior to experimentation. Fed fish were fed thawed, previously frozen anchovies, then moved to a separate tank and experimented upon within 48 h. Only those that were caught in 2019 were measured for total body length and GIT length ($N = 14$). With respect to individual measurements, occasional measurements could not be made because of experimental problems. All experiments were approved by the Animal Care Committees of the University of British Columbia (AUP 14-0251 and 18-0271) and Bamfield Marine Science Centre (RS-18-20 and RS-19-15).

2.2. Cannulation and blood measurements

The fish were anaesthetized with 0.1–0.2 g L^{-1} NaOH-neutralized MS-222 (Syndel Laboratories, Parksville, British Columbia, Canada) and placed on a flat surgery table. Due to the anatomical orientation of the fish, the gills were irrigated through the upper operculum with temperature-controlled water (10–12 °C). The anaesthetic level was adjusted as necessary so as to maintain Stage 5 anaesthesia (*i.e.* "loss of reflex activity, total loss of reactivity, shallow opercular movements" - McFarland, 1959) throughout the following cannulation and subsequent experiment. The caudal artery was cannulated for blood sampling as described by Watters and Smith (1973), using polyethylene tubing (Clay-Adams™ PE50, Becton and Dickinson Co., Franklin Lakes, NJ, USA) filled with Cortland saline (Wolf, 1963). The Cortland saline was modified for SW teleosts by the addition of 20 mmol L^{-1} NaCl, and was heparinized at 100 i.u. ml^{-1} with lithium heparin (Sigma-Aldrich, St. Louis, MO, USA). Approximately 1-ml samples of blood were collected from the catheter by blood pressure-driven flow into 2-ml microcentrifuge tubes. Measurements of pH, PO_2, and PCO_2 were made by inserting the micro-optodes to the bottom of the tube during and after collection. The Hct was determined by drawing blood into heparinized microhematocrit capillary tubes that were then centrifuged at 10,000 *g* for 5 min. The [Hb] was measured as described by Kampen and Zijlstra (1961).

The pH of the blood sample was measured using an oesophageal pH microelectrode (MI-508; 1.4–1.6 mm OD) and a flexible micro-reference electrode (MI-402; Microelectrodes Inc., Bedford, NH, USA) connected to a model 220 pH meter (Corning Instruments, Corning, NY, USA) in 2018, and using a thermo-jacketed Orion ROSS glass combination micro-electrode (Fisher Scientific, Toronto, ON, Canada) coupled to an Accumet™ meter (Fisher Scientific) in 2019. The electrodes were calibrated with precision buffers (Fisher Scientific and Radiometer-Copenhagen, Copenhagen, Denmark). The partial pressures of gases (PO_2, PCO_2) were measured using micro-optodes (manufactured by PreSens Precision Sensing GmbH Regensburg, Germany) mounted in #23 hypodermic needles. The micro-optodes were precisely positioned to the bottom of the collection vials using micro-manipulators (World Precision Instruments, Sarasota, FL, USA), and the blood samples were kept at experimental temperature and measured immediately during and after collection. The PO_2 micro-optodes were calibrated with air-equilibrated and sodium sulfite saturated saline kept in gas-tight bottles. The PCO_2 needle-housing type micro-optodes were prototype devices (PreSens 200 001 368) connected to an electronic transmitter (PreSens 300 000 114), with the output displayed on a personal computer running prototype software (PreSens 200 001 488). These PCO_2 optodes were calibrated with physiological salines equilibrated to 0.04, 0.3, 1, 3, 5, and 8% CO_2 (0.04–7.93 kPa, 0.3–59.5 mmHg) using CO_2/air mixtures created by a 301aF precision gas-mixing pump (Wösthoff Messtechnik GmbH, Bochum, Germany). The higher PCO_2 levels were needed to cover the range of values found in the GIT. All calibration solutions were in modified Cortland saline kept at the experimental temperature. After measurement of the gases, blood and gastrointestinal fluid samples (sampled as described below) were centrifuged (2 min, 5000 *g*), and the plasma and supernatant were flash-frozen in liquid N_2. The samples were later thawed over ice and measured for total CO_2 content (TCO_2) using a Corning 965 CO_2 analyser (Ciba-Corning Diagnostics, Halstead, Essex, UK) calibrated with $NaHCO_3$ standards. Previous validation tests in the lab demonstrated that the flash-frozen samples thawed on ice and assayed quickly thereafter exhibited TCO_2 values that were unchanged from those of fresh samples.

2.3. GIT measurements

Immediately following blood collection, the peritoneal cavity was surgically opened for direct *in vivo* measurements of PO_2, PCO_2 and pH in the GIT fluids. Similar measurement and gastrointestinal fluid sampling procedures were used as in Wood and Eom (2019). The same micro-optodes were directly inserted into stomach, pyloric caeca, and anterior, mid and posterior intestines of the anaesthetized fish. In fed

fish, the PO_2 and PCO_2 micro-optodes were additionally inserted into the engorged subintestinal vein for gas measurements in the venous blood draining from the GIT to the liver. These measurements were made immediately following the measurements in the lumen of the adjacent intestinal segment. In fasted fish, the subintestinal vein was generally collapsed so we were not able to make the same measurements. After gas measurements, small incisions were made in each segment for pH microelectrode insertion. Again, all insertions were made using micro-manipulators to correctly position the tips of the probes.

At the end of the experiment, the fish was euthanized by an overdose of neutralized MS-222, weighed and measured for total length. The length of the GIT from stomach to the end of posterior intestine was measured. The four segments of the GIT (stomach, anterior intestine, mid intestine, posterior intestine) were ligated and excised, and the gastrointestinal fluid was collected into 2-ml centrifuge tubes. The samples were centrifuged (2 min, 5000 *g*), and the supernatants were flash-frozen for TCO_2 assay as described above.

2.4. Calculation and statistical analyses

All graphs were made and statistical analyses were performed using Graphpad Prism software (version 7.0a). Data are expressed as means ± SEM (N = number of fish). Calculated PCO_2 and $[HCO_3^-]$ values were derived from rearrangements of the Henderson-Hasselbach equation (see Wood et al., 1983) using values for pK' and CO_2 solubility for teleost plasma (Boutilier et al., 1984). The limitations with respect to this calculation for gastrointestinal fluid are assessed in the Discussion. Relative GIT length was calculated as: GIT length*total fish length^{-1}.

Two-way ANOVA and *post hoc* Tukey's multiple comparison tests were performed on all GIT measurements. Differences in blood gas values between treatment groups and between arterial and venous blood were analyzed using two-tailed Student's unpaired and/or paired *t*-test as appropriate. The PCO_2 and PO_2 measurements of subintestinal venous blood and the lumen of the nearby GIT segment from which it was draining were compared using two-tailed Student's paired t-test. Blood pH, $[HCO_3^-]$, Hct, [Hb], and relative GIT length were also compared between treatment groups using the Student's unpaired t-test. A significance level of $p < 0.05$ was used in all tests.

3. Results

The total body length and body weight between fasted (31.7 ± 1.0 cm; 391.7 ± 24.7 g; N = 8) and fed (32.9 ± 1.0 cm; 497.3 ± 47.3 g; N = 6) groups were not significantly different (p = 0.40; p = 0.08 respectively). The relative GIT length was also not significantly different: fasted group 0.92 ± 0.03 (N = 6) and fed group 0.97 ± 0.06 (N = 6) (p = 0.50). Feeding had no effect on either hematocrit or hemoglobin concentrations (p = 0 .26 and 0.70 respectively). The Hct value of the fasted group was 21.8 ± 1.5% and of the fed group was 24.4 ± 0.9% (N = 5–6). The [Hb] of the fasted group was 6.2 ± 0.4 g dL^{-1} (N = 7) and of the fed group was 6.4 ± 0.4 g dL^{-1} (N = 7).

3.1. PCO_2

The mean stomach PCO_2 of the fasted fish was the lowest (13.5 mmHg) measured among all GIT segments in both groups but was not significantly different from measurements in the other segments of fasted fish (Fig. 1). All other GIT segments of fasted fish exhibited similar mean PCO_2 values (16.9–17.3 mmHg). Using two-way ANOVA, we found a significant overall effect of feeding on luminal PCO_2 of all GIT segments ($p < 0.0001$), but no effect of GIT segment on luminal PCO_2 (p = 0.71) and no significant interaction (p = 0.62). Among the intestine segments only, there was also a significant overall effect of feeding ($p < 0.0001$), but no effect of intestine segment (p = 0.56) and no significant interaction (p = 0.57). In both fasted and fed groups, we found that the inter-individual values tended to be more variable than intra-individual values across GIT sections. We varied the measurement order of GIT segments among individuals, and detected no influence on the order of measurement. The subintestinal blood was always measured after the final GIT measurement to ensure all tissue was getting sufficient blood supply.

The mean luminal PCO_2 values in all segments, except for the pyloric caecae, were significantly higher in the fed fish than the corresponding levels of the fasted group ($p < 0.05$). In the fed group, the mean pyloric caeca PCO_2 was the lowest measured value (34.1 mmHg) and was not significantly different from that of the fasted group. The mean PCO_2 values of all other segments were similar (49.4–50.3 mmHg) and about 2.7–3.6 fold higher than those of the fasted group.

Relative to a substantial increase in luminal PCO_2 after feeding, the difference between mean arterial blood PCO_2 in fasted (4.9 mmHg) and fed (9.3 mmHg) groups was modest and not significant (p = 0.06) (Fig. 1). After feeding, the PCO_2 values in the subintestinal venous blood and arterial blood were significantly different (p = 0.014). The subintestinal PCO_2 values were quite variable among the 5 fed individuals measured: one fish at 4 mmHg; 2 fish at ~17 mmHg; 2 fish at ~50 mmHg (Table 1). A comparison of the PCO_2 of the subintestinal venous blood and the lumen at the point of measurement by paired *t*-test revealed no significant difference (p = 0.15; N = 5).

3.2. pH

There was a significant overall effect of feeding (p = 0.007) and a significant overall effect of GIT segment on luminal pH ($p < 0.001$), as well as a significant interaction ($p < 0.0001$) (Fig. 2). However, the only GIT segment that exhibited a statistically significant change in pH associated with feeding was the stomach ($p < 0.001$): mean gastric pH decreased from 6.8 to 4.9 after feeding. The stomach pH after feeding was also significantly lower than any other intestinal segments in fed fish ($p < 0.05$). In the intestine alone (stomach excluded from the analysis), there was no significant effect of feeding (p = 0.87), no significant effect of the intestinal segment (p = 0.84), and no significant interaction (p = 0.30) for pH. The arterial blood pH did not change after feeding (p = 0.35), with mean values of ~7.6 in both treatments.

3.3. Calculated $[HCO_3^-]$

Mean $[HCO_3^-]$ values as calculated by the Henderson-Hasselbalch equation were close to zero in the stomach, but ranged from about 40 to over 100 mmol L^{-1} in the intestinal segments of both fasted and fed fish (Fig. 3). There was no overall significant effect of feeding (p = 0.18) on the GIT $[HCO_3^-]$ (Fig. 3) and no significant interaction (p = 0.29), but there was a significant effect of GIT segment on $[HCO_3^-]$ (p = 0.0002). In the fed group, the posterior intestine $[HCO_3^-]$ was significantly higher than that of other segments. There were no differences among segments in the fasted group. In the intestine alone (stomach excluded from the analysis) there was also no significant effect of feeding (p = 0.17) and no significant interaction (p = 0.33), but a significant effect of GIT segment on $[HCO_3^-]$ (p = 0.028), with a tendency for higher values in posterior regions. The arterial blood $[HCO_3^-]$ was not significantly different between fasted and fed groups (p = 0.32).

3.4. PO_2

All segments of the GIT were almost anoxic with mean values ≤0.3 mmHg in both groups (Fig. 4). Nevertheless there was a significant overall effect of feeding on GIT PO_2(p = 0.008), but no effect of GIT segment on PO_2 (p = 0.67) and no significant interaction (p =

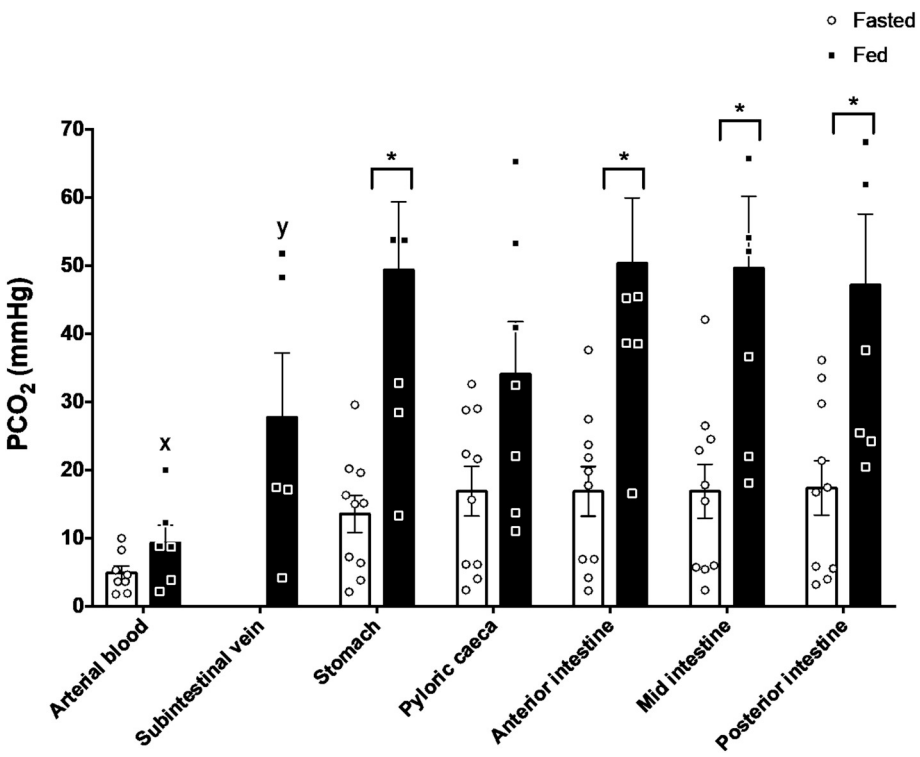

Fig. 1. Measurements of PCO_2 in the arterial blood, subintestinal venous blood, and GIT segments of fasted (N = 8–10) and fed (N = 5–7) English sole treatment groups. The subintestinal venous blood PCO_2 was not measured in the fasted group. Values are means ± SEM. Asterisk indicates a significant difference between fasted and fed groups within the respective GIT segment. Letters that differ indicate significant differences between arterial and venous blood within the fed group. There were no significant differences among GIT segments within a given treatment.

Table 1
The subintestinal venous blood PCO_2 values of 5 fed fish, and the intestinal PCO_2 values of the intestinal segment at the point of measurement.

Fed fish	Subintestinal vein PCO_2 (mmHg)	Intestinal segment PCO_2 (mmHg) at the point of measurement
#1	17.5	24.2; posterior
#2	48.3	76.5; anterior
#3	51.8	45.2; anterior
#5	17.1	21.9; mid
#7	4.1	38.6; anterior

0.67) (Fig. 4). The fasted group had higher mean PO_2 values (0.06–0.24 mmHg) than the fed group (0.00 mmHg throughout). The mean arterial blood PO_2 after feeding (27.5 mmHg) was significantly lower by 50% (p = 0.022) than that of the fasted group (54.7 mmHg). In the fed group, the mean PO_2 of the subintestinal venous blood (8.8 mmHg) was approximately one third of that of arterial blood (27.5 mmHg) (p = 0.033).

4. Discussion

4.1. Overview

In this study, we have made the first direct measurement of PCO_2 inside the GIT of a SW teleost (Fig. 1). Consistent with our original hypotheses, PCO_2 values in the GIT lumen of the English sole were greatly elevated relative to arterial blood levels, and were higher even than recent direct measurements in the carnivorous FW rainbow trout, and much higher than in the omnivorous FW goldfish (Wood and Eom, 2019). Our fasting mean values were 14–17 mmHg, whereas those in the trout were 7–13 mmHg. Furthermore, after feeding, GIT PCO_2 values increased to 34–50 mmHg in the English sole, relative to 20–41 mmHg in the post-prandial trout (Wood and Eom, 2019). These internal PCO_2 levels were much higher than environmental values of common concern in aquaculture (Skov, 2019) and global climate change scenarios (McNeil and Matsumoto, 2019). Moreover, the projected levels of water PCO_2 associated with climate change have been shown to significantly increase luminal HCO_3^- secretion to maintain water balance in another seawater teleost (Heuer and Grosell, 2016), and water PCO_2 levels in intensive aquaculture can be even greater. Clearly, these could further exacerbate luminal PCO_2 conditions. The present measurements support our hypotheses that greater acid-base transport rates associated with both life in SW and feeding result in higher luminal PCO_2 levels. Certainly, as predicted, pH in the stomach dropped markedly after feeding in the English sole, opposite to the pattern in the trout, and pH values then increased again to fasting levels in the intestine. This presumably reflected greatly increased gastric secretion of HCl and greatly elevated intestinal HCO_3^- secretion, respectively. With respect to whether the blood in the subintestinal vein equilibrates with the high PCO_2 levels in the GIT lumen, our results are not clear-cut but suggest, contrary to our hypothesis, that there may be some degree of equilibration, because most of the subintestinal vein PCO_2 values were very high and close to GIT lumen values. Finally, by making the first direct measurements of PO_2 in the GIT lumen, we have confirmed our hypothesis that the inside of the teleost digestive tract is virtually anoxic throughout, consistent with that observed in mammals (Espey, 2013; Kurbel et al., 2006).

4.2. CO_2 in the GIT

Our data on English sole are in agreement with a recent review by Wood (2019) that surveyed the PCO_2 values calculated *via* the Henderson-Hasselbalch equation from pH and total CO_2 measurements in the intestinal fluids reported for a variety of fish species. While there are uncertainties in this approach (discussed below), 11 of the 15 SW species had values greater than 5 mmHg, with a few above 20 mmHg. A similar phenomenon is known to occur in the human intestine, with luminal PCO_2 levels up to 8-fold greater than blood levels (Steggerda, 1968; Suarez et al., 1997). In teleosts, the high PCO_2 is presumably a result of the metabolism of the GIT tissue itself and of the gut microbiome, as well as osmoregulatory processes occurring in the GIT in SW species.

The GIT has a very high mass-specific metabolic rate relative to other tissues in teleosts (Brijs et al., 2018; Taylor and Grosell, 2009) and

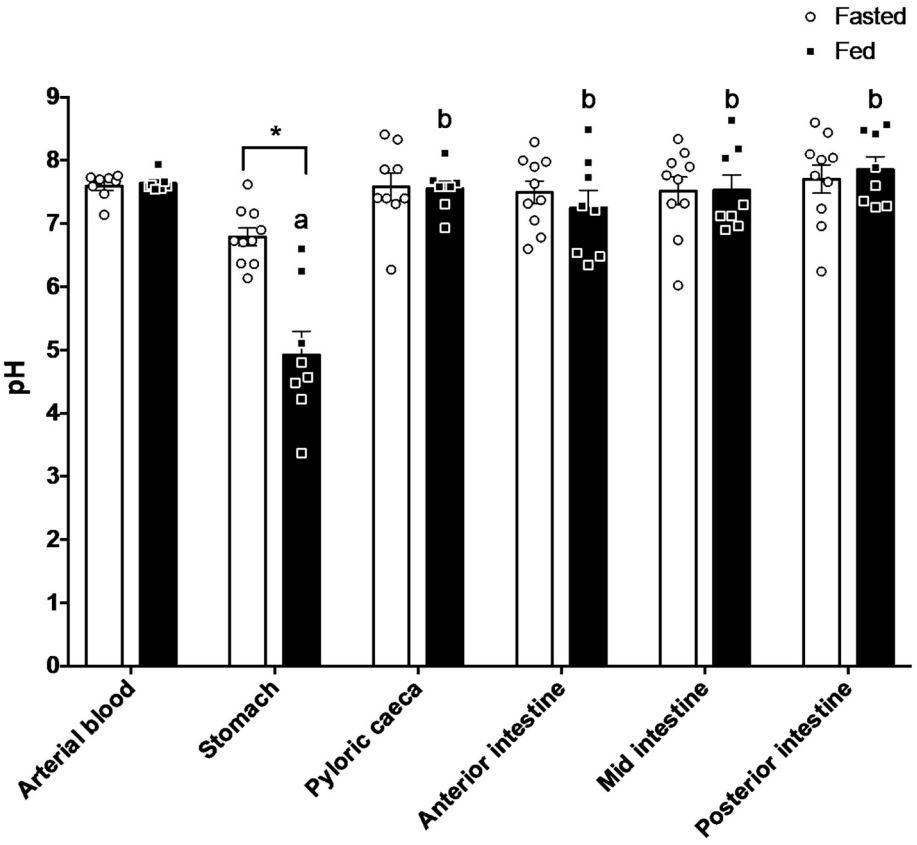

Fig. 2. Measurements of pH in the arterial blood and GIT segments of fasted (N = 8–10) and fed (N = 7–8) English sole treatment groups. Values are means ± SEM. Asterisk indicates a significant difference between fasted and fed groups within a GIT segment. Letters that differ represent significant differences among GIT segments within a fed group. There were no significant differences among GIT segments in the fasted group.

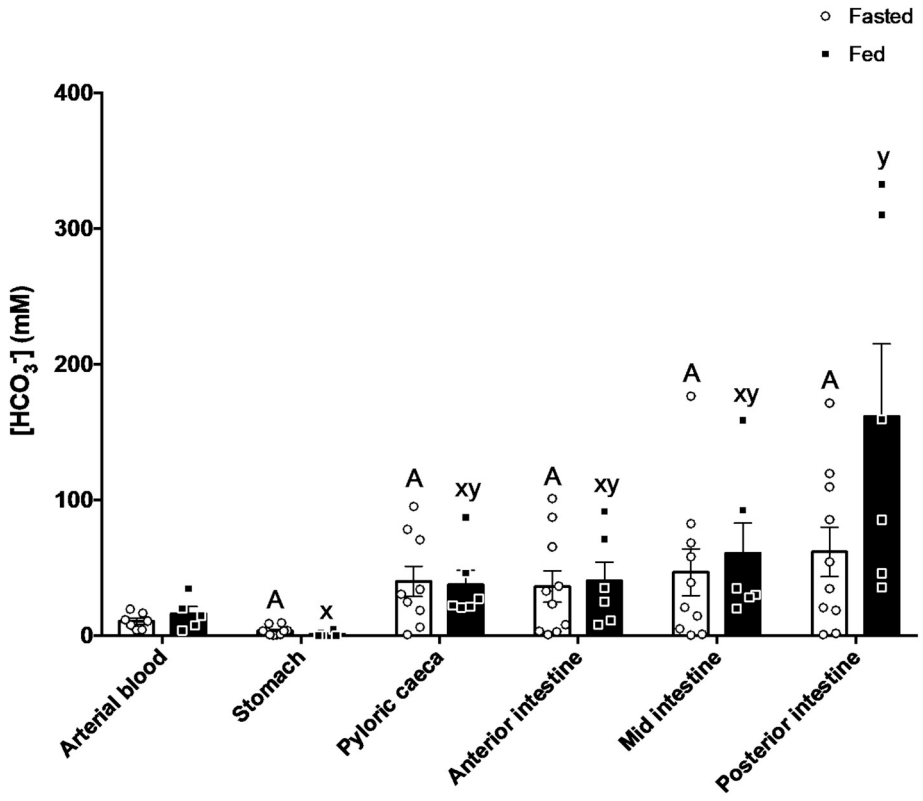

Fig. 3. Calculated [HCO_3^-] values using measured *in vivo* PCO_2 (Fig. 1) and pH (Fig. 2) values of fasted (N = 7–10) and fed (N = 5–6) English sole treatment groups. Values are means ± SEM. Letters that differ indicate significant differences among GIT segments within a treatment group.

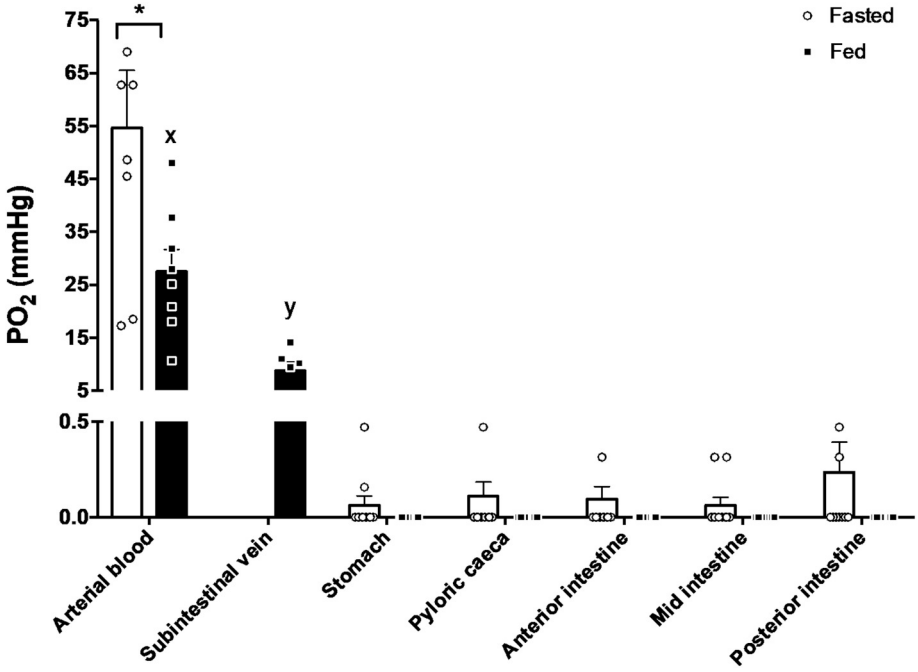

Fig. 4. Measurements of PO_2 in the arterial blood, subintestinal venous blood and GIT segments of fasted (N = 8–10) and fed (N = 6–9) English sole treatment groups. The subintestinal venous blood PO_2 was not measured in the fasted group. Values are means ± SEM. Asterisk indicates a significant difference of arterial blood values between treatment groups. Letters that differ indicate significant differences between arterial and venous blood values in the fed group. Note the break in the scale on the Y-axis. All mean GIT PO_2 values were ≤ 0. 3 mmHg in the fasted fish, and 0.0 mmHg in the fed group, and there were no significant differences among GIT segments or between treatment groups.

in mammals (Britton and Krehbiel, 1993; Duée et al., 1995). In unfed teleosts, the blood flow to the GIT typically ranges from 10 to 40% of cardiac output, and may increase greatly after feeding (Seth et al., 2011), indicating a significant functional importance. As a result, the respiratory CO_2 production by the enterocytes is presumably high. There are also some reports (Clements et al., 1994; Mountfort et al., 2002) of intestinal microorganisms which ferment amino acids and carbohydrates in fish to produce short chain fatty acids. Recent studies suggest that GIT microbiome composition, metabolic activity and enzyme activities change depending on the diet (Desai et al., 2012; Liu et al., 2016) as well as a variety of other environmental conditions such as geographical location (reviewed by Talwar et al., 2018). Fine flounder (*Paralichthys adspersus*) raised in the aquaculture setting were found to have different GIT microbiomes from those in the wild. However, in another flounder species (*Pleuronectes platessa*), microbiome composition was uniform and was not influenced by environmental parameters (Heindler et al., 2019). In the human large intestine, CO_2, along with H_2, CH_4, and H_2S, is also produced by the breakdown of these fatty acids (Macfarlane and Macfarlane, 2003) but this area has not been fully elucidated in teleosts.

The GIT of SW teleosts has an additional osmoregulatory function (Carrick and Balment, 1983; Fuentes and Eddy, 1997; Lin et al., 2001; Perrott et al., 1992; Smith, 1930). With an increase in ambient water salinity, there is an increase in drinking rate (see references within Grosell, 2007) and increase in blood flow to the GIT (Brijs et al., 2015). The ingested SW, along with additional [HCO_3^-] secreted in the esophagus during the desalination process (Esbaugh and Grosell, 2014; Takei et al., 2017), enters the stomach. Note that the calculated stomach [HCO_3^-] of fasted sole (3.7 mM; Fig. 3) was actually higher than typical SW levels (~2.3 mM).

Following feeding, the secretion of HCl reduces stomach pH (Koelz, 1992; Krogdahl et al., 2011) to values between 1 and 5 depending on a number of factors including species and time after a meal (reviewed by Bakke et al., 2011). In a non-digesting state, some species such as trout maintain an acidic stomach (*e.g.*Bucking and Wood, 2009; Wood and Eom, 2019) and some species keep the stomach pH high (*e.g.*Papastamatiou and Lowe, 2005; reviewed by Wood, 2019). The latter appears to be the case in English sole, as its stomach pH decreased from circumneutrality by almost 2 units following feeding (Fig. 2). In a previous study of SW European flounder (*Platichthys flesus*), stomach pH decreased to an even greater degree with time after feeding (Taylor et al., 2007), suggesting that flatfish in general may maintain stomach pH high during the non-digesting state, and decrease it with HCl secretion following food intake. The low pH favors dehydration of HCO_3^- to CO_2, both from ingested SW and carbonates in food, producing a significant increase in PCO_2 in the stomach after feeding (Fig. 1). This then suggests that the high [HCO_3^-] levels in the intestine arise mainly from endogenous intestinal HCO_3^- secretion that occurs posterior to the stomach to neutralize the GIT fluid (Fig. 3).

The gastrointestinal fluid entering the intestine is progressively depleted of Na^+ and Cl^- by transport processes of solute-coupled water absorption, leaving behind high concentrations of divalent ions such as Ca^{2+} and Mg^{2+}. The intestine secretes HCO_3^- to precipitate these ions (Genz et al., 2008; Marshall and Grosell, 2005; McDonald and Grosell, 2006), reducing the osmotic pressure and facilitating intestinal water absorption (Wilson et al., 2002). Thus, the SW teleosts typically have higher [HCO_3^-] in the lumen than FW teleosts (Wood, 2019). Fasted English sole had high calculated [HCO_3^-] in the intestine (36–62 mM; Fig. 3), similar to direct measurements in a previous study on the same species (23–42 mM; Grosell et al., 2001). The secreted HCO_3^- seems to be supplied by the respiratory CO_2 that is reabsorbed into the enterocytes and hydrated by intracellular carbonic anhydrase (reviewed by Grosell, 2019). In SW European flounder, a significant part of the endogenous enterocyte CO_2 produced from aerobic respiration supplies the HCO_3^- that is secreted *via* apical Cl^-/HCO_3^- exchangers (Grosell et al., 2005). The additional HCO_3^- secretion for osmoregulation may also then contribute to high PCO_2 in the intestine, especially since some H^+ secretion by the intestinal epithelium occurs in parallel to the HCO_3^- secretion (Grosell, 2011; Grosell, 2019; Guffey et al., 2011; Wood et al., 2010). In fact, *in vitro* gut sac experiments of killifish (*Fundulus heteroclitus*) showed higher mucosal PCO_2 when the fish were acclimated to SW than FW, and this increased further after feeding (Wood et al., 2010). Furthermore, our *in vivo* measurements of intestinal PCO_2 and calculations of intestinal [HCO_3^-] (~17 mmHg and 36–62 mM and respectively; Figs. 1 and 3) in the fasted English sole were higher than those found in unfed FW rainbow trout (7–13 mmHg and 6–15 mM respectively) (Wood and Eom, 2019). The presence of high PCO_2 in the intestinal fluid of English sole then likely helps to promote CO_2 recycling by diffusion back into the enterocytes (Grosell, 2019; Wood, 2019).

The GIT PCO_2 of English sole is further elevated following feeding, which probably is associated with the significant doubling in the calculated [HCO_3^-] in the posterior intestine (62–131 mM; Fig. 3). To neutralize acidic stomach fluid and allow digestive enzymes to function optimally in the intestine, fish secrete HCO_3^-. At present most evidence (*e.g.*Bucking et al., 2009; Ferlazzo et al., 2012; Grosell and Genz, 2006; Perry et al., 2010; Taylor and Grosell, 2009) points to the intestinal tissue itself as the major source of this HCO_3^- secretion (Wood, 2019). The role of the liver (*e.g.*Boyer et al., 1976; Grosell et al., 2000) appears modest, while exocrine HCO_3^- secretion by the fish pancreas has not been detected (reviewed by Bakke et al., 2011). The rate of [HCO_3^-] secretion into the intestine in both SW rainbow trout (Bucking et al., 2009) and SW gulf toadfish (*Opsanus beta*) (Taylor and Grosell, 2009) increased after feeding. Similarly, SW European flounder had high total CO_2 (11–45 mM) in the intestine 12 h following feeding (Taylor et al., 2007). Moreover, feeding also significantly increased the calculated intestinal [HCO_3^-] and measured PCO_2 of fed FW rainbow trout (43–50 mM and 36–41 mmHg, respectively) (Wood and Eom, 2019), but to a lesser extent than in fed English sole (38–162 mM and 34–50 mmHg, respectively). It is, however, important to note that the English sole were fed whole anchovies containing carbonate-rich bones, which may have resulted in an even greater PCO_2 than that associated with the use of commercial pellets used by Wood and Eom (2019) in rainbow trout. The increased PCO_2 could also be a result of increased O_2 consumption of the GIT tissue (Taylor and Grosell, 2009) and increased metabolism of bacterial colonies associated with feeding (Altman, 1986; Kurbel et al., 2006; Tomlin et al., 1991), however, it was beyond the scope of this study to investigate these different potential contributors.

4.3. Measurements versus calculations of PCO_2 and [HCO_3^-] in gastrointestinal fluids

In the present study, we have made direct measurements of gastrointestinal fluid PCO_2 levels using micro-optodes. With the exception of Wood and Eom (2019), all previous values of intestinal fluid PCO_2 levels in fish (summarized by Wood, 2019) were calculated *via* the Henderson-Hasselbalch equation from terminal measurements of pH and total CO_2. In the present study, we made some terminal measurements of these same parameters, so we could make paired comparisons with the directly measured *in vivo* PCO_2 values (Fig. 5A). While calculated and measured PCO_2 values were significantly correlated ($p = 0.007$, $r^2 = 0.41$, $N = 16$), quantitative agreement for any individual point was generally poor. Similarly, when calculated [HCO_3^-] values (computed from directly from measured PCO_2 and pH) were regressed against measured [HCO_3^-] values (from terminally sampled total CO_2), the relationship was again significant (Fig. 5B; $p = 0.022$, $r^2 = 0.32$, $N = 16$), but agreement for individual points was poor. Similar conclusions were reached by Wood and Eom (2019) for parallel comparisons in FW trout and goldfish. As discussed by these authors and by Wood (2019), possible reasons include uncertainties in pK' and CO_2 solubility coefficients for gastrointestinal fluid, micro-heterogeneity in the gastrointestinal fluid, absence of equilibrium conditions due to insufficient carbonic anhydrase activity in the gastrointestinal fluid, and post-mortem changes. The conservative conclusion is that while calculated values provide useful semi-quantitative indications, directly measured values are far more reliable.

4.4. O_2 in the GIT

We measured PO_2 in the GIT of English sole *via* direct insertion of PO_2 micro-optodes into the lumen and found very low PO_2 throughout the tract (Fig. 4), similar to the virtually anoxic mammalian GIT (Kurbel et al., 2006). The mammalian tract exhibits a steep PO_2 gradient from very low PO_2 (relative to arterial blood) at the outer margins, dropping to anoxia at the luminal midpoint. While usually attributed to respiration by aero-tolerant microbes (Espey, 2013), a recent study suggests that the unusual oxidative chemistry of the GIT fluids also contributes to O_2 depletion (Friedman et al., 2018). We did not attempt to measure a PO_2 gradient in English sole, and our measurements were made by blind puncture, with most micro-optode placements probably close to the midpoint of the lumen. Clearly, virtual anoxia predominates, but in future, it will be of interest to investigate potential PO_2 gradients (as well as pH, PCO_2, ion, and ammonia gradients) from margin to mid-lumen in both fed and fasted fish. Our findings raise questions about the oxygenation status of the enterocytes (discussed in the next section), and also call into question the relevance of gassing salines on the luminal surface of the gut with normoxia or hyperoxia, as is commonly done in transport studies *in vitro* (*e.g.*Bucking et al., 2013; Grosell et al., 2001; Grosell and Genz, 2006; Rubino et al., 2014).

4.5. Blood

We found the arterial blood pH (Fig. 2) and [HCO_3^-] (Fig. 3) of English sole did not change significantly following feeding, despite a significant decrease in stomach pH (Fig. 2). In many fish, as a consequence of H^+ secretion into the stomach for HCl formation, there is an equimolar increase in plasma HCO_3^- after feeding, causing an alkalinisation of the blood ("alkaline tide") (Bucking and Wood, 2009; Cooper and Wilson, 2008; Wood et al., 2005). Some fish such as the SW dogfish shark and FW rainbow trout compensate by excreting metabolic base across the gills (Bucking and Wood, 2008; Tresguerres et al., 2007; Wood et al., 2007, 2009). However, SW species may be equipped to deal with the elevated metabolic base load of the alkaline tide due to their higher capacity for HCO_3^- secretion into the intestinal lumen for osmoregulation as mentioned above. This has been seen previously in SW gulf toadfish (Taylor and Grosell, 2006) and SW European flounder (Taylor et al., 2007) that appear to not exhibit a postprandial alkaline tide. The same may be true in English sole, as indicated by the very high [HCO_3^-] in the posterior intestine (Fig. 3).

We are aware of no previous investigation on blood O_2 transport in English sole, but the starry flounder (*Platichthys stellatus*), with which it commonly hybridizes (Garrett et al., 2007), has been well studied (*e.g.*Milligan and Wood, 1987; Watters and Smith, 1973; Wood et al., 1979). Based on blood gases and blood O_2 dissociation curves reported by Wood et al. (1979) and Milligan and Wood (1987) for *P. stellatus*, our arterial blood measurements (PO_2, PCO_2, pH) in fasted animals were in the normal range for resting animals, yielding about 90% arterial O_2 saturation. However the increases in arterial PCO_2 (Fig. 1) and decreases in arterial PO_2 (Fig. 4) seen in fed fish would have depressed arterial O_2 saturation to about 70%. As there appear to be no previous data on fed flatfish, we cannot determine whether these are artifacts of anaesthetization and artificial ventilation, or a normal response to feeding. The mean PO_2 (8.8 mmHg; Fig. 4) measured in the subintestinal vein draining the GIT of fed English sole was lower than mixed venous PO_2 in the starry flounder (13.4 mmHg, from the caudal vein) which gave 66% O_2 saturation in resting, fasted *P. stellatus* (Wood et al., 1979). In combination with the very high (but variable) PCO_2 in the subintestinal vein (mean = 27.8 mmHg, Table 1, Fig. 1) of English sole, this would have greatly reduced the O_2 saturation in the blood leaving the intestine to less than 20%, based on blood O_2 dissociation curves reported by Milligan and Wood (1987) for *P. stellatus*. This could partly be due to the increase in metabolism of the GIT, an artifact of the anaesthesia and artificial gill irrigation, or a combination of both. Certainly the high PCO_2 would be beneficial in releasing blood O_2 from the venous reserve to the enterocytes by Bohr and Root effects (Jensen, 2004; Nikinmaa, 2006; Wells, 2009). However these elevated subintestinal PCO_2 values (Table 1) were quite variable among animals, and did not agree with generally low subintestinal vein PO_2 values reported in fasted (Cooper et al., 2014) and fed salmonids (Eliason et al., 2007; Wood and Eom, 2019), so it would be premature to draw firm conclusions. The diffusion of both O_2 and CO_2 between the lumen

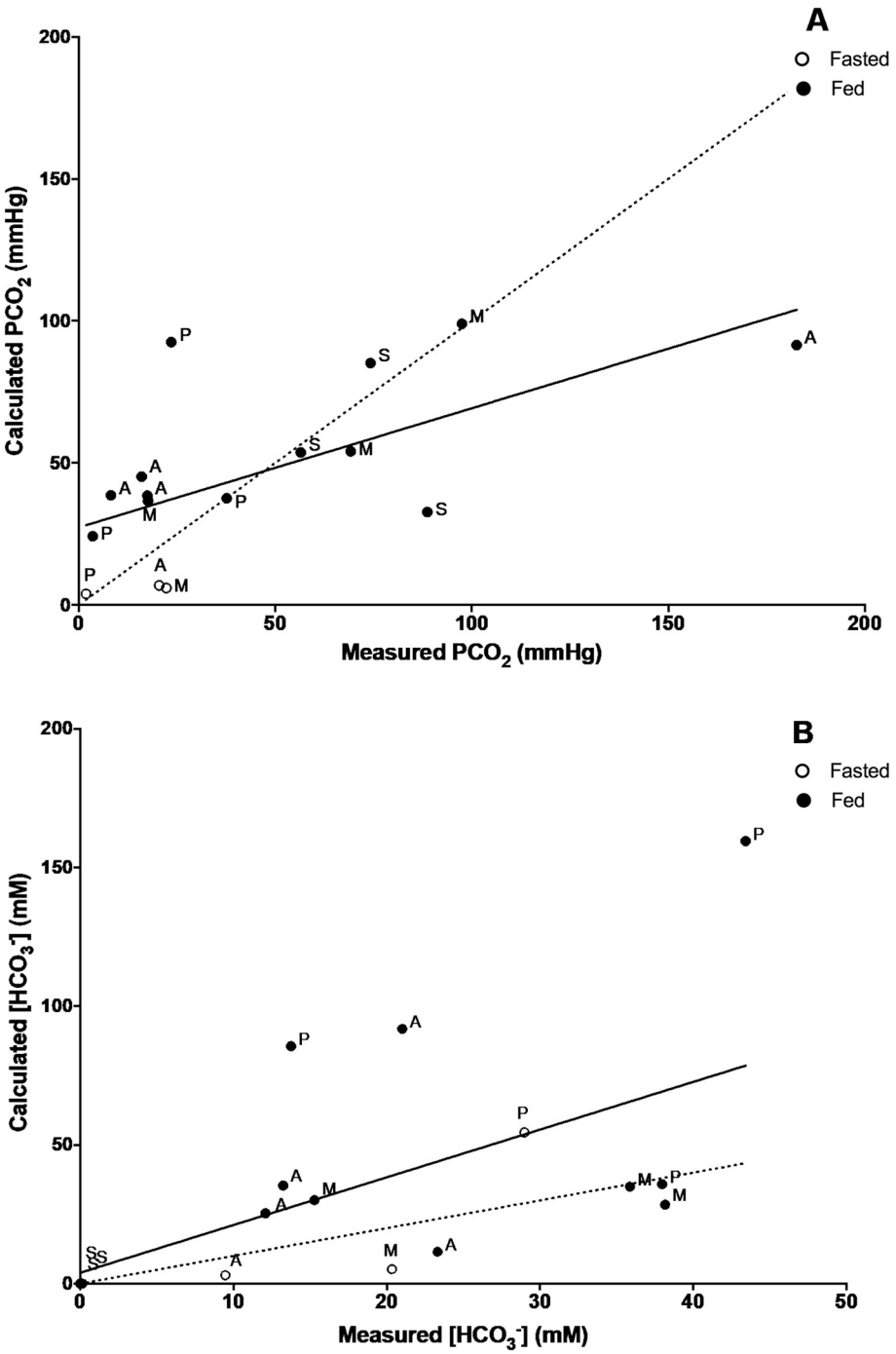

Fig. 5. Scatter plots of the calculated and measured (A) PCO_2 and (B) $[HCO_3^-]$ values of different GIT segments (S = stomach; A = anterior intestine; M = mid intestine; P = posterior intestine). The dotted lines represent the lines of equality and the solid lines represent best-fit linear regressions. (A: slope = 0.4195 ± 0.13; b = 27.31 ± 8.72; r^2 = 0.413; p = .0073; N = 16) (B: slope = 1.719 ± 0.67; b = 3.93 ± 16; r^2 = 0.32; p = .0224; N = 16).

and blood as well as their consequent effects on the O_2 delivery to the metabolically active enterocytes still requires further investigation.

4.6. Future perspectives

This study investigated the CO_2 and O_2 profiles in the GIT and part of its circulatory system in the marine English sole. We found that this SW teleost has relatively higher PCO_2 levels than two FW species, with levels increasing even further with feeding. We also found extremely low PO_2 levels throughout the GIT, a novel finding in teleosts. Thus, the influence of the high PCO_2 in the GIT on blood O_2 transport, especially given the anoxic lumen, would be of interest in the future. This study had a limitation of working with fish artificially ventilated under anaesthesia, which may have had some influence on the gas levels. Thus *in vivo* gas measurements in the GIT and blood of unanaesthetized free-swimming fish prior to and following feeding would be a very informative next step. The possible presence of PO_2 gradients in the lumen, the extent of gas diffusion between the GIT and blood, the fine details of the vasculature of the GIT villi, and the effect of various feeds on gas levels would all be of potential interest in future studies. Furthermore, in accord with a few previous reports on other teleosts (see Introduction), we have measured levels of ammonia, which is

another respiratory gas in fish (Randall and Ip, 2006), in the gastrointestinal fluid of the English sole that were much higher than blood levels (E. Jung and C.M. Wood, unpubl. results). Therefore, in future, the ammonia profile in the GIT and its influence on O_2 and CO_2 exchange dynamics would also be of great interest.

Authors' contributions

E.H.J. and C.M.W and devised the study. The experiments were conducted by E.H.J. with the help of J.E. C.M.W. and C.J.B. provided supervision. E.H.J analyzed data and wrote the first draft. C.M.W., C.J.B., J.E., and F.M. edited it. F.M. provided PCO_2 micro-optodes and advice on their use.

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Declaration of competing interest

We declare we have no competing interests.

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