www.sciencemag.org/cgi/content/full/340/6138/1327/DC1

Supplementary Material for

Roof Effect Hemoglobin May Have Evolved to Enhance General Tissue Oxygen Delivery

Jodie L. Rummer,* David J. McKenzie, Alessio Innocenti, Claudiu T. Supuran, Colin J. Brauner

*Corresponding author. E-mail: jodie.rummer@jcu.edu.au

Published 14 June 2013, *Science* **340**, 1327 (2013)
DOI: 10.1126/science.1233692

This PDF file includes:

Materials and Methods

Experimental animals, holding conditions

Rainbow trout, (*O. mykiss*, mean wet body mass ±SD: 1,291 ±390 g, $N = 13$), obtained from Spring Valley Trout Farm (Langley, British Columbia, Canada), were maintained in outdoor aquatic facilities in the Department of Zoology at the University of British Columbia until used for experiments. Fish were held in 8,000 l tanks supplied with flow-through Vancouver dechlorinated municipal tap water (average 12°C) under a natural photoperiod at densities no greater than 10kg/m^3 (*24*). Fish were fed every other day to satiation using commercial trout pellets (Skretting, Orient 4-0), but food was withheld 24 h prior to experimentation. All protocols complied with the guidelines approved by the Canadian Council on Animal Care, protocol # A07-0080/A11-0235.

Surgical procedures

Rainbow trout are well described in terms of their Hb-O_2 transport characteristics, they possess a pronounced Root effect (*12, 25-29*), and they have anatomically distinct slow-twitch oxidative "red" muscle (RM) that appears to contain plasma-accessible CA (*14, 15, 30-32*). Therefore, we implanted fiber optic O_2 sensors into rainbow trout RM (Fig. S1) so that we could directly monitor $RMPO_2$ in real-time. To do this, fish were anaesthetized in 0.1 $g \cdot l^{-1}$ MS-222 buffered with 0.1 $g \cdot l^{-1}$ $NaHCO_3$, and then immediately transferred to a surgery table. Gills were continuously irrigated with appropriately chilled, aerated, dechlorinated freshwater containing diluted anaesthetic (0.075 $g \cdot l^{-1}$ MS-222 and $NaHCO_3$). An indwelling cannula (PE50) was surgically implanted into the dorsal aorta (DA) according to Soivio et al. (*33*), following which a fiber optic O_2 sensor was implanted into the red muscle (RM) as described in Fig. S1. A fiber optic O_2 sensor (PreSens; Precision Sensing GmbH, Loligo Systems ApS, Denmark), with a 10 mm tapered Teflon-coated tip was connected to an Oxy-4 micro four-channel DAQ-TEMP O_2 meter and signal amplifier (cat #OX11700 Loligo Systems ApS, Denmark) via RS-232 serial cable and calibrated in humidified 12°C air (100% saturation) and a saturated sodium sulfite (Na_2SO_3, Sigma-Aldrich cat. no. 239321; St. Louis, MO, USA) solution (0% saturation). Phase angles were recorded and integrated into the algorithms provided with the manufacturer's software package, FibSoft™, for PC Windows. Guide marks were made with a permanent marker on the coated portion of the optode, see Fig. S1 for further details. Each calibrated optode remained soaking in heparinized Cortland's saline (100 IU ml^{-1}) until it was surgically implanted into the fish as described below, similar to the protocol of McKenzie et al. (*17*).

The fish, with DA cannula in place, was repositioned on the surgery table, and irrigation tubes were moved from the opercula/gills into the buccal cavity exposing the left lateral side for optode insertion. Moist sponges and paper towels were used to cover the rest of the fish throughout the surgical procedure. See Fig. S1 for a detailed description. The position of the optode in the RM was confirmed *post-mortem* under a dissection microscope upon completion of the experiment, and each optode was removed and recalibrated to account for drift over the experimental period.

Following surgery, fish were transferred to a black Perspex box to recover, during which time $RMPO_2$ was continuously monitored using the manufacturer's software. The DA cannula was flushed with heparinized (10 IU ml^{-1}) saline and then attached to a pre-

calibrated (as described above) stainless steel flow-through fiber optic O_2 sensor (FTCH-MICRO Loligo Systems ApS, Denmark) to measure arterial blood PO_2 during each blood-sampling interval as specified below. The open end of the DA cannula was filled with heparinized Cortland's saline, and sealed for the overnight recovery period. Both the $RMPO_2$ and arterial blood PO_2 sensors were reconnected to the Oxy-4 micro four-channel system. The $RMPO_2$ was logged every 2 min overnight, during which time fish were allowed to recover in black Perspex boxes in normoxic water.

Neuromuscular blockade and immobilization

Prior to experimentation, a baseline blood sample was collected from resting fish as described above. Then, fish were lightly anaesthetized over the duration of the following experimental protocol and fish were injected, via the DA, with a skeletal muscle relaxant, tubocurarine ((+)-tubocurarine chloride hydrate, Sigma-Aldrich cat. no. T2379; St. Louis, MO, USA) an anti-nicotinic, neuromuscular blocking drug (*34-37*). Fish were immobilized to reduce variations in muscle $\dot{M}O_2$ and reduce muscle activity (spontaneous activity in trout causes sudden large, prolonged changes in tissue O_2 recordings (*17*)) and to maintain the integrity and placement of the implanted optode during experimentation. It is understood that surgical procedures but not anaesthesia will elevate plasma catecholamines (*38*). Therefore it was assumed that these fish prior to, during, and following neuromuscular blockade would exhibit resting catecholamine levels because they were allowed overnight recovery from surgery. This was later confirmed upon plasma catecholamine analyses (Fig. S4).

Tubocurarine was prepared with Cortland's saline and administered for a final concentration of 0.1 mg ml^{-1} in the fish (assuming 5% blood volume). The concentration of tubocurarine used was chosen to cause complete immobilization of the animal (*37*). However, this would also impede normal opercular movements, and therefore fish were force-ventilated (1,600 ml min^{-1} kg^{-1}) in the black Perspex boxes during the experiments. This would also control for any changes in ventilation patterns that may have otherwise occurred during experimental treatments. Upon re-establishment of baseline physiological parameters (which ranged from 70-110 min), the experimental protocol commenced.

Experimental protocol

Once baseline $RMPO_2$ measurements had been recorded (Fig. 2, S3; Table S1), rainbow trout were exposed to an experimental series consisting of hypercarbia, normocarbia, C18, and hypercarbia with C18. Hypercarbic conditions (1.5% CO_2, balance air) were created in a header tank and regulated using a Cameron gas-mixer (Cameron, prototype U.S.A.). The PCO_2 (P_wCO_2) of the water flowing into the black Perspex box where experiments commenced was monitored continuously using a Clark-type PCO_2 electrode (Model #E201, Loligo Systems ApS, Denmark) and Radiometer PHM 71 Acid-Base Analyzer (Radiometer, Denmark). Fish were exposed to hypercarbia for approximately 20 min, during which time $RMPO_2$ was continuously monitored and logged every second. Following a 20-min hypercarbia exposure, blood was sampled for arterial blood PO_2, TO_2, pH_e, [Hb], Hct, whole blood TCO_2, plasma TCO_2 pH_i, and plasma catecholamines (described below, Fig. 2, S3, S4; Table S1). Then normocarbia was reinstated for at least 20 min, during which time $RMPO_2$ was monitored for

recovery. Upon recovery, blood was sampled and a bolus of C18 prepared in Cortland's saline and DMSO (20% by volume) was injected into the DA cannula to achieve a final concentration in the blood (assuming 5% blood volume) of 4 mg kg^{-1} or 200 μM (404.87 g mol^{-1} molecular weight) (*39, 40*). $RMPO_2$ was continuously monitored, and following 20 min., blood was sampled. Fish were then re-exposed to hypercarbia, as described above, $RMPO_2$ was continuously monitored and blood was sampled after 20 min. All blood samples withdrawn (≤ 9 ml (15% of the total blood volume) over the course of the experiment) were replaced with an equal volume of Cortland's saline administered over the course of 1 min.

Experiment and sampling procedures

For each blood-sampling interval approximately 1.5 ml of whole blood was withdrawn from the DA through a flow-through O_2 optode (to measure arterial blood PO_2) and collected in an Eppendorf™ tube. Total O_2 (TO_2) was measured according to Tucker (*41*) in duplicate on 50 μl aliquots taken from the blood sample via gas-tight Hamilton™ syringe. Whole blood [Hb] (mM per tetramer) was measured by adding 10 μl of whole blood to 1 ml Drabkin's solution (Sigma-Aldrich cat. no. D5941; St. Louis, MO, USA), measuring absorbance at 540 nm, and applying a millimolar extinction coefficient of 11. Haematocrit was determined by centrifuging 60 μl of whole blood in heparinized micro-capillary tubes for 3 min at 17,000 g. Blood pH (pH_e) was measured using a thermostatted capillary pH electrode (model BMS 3 MK 2 Blood Microsystem), in conjunction with a PHM 84 meter (Radiometer, Denmark). Duplicate 50 μl aliquots of whole blood were analyzed for TCO_2 (model 965 Analyzer; Corning). The remaining blood was centrifuged (3 min at 17,000 g), and plasma was removed to measure plasma TCO_2. The remaining plasma was then flash frozen in liquid N_2 and stored at -80°C for later analysis of catecholamine concentrations by HPLC with electrochemical detection (*42*). The red blood cell pellet was frozen in liquid N_2 and pH_i was later measured, according to the freeze-thaw technique (*43*), using the BMS 3 Mk2 Blood Microsystem and PHM 84 meter. Unless noted, all assays were performed in triplicate, and the grand means were reported and statistically compared.

Characterizing C18 for use in rainbow trout

A modified version of the electrometric delta pH CA assay (*44-48*) was used to compare the effectiveness of C18 with those of known teleost CA sulfonamide inhibitors (acetazolamide and benzolamide). The baseline reaction rates for the uncatalyzed and CA-catalyzed (using bovine CA) CO_2 hydration reactions were determined. The uncatalyzed reaction rate was significantly slower than CA-catalyzed rates ($p<0.001$; Fig. S6A), and all three inhibitors, C18, benzolamide, and acetazolamide also significantly slowed the CA-catalyzed reaction (Fig. S6B). Based on these trials, a C18 concentration of 200μM was used for experiments in this study, as this concentration most closely resembled the inhibition profiles for benzolamide and acetazolamide, where concentrations commonly used to inhibit CA *in vivo* and *in vitro* for rainbow trout have been used (Fig. S6).

To determine the degree to which C18 permeated the RBCs, a bolus of stock C18 prepared in Cortland's saline and DMSO (20% by volume, as used *in vivo*) was added to rainbow trout blood (collected from a separate group of fish 24h following cannulation,

techniques described above), maintained at 12°C, and equilibrated with 100% air in Eschweiler tonometers (final C18 concentration of 200 μM). A 1-ml sample was withdrawn after 30, 60, 150, 210, and 260 min and centrifuged. The resulting pellet was rinsed with physiological saline, and later analyzed to determine the degree to which RBC CA activity was inhibited if C18 had entered the RBC according to the methods of (*49, 50*).

To ensure that C18 would not enter the RBC over the duration of this study, the time required for C18 to significantly impair RBC CA activity was determined (*49, 50*). First, the catalytic rate of CA in rainbow trout RBCs (153.6 ±9.1 μM and 7.2 x 10^4 s^{-1}, expressed as K_{cat}) was measured; this value was assumed to represent 100% RBC CA concentration and activity. Blood samples (collected as described above) were then incubated with C18 for up to 260 min. and the catalytic rate of CA was measured (Fig. S7). Following incubation of RBCs in the presence of C18, RBC CA K_{cat} was not significantly reduced at 30 min (86% (6.1 x 10^4 s^{-1}) but was significantly reduced at 60, 150, 210, and 260-min (73, 63, 53, and 43% activity, respectively) ($F(5, 3) = 23.90$, $p<0.001$; Fig. S7). Thus, minimal C18 would be expected to enter the RBC over the 30 min exposure duration employed in this study.

Plasma catecholamines

Catecholamine levels, noradrenaline (NA), adrenaline (AD), and combined, were measured in rainbow trout plasma samples collected as described above (*N*= 7-11). After this time, 1.5 ml of blood was collected from each fish into heparinized syringes, withdrawn into an Eppendorf™ tube, centrifuged at 6,000 rpm for 3 min, plasma removed and expired into a separate Eppendorf™ tube, and immediately frozen in liquid N_2. All samples were shipped on dry ice to be analyzed by Dr. S.F. Perry at the University of Ottawa, Canada.

Plasma catecholamine levels were determined on alumina extracted samples (75-200 μl) using HPLC with electrochemical detection (*42*) and 3,4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard. The HPLC incorporated a Varian ProStar 410 solvent delivery system (Varian Chromatography Systems, Walnut Creek, CA) coupled to a Decade II electrochemical detector and VT-03 electrochemical flow cell (Antec USA, Hanover MD).

To compare these (Fig. S4) with true resting catecholamine levels, an additional group of fish (300-600 g wet body mass) was equipped with a DA cannula, as described above, and allowed to recover for 24-48 h (*51-53*). Plasma catecholamines for these fish were 4.9 ±2.8 and 0.4 ±0.2 nM for noradrenaline (NA) and adrenaline (AD) respectively (*N*=13).

Calculations and statistical analyses

Data are presented as mean ±SEM. Mean cell haemoglobin concentration (MCHC) was calculated as Hb/(Hct/100). Haemoglobin saturation (SO_2) was calculated by dividing TO_2 (after subtracting physically dissolved O_2 according to Boutilier et al. (*54*)) by the theoretical maximum carrying capacity of the rinsed RBCs based upon the tetrameric Hb concentration obtained spectrophotometrically according to Tucker (*41*). Blood PCO_2 was calculated from plasma TCO_2 and pH_e as described previously (*55*) using the CO_2 solubility coefficient and pK published for rainbow trout (*54*) and by

rearranging the Henderson-Hasselbalch equation. All PO_2 (RM and arterial) data were saved as text files and analyzed using Acqknowledge® Data Acquisition Software (Version 3.7.3, BIOPAC Systems, Inc.). Catecholamine concentrations for both noradrenaline (NA) and adrenaline (AD) were reported for each individual fish at each sampling interval (Fig. S4) and statistically compared to the mean resting levels derived from the parallel study performed on resting fish 24-48h post-cannulation (n=13, 4.9 ±2.8 and 0.4 ±0.2 nM, respectively). All other data were compared statistically between treatments, and statistical differences were detected via paired t-test or repeated measures ANOVA and, when necessary, a post-hoc Holm-Sidak multiple comparisons test. All statistical analyses were conducted using SigmaStat for Windows 3.5.0.54 (Systat Software, Inc., 2006), and all analyses were interpreted using $\alpha < 0.05$ to determine statistical significance.

Supplementary Text

Techniques used

The response to hypercarbia in this study was as predicted, and changes in blood PCO_2 and pH were relatively modest and consistent with those reported in previous studies. The pH changes that Smith and Jones (*56*) demonstrated in hypercarbic rainbow trout (from 7.99 to 7.79) were similar to the pH changes observed here after 20-30 min. However, a 20-30 min hypercarbia exposure decreased arterial blood pH_e from 8.09 to 7.79 and increased blood PCO_2 from 1.7 to 4.0 mmHg (Fig. 2, S3; Table S1). McKendry and Perry (*57*) observed that arterial blood pH decreased to 7.43, and blood PCO_2 increased to 6.15 mmHg during a similar 20-min 1.5% CO_2 hypercarbic exposure. The changes induced in this study were consistent with previous studies on non-immobilized fish and only slightly below those expected at 90% U_{crit} or post-exercise for rainbow trout (*58, 59*). Thus, disturbances to blood acid-base status of the magnitude chosen in this study are likely encountered *in vivo* in rainbow trout and therefore the Root effect could enhance tissue O_2 delivery under such conditions.

While hypercarbia typically induces hyperventilation in elasmobranchs and teleosts (*60-64*), we chose to control for this by immobilizing and force-ventilating the fish. In this study, fish were force-ventilated at a water flow rate of approximately 1,600 ml min^{-1} kg^{-1}, a rate 6-fold greater than that of previously measured hypercarbia-exposed fish (*56*), but this was necessary to recover tissue PO_2 following tubocurarine injection.

Hypercarbia was also chosen as the treatment to induce an acid-base disturbance because it has only minor, if any, affects on metabolic rate (*65*). Therefore, any changes in $RMPO_2$ could more easily be detected over an unchanging background O_2 consumption rate. Ultimately, this preparation was chosen to maximize the chances of observing the proposed phenomenon.

Verifying the role of plasma-accessible carbonic anhydrase

Results from this study indirectly support the presence of a plasma-accessible CA in rainbow trout RM. Hypercarbia increased $RMPO_2$ with a plasma-accessible CA-dependent mechanism (Fig. 2, 3, S3, S5). The C18 inhibitor does not enter the RBC for at least 30 min, and by 60 min, at least 75% of RBC CA activity still remains functional, which was well within the duration over which experiments were completed following

C18 injection (Fig. S7). Until this study, C18 had only been used in rats, and with great success. C18 is a potent CA inhibitor (Fig. S2), relatively impermeant to RBC membranes, and does not affect other physiological variables *in vivo* (*66*). Benzolamide, another low-molecular weight CA inhibitor that has been commonly used in fish, easily permeates the rat RBC such that after 2 h, the final concentration in the rat RBCs was 15,500-times the trace concentration of C18 (*66*). Successful use of C18 in fish is a great advance because to date localizing and determining function of plasma-accessible CA isoforms has been difficult in fish, despite their ubiquity among vertebrates (*67, 68*). Further research is necessary to localize and characterize these functionally significant CA isoforms.

Modeling the benefit to tissue oxygen delivery

To date, only two studies have monitored real-time $RMPO_2$ in a teleost. Both provided evidence that tissue O_2 delivery may be enhanced relative to other vertebrates, in part due to the presence of Root effect Hbs. $RMPO_2$ was measured in trout in normoxia and mild hypoxia, and during both sustained and exhaustive exercise (*17*). The $RMPO_2$ values (between 50 and 60 mmHg) were consistent with the values reported here (Fig. 2, 3, S3), being significantly higher than those in mammals (25-35 mmHg; (*2, 3, 69-72*), despite similar arterial and mixed venous blood PO_2 values (*3, 17, 73, 74*). Especially compared to rats and humans (*2, 3, 70-72, 75*), $RMPO_2$ is consistently high in teleost fishes such as the trout. Furthermore, $RMPO_2$ remains elevated during stress (hypoxia, exercise, and hypercarbia) (present study and (*17*)) and above P_vO_2 (approximately 20 mmHg; (*73*)), despite decreases in P_aO_2. All of these observations are associated with the presence of a Root effect Hb (*17*).

Given the substantial (31 mmHg) increase in $RMPO_2$ following exposure to hypercarbia, it is clear that a mild acidosis in the presence of a pH-sensitive Hb (Root effect) and plasma-accessible CA can increase the driving force for O_2 diffusion and potentially benefit tissue O_2 delivery. The *degree* to which O_2 delivery may be enhanced, however, is more difficult to determine. Changes in $RMPO_2$ at any given time or during any experimental condition will be influenced by myriad factors including tissue metabolic rate ($\dot{M}O_2$), tissue perfusion rate, and changes in Hb-O_2 affinity within the tissue. Assuming neither tissue $\dot{M}O_2$ nor tissue perfusion changed in this study – because fish were immobilized (to abolish skeletal muscle activity and variations in muscle $\dot{M}O_2$) and force-ventilated (to ensure constant P_aO_2) – the increase in $RMPO_2$ during hypercarbia likely reflected a right shift in the oxygen equilibrium curve (OEC) at the level of the muscle. In a similar experimental preparation where resting dogs were artificially ventilated and immobilized, arterial injection of an allosteric modifier of Hb-O_2 affinity (RSR13) resulted in a 12 mmHg increase in blood P_{50} (PO_2 at which the blood is 50% saturated with O_2) which in turn was associated with a 9 mmHg increase in mean muscle PO_2 (*76*). These results are consistent with the assumption that, in a preparation of this nature, a change in blood P_{50} (and thus Hb-O_2 affinity) will manifest as an increase in muscle PO_2. In the present study, we did not measure tissue perfusion but, given that catecholamines were not significantly elevated over published resting values, it is unlikely that hypercarbia had an effect (*57, 77*) and there was no difference in $RMPO_2$ between control values and C18+hypercarbia. Thus, assuming the increase in $RMPO_2$ during exposure to hypercarbia was predominantly associated with the right shift of the

OEC within the RBC during capillary transit, the potential benefit to O_2 delivery can be interpolated by plotting arterial and $RMPO_2$ values onto the OECs of rainbow trout blood incubated at various CO_2 tensions and thus pH values (re-plotted from (*12*); Fig. S8).

Modeling O_2 extraction from the OEC in this manner would normally apply to differences between P_aO_2 and P_vO_2; $RMPO_2$ is lower than P_aO_2, and while higher than P_vO_2, probably more closely resembles P_vO_2 values and will be regarded as such to model O_2 delivery here (Fig. 6). Arterial O_2 content did not differ significantly among any treatments and is indicated by "a" in Fig S8. The mean $RMPO_2$ was 45-47 mmHg during normocarbia, which is very similar to that following C18 injection with and without hypercarbia. Assuming tissue $\dot{M}O_2$ and perfusion did not change in any of these treatments, and there was no change in the position of the OEC used *in vivo*, all these $RMPO_2$ data points (normocarbia and C18 with and without hypercarbia) would fall on the OEC at the position labeled "M" (Fig. S8). This would correspond to an "a" to "M" O_2 extraction of approximately 22% (as per the vertical distance between the two points, indicated by the corresponding arrow) (Fig. S8). The benefit to tissue O_2 delivery associated with hypercarbia (i.e. in the absence of C18) can then be estimated. If it is assumed that tissue $\dot{M}O_2$, perfusion, and thus "a" to "M" O_2 extraction remain constant (*65*) whereby the resulting $RMPO_2$ value of 77 mmHg can be traced horizontally from "M" to "M_{CO2}" (Fig. S8). This point falls nicely onto the right-shifted OEC from a previous study when rainbow trout blood was incubated under 4 mmHg (0.5%) CO_2 (*12*) (Fig. S8). The degree to which O_2 delivery could be enhanced can then be estimated by moving down this new OEC to the point that $RMPO_2$ is 45-47 mmHg, corresponding with M' on the model – the same driving force for O_2 delivery as in the other treatments (Fig. S8). Now, the "a" to "M" O_2 extraction is increased to 39%, nearly double that described above (22%) (Fig. S8). Under a more severe acidosis, catecholamines may be released to stimulate RBC βNHE and the right shift of the OEC in the presence of plasma-accessible CA at the tissue may be even greater, further enhancing conditions for O_2 delivery corresponding to M'' or M''' based on the preceding assumptions and OECs previously generated for this species (*12*) (Fig. S8). Ultimately, this analysis serves to illustrate that the 31 mmHg increase in $RMPO_2$ during exposure to a moderate stressor could almost double tissue O_2 delivery with no change in tissue perfusion. This is a remarkable accomplishment for this system unique to teleosts, especially because similar analyses in mammalian systems reveal that the increase in oxygen delivery may be only 5% (*2, 3, 12, 69, 70*). This may be particularly important to increase aerobic metabolism in aquatic species during exposure to environmental stress or exercise and to speed up post-exercise recovery, for example, following a predator-prey encounter or during long, upstream migrations as exhibited in Pacific salmon (*78*).

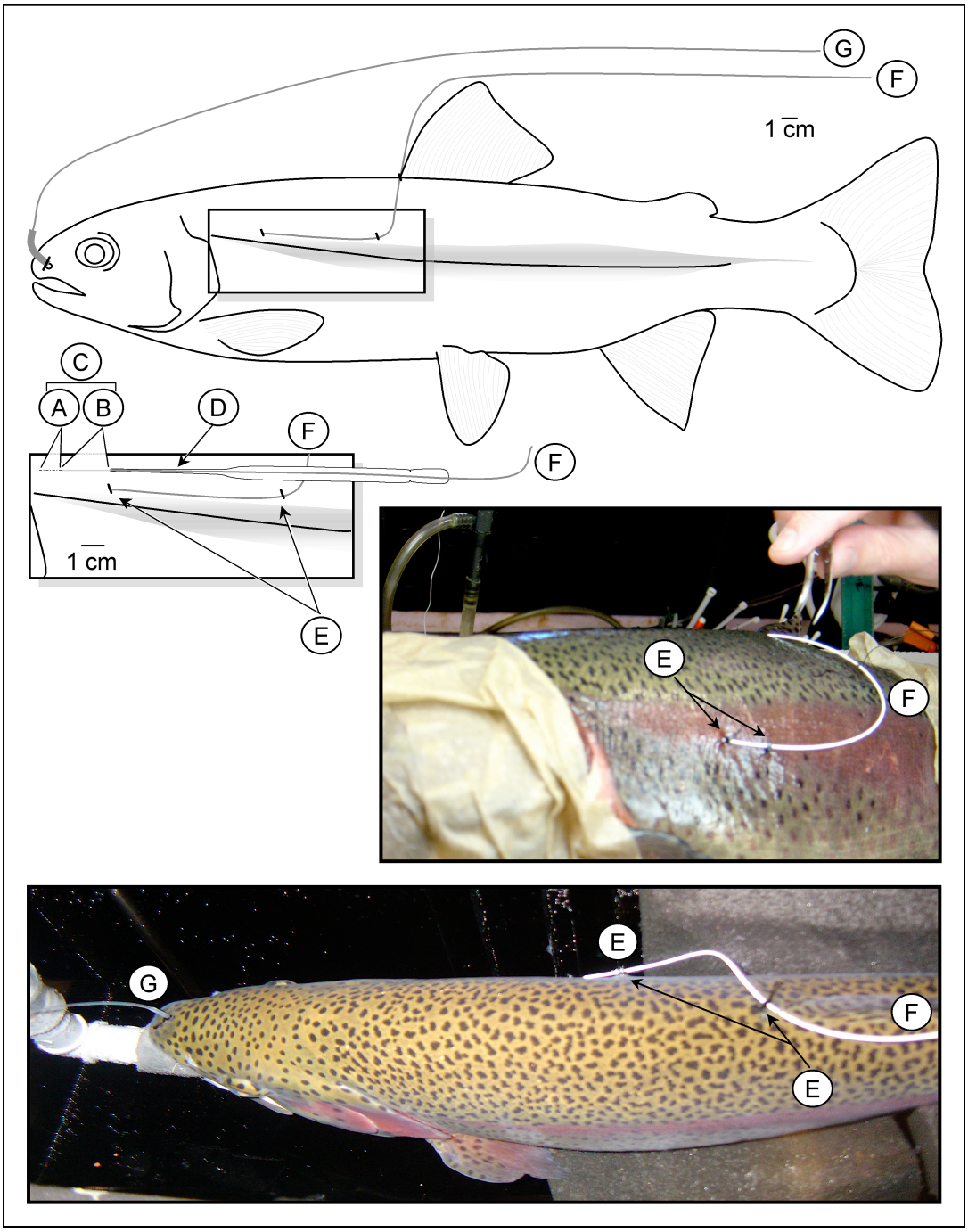
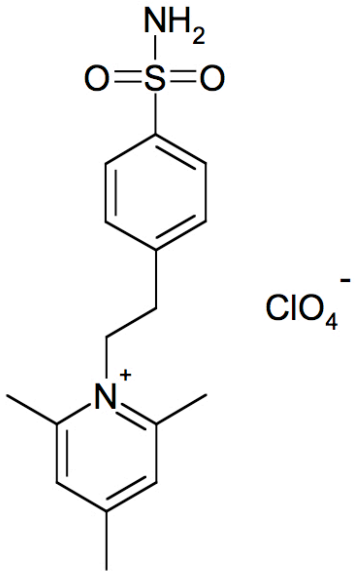


Fig. S1.

A schematic and photographic representation of the surgical procedure employed to implant the fiber optic O_2 sensor into the red muscle (RM) of rainbow trout and the recovery apparatus. First, a small incision was made in the skin just dorsal to the lateral line and 40 mm posterior to the periphery of the opercular plate to reveal the underlying

thin layer of RM. Then, the fiber optic O_2 sensor with a 10 mm tapered Teflon-coated tip (A) and an additional 25 mm of the coated portion of the optode (B) with guide marks made at 5 mm as well as 20 mm beyond the exposed glass, was secured inside a sterile glass Pasteur pipette (D). Two sutures (E) were secured to the tissue, dorsal to the lateral line, approximately 20 and 80 mm posterior to the periphery of the operculum. A third suture was prepared on the dorsal side of the fish, immediately anterior to the dorsal fin (E). The exposed glass tip of the sensor (A) as well as 10 mm of the coated part of the lead (B) were collectively (C) advanced under the skin into the RM for approximately 10 mm ensuring that the blunted end was flush with the underside of the skin to avoid damaging the musculature. Gentle pressure was applied to the skin atop the implanted optode, and the glass pipette was withdrawn and the optode was secured with the sutures (E) (as seen in upper photograph). The previously implanted DA cannula (G) was connected to a 1cc tuberculin syringe and flushed periodically with heparinized Cortland's saline. The entire lead and fiber optic O_2 sensor (F) was connected to an Oxy-4 micro four-channel DAQ-TEMP O_2 meter and signal amplifier via RS-232 serial cable (not shown). Post-surgery, fish were transferred to a black Perspex box to recover overnight, during which time they were force ventilated through Tygon® tubing (12.7mm inner diameter, 18mm outer diameter; bottom photograph) until regular opercular movement resumed. This force-ventilation apparatus (bottom photograph) was used again on fish following exposure to the neuromuscular blockade just prior to the experimental procedure.



C18

Fig. S2

Structure of compound-18 (C18), the low molecular weight (404.87 g mol^{-1}), membrane-impermeant, sulphonamide. The CA inhibitor, C18, has been demonstrated to inhibit human (cloned) CA I and II isozymes as well as CA IV isolated from bovine lung microsomes (Inhibition, K_i = 3, 13, and 10 nM respectively) (*16*).

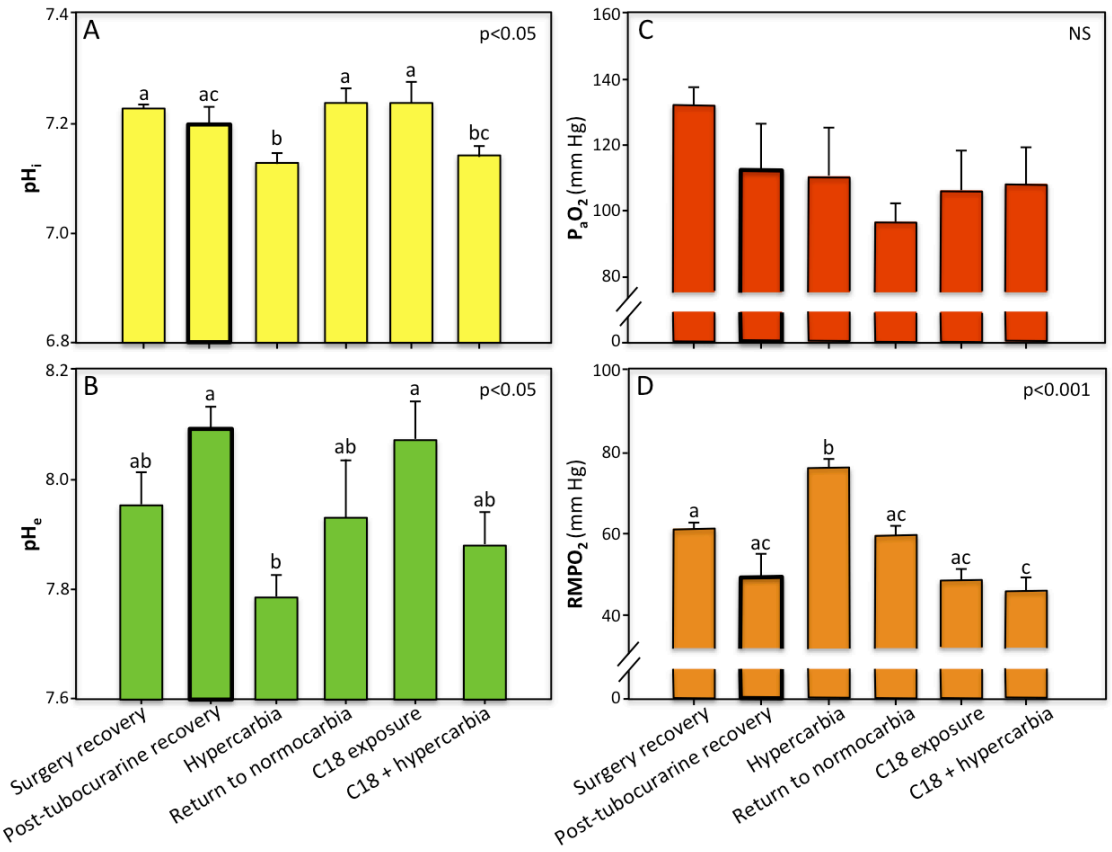


Fig. S3

The effect of hypercarbia, membrane-impermeant carbonic anhydrase inhibitor (C18) and combined hypercarbia and C18 on haematological and O_2-related variables. Panels are as follows: (A) intracellular pH (pH_i), (B) extracellular pH (pH_e), (C) arterial PO_2 (P_aO_2 in mmHg), and (D) red muscle PO_2 ($RMPO_2$ in mmHg). As a reference, post-tubocurarine recovery data are outlined with a bold outline, as these data represent the new baseline values for each variable to which subsequent treatment values are compared. Lower-case letters that differ demarcate significant differences between treatment groups within a panel. Statistical significance is indicated with p-values, if applicable (non-significance is indicated "NS"). All data are presented as means ±SEM, *N*= 7-11 per treatment. Statistical significance (repeated measures ANOVA) was assessed using $\alpha < 0.05$.

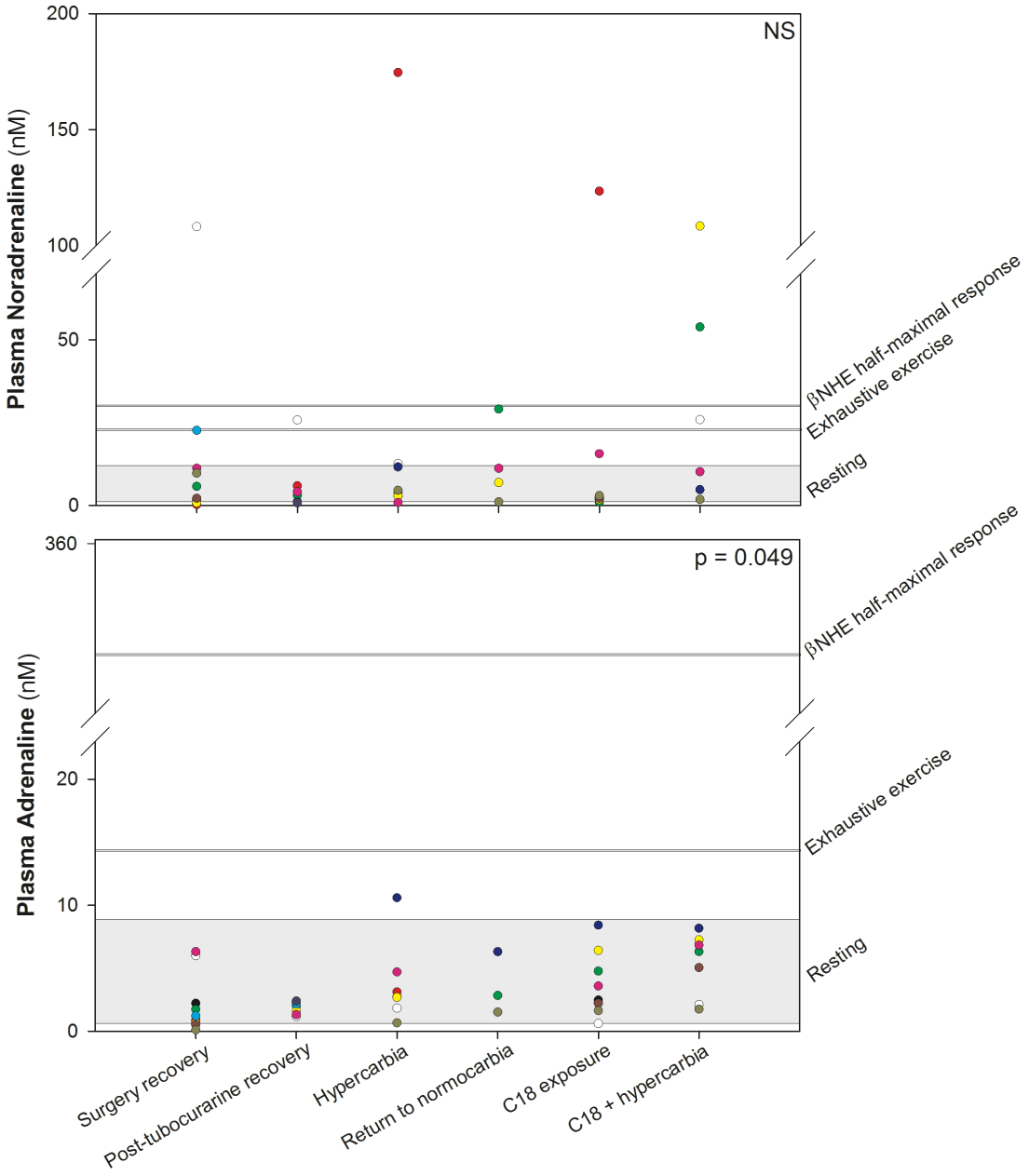


Fig. S4

Plasma noradrenaline (NA) and adrenaline (AD) levels (nM) in individual rainbow trout (a single colour represents the same animal) upon recovery from surgery (starting conditions), post-tubocurarine recovery, and following each experimental treatment. *N*= 7-11 per treatment. Published NA and AD levels for resting fish (indicated by the bottom gray bar), following exhaustive exercise, and values associated with βNHE half maximal response are indicated by gray lines as indicated on the right y-axis for both panels (see text above for references and further details). Statistical significance (relative to resting levels measured in this study: 4.9 ±2.85 nM and 0.42 ±0.23 nM respectively) is indicated in the top right corner of each panel.

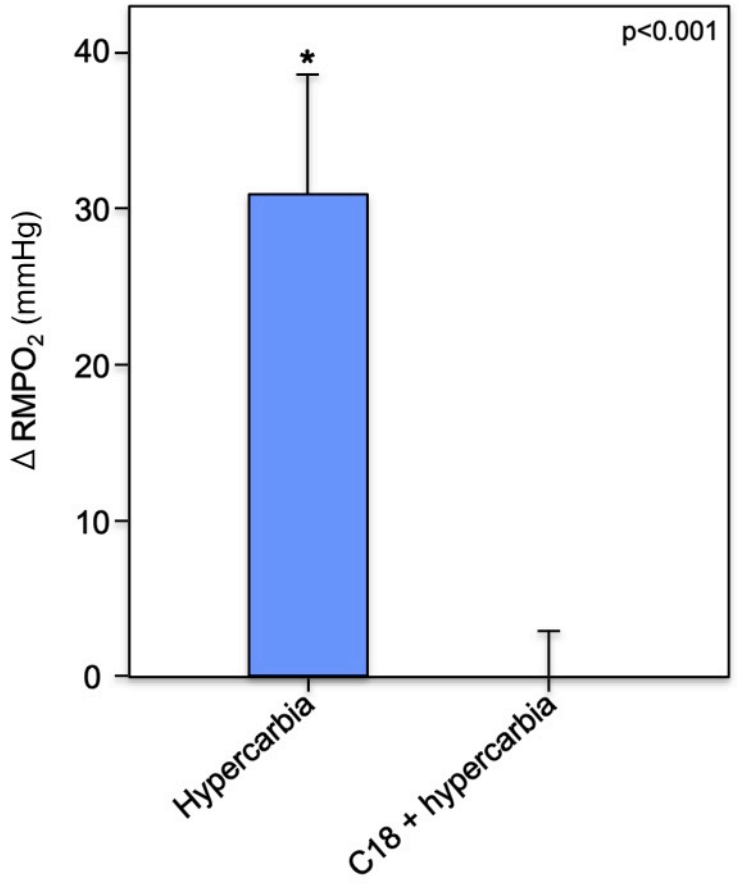


Fig. S5

The absolute change in $RMPO_2$ upon exposure to hypercarbia before and after carbonic anhydrase (CA) was inhibited. An asterisk demarcates a significant difference from post-tubocurarine recovery values ($p<0.001$).

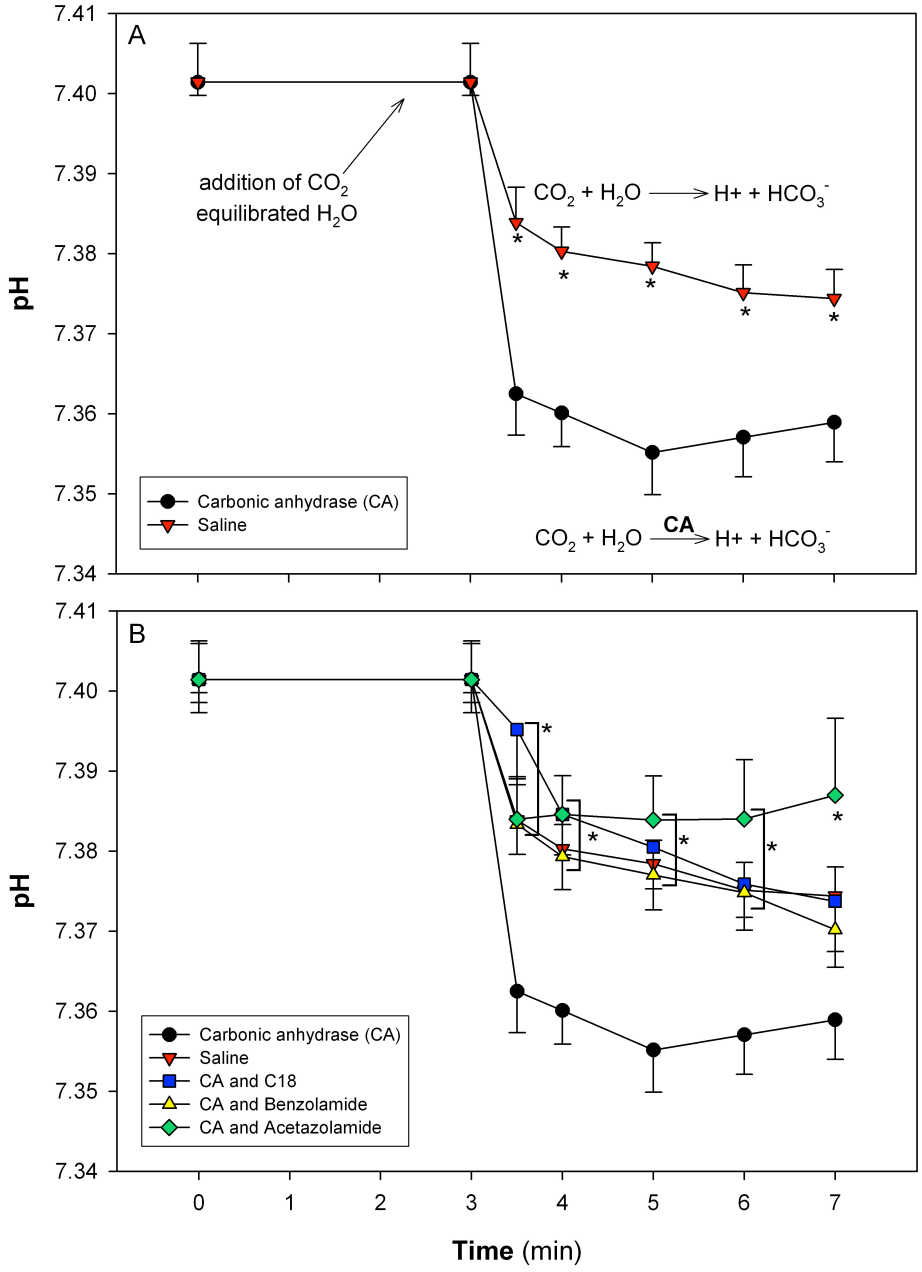


Fig. S6

The electrometric delta pH carbonic anhydrase (CA) assay reporting the change in pH over time following addition of CO_2 equilibrated water in the presence (solid circles) and absence (red inverted triangles) of carbonic anhydrase (CA) (Panel A) and with CA in the presence of CA inhibitors, C18 (blue squares), benzolamide (yellow triangles), and acetazolamide, (green diamonds), final concentrations 200 µM, 94 µM, and 100 mM respectively (Panel B). Data are mean ±SEM. Upon the addition of CO_2-equilibrated water at t = 3 min (x-axis), asterisks represent significant differences from the CA-catalyzed reaction at 3.5, 4, 5, 6, and 7 min.

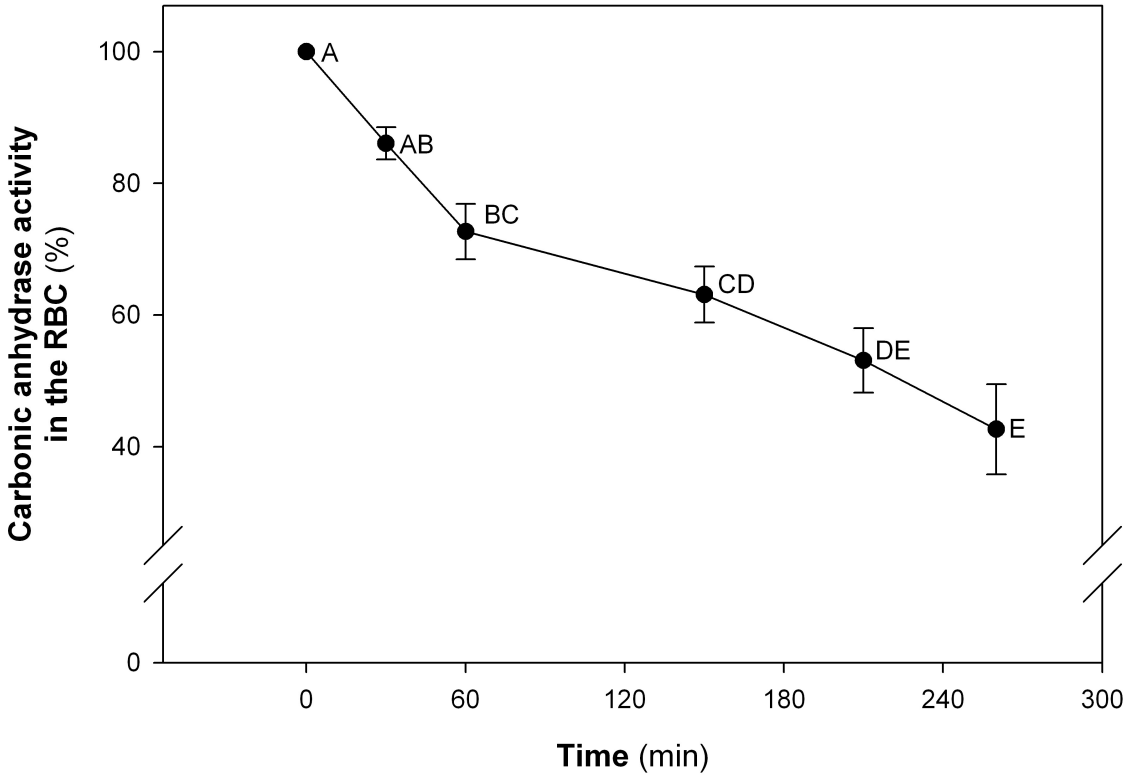


Fig. S7

Red blood cell (RBC) CA activity in rinsed RBCs exposed to C18 (final concentration 200 μM) in vitro. Letters that differ indicate significant differences, symbols indicate mean ±SEM. Note: RBC CA activity is not significantly reduced until 60 min following exposure to C18.

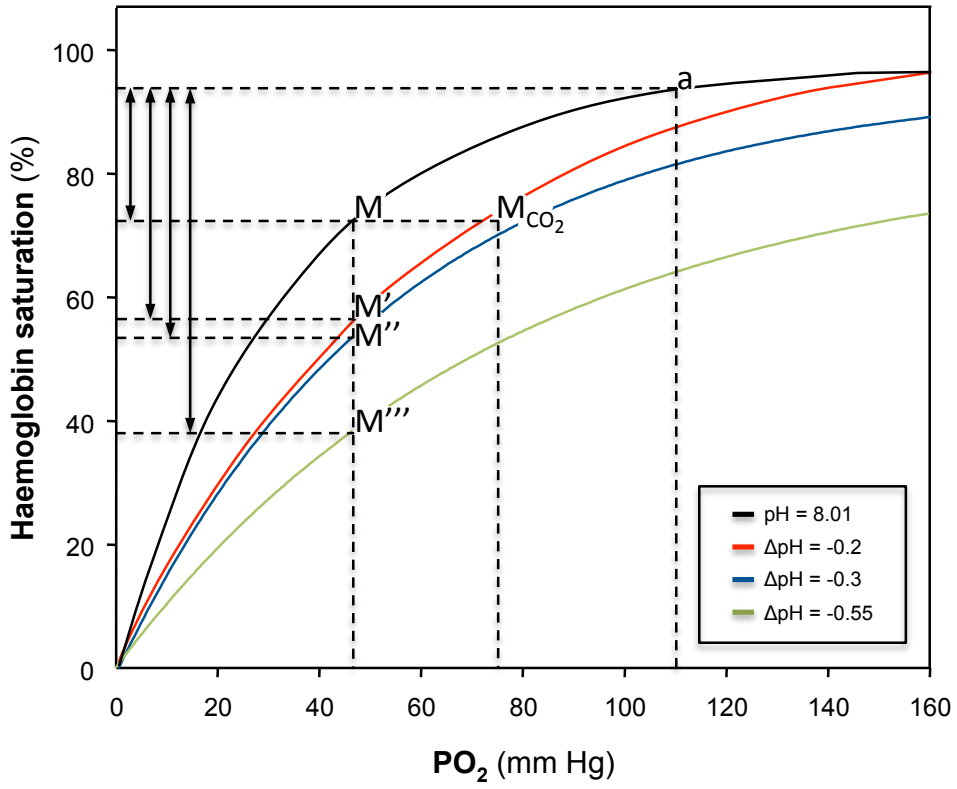


Fig. S8

A model depicting the degree to which O_2 delivery is enhanced as estimated from the results obtained in this study and assuming constant tissue metabolic rate ($\dot{M}O_2$) and perfusion of the fish (see above section on "modeling the benefit for oxygen delivery" for details).

Table S1.

The effect of hypercarbia, membrane-impermeant carbonic anhydrase inhibitor (C18) and combined hypercarbia and C18 on haematological and CO_2-related variables. Haemoglobin concentration, [Hb]; mean cell haemoglobin concentration, MCHC; red blood cell, RBC; total CO_2, TCO_2. Lower-case letters that differ denote significant differences among treatment groups within a given physiological variable. As a reference, post-tubocurarine recovery data are outlined with a bold black line, as these data represent the new baseline values for each variable to which all subsequent values are compared. All data are presented as means ±SEM, *N*= 7-11 per treatment, and statistical significance was assessed via repeated measures ANOVA and using $\alpha < 0.05$.

	Treatment					
	Surgery recovery	**Baseline** Post-tubocurarine recovery	**Hypercarbia**	**Return to normocarbia**	**C18 exposure**	**C18 + hypercarbia**
[Hb]	1.03^{a}	1.35^{ab}	1.08^{ab}	1.56^{b}	1.26^{ab}	1.12^{ab}
(mM)	±0.10	±0.06	±0.15	±0.11	±0.15	±0.08
Hematocrit	25.93	31.22	31.61	34.62	25.87	28.28
(%)	±2.37	±1.45	±3.25	±1.30	±1.66	±2.79
MCHC	4.34	4.44	3.64	4.54	4.25	3.68
(mM)	±0.83	±0.22	±0.55	±0.64	±0.29	±0.48
RBC TCO_2	3.2^{ab}	0.9^{a}	2.8^{ab}	1.9^{ab}	1.8^{ab}	4.4^{b}
(mM)	±1.1	±0.5	±0.3	±0.6	±0.9	±0.9
Plasma TCO_2	9.9	9.7	11.2	9.8	10.0	11.4
(mM)	±0.6	±0.8	±0.8	±2.3	±1.1	±0.9
Blood PCO_2	2.4^{ab}	1.7^{a}	4.0^{c}	2.5^{ab}	1.8^{a}	3.3^{b}
(mm Hg)	±0.2	±0.1	±0.3	±0.6	±0.2	±0.3

References and Notes

1. C. Bohr, K. Hasselbalch, A. Krogh, Über einen in biologischer Beziehung wichtigen Einfluss, den Kohlen- säurespannung des Blutes auf dessen Sauerstoffbindung übt. *Skand. Arch. Physiol.* **16**, 402 (1904). doi:10.1111/j.1748-1716.1904.tb01382.x

2. B. J. Behnke, C. A. Kindig, T. I. Musch, S. Koga, D. C. Poole, Dynamics of microvascular oxygen pressure across the rest-exercise transition in rat skeletal muscle. *Respir. Physiol.* **126**, 53 (2001). doi:10.1016/S0034-5687(01)00195-5 Medline

3. J. Hutter *et al*., Effect of acute normovolemic hemodilution on distribution of blood flow and tissue oxygenation in dog skeletal muscle. *J. Appl. Physiol.* **86**, 860 (1999). Medline

4. J. L. Rummer, C. J. Brauner, Plasma-accessible carbonic anhydrase at the tissue of a teleost fish may greatly enhance oxygen delivery: *in vitro* evidence in rainbow trout, *Oncorhynchus mykiss*. *J. Exp. Biol.* **214**, 2319 (2011). doi:10.1242/jeb.054049 Medline

5. G. S. Helfman, B. B. Collette, D. E. Facey, *The Diversity of Fishes* (Blackwell, Oxford, 1997).

6. R. W. Root, The respiratory function of the blood of marine fishes. *Biol. Bull.* **61**, 427 (1931). doi:10.2307/1536959

7. P. F. Scholander, L. Van Dam, Secretion of gases against high pressures in the swimbladder of deep sea fishes I. Oxygen dissociation in blood. *Biol. Bull.* **107**, 247 (1954). doi:10.2307/1538611

8. J. B. Wittenberg, B. A. Wittenberg, The choroid *rete mirabile* of the fish eye. I. Oxygen secretion and structure: Comparison with the swimbladder *rete mirabile*. *Biol. Bull.* **146**, 116 (1974). doi:10.2307/1540402 Medline

9. A. Baroin, F. Garcia-Romeu, T. Lamarre, R. Motais, A transient sodium-hydrogen exchange system induced by catecholamines in erythrocytes of rainbow trout, *Salmo gairdneri*. *J. Physiol.* **356**, 21 (1984). Medline

10. S. F. Perry, R. Kinkead, The role of catecholamines in regulating arterial oxygen content during acute hypercapnic acidosis in rainbow trout (*Salmo gairdneri*). *Respir. Physiol.* **77**, 365 (1989). doi:10.1016/0034-5687(89)90123-0 Medline

11. D. R. Primmett, D. J. Randall, M. Mazeaud, R. G. Boutilier, The role of catecholamines in erythrocyte pH regulation and oxygen transport in rainbow trout (*Salmo gairdneri*) during exercise. *J. Exp. Biol.* **122**, 139 (1986). Medline

12. J. L. Rummer, thesis, University of British Columbia, British Columbia, Canada (2010).

13. S. M. Rahim, J. P. Delaunoy, P. Laurent, Identification and immunocytochemical localization of two different carbonic anhydrase isoenzymes in teleostean fish erythrocytes and gill epithelia. *Histochem. Cell Biol.* **89**, 451 (1988). doi:10.1007/BF00492602 Medline

14. R. P. Henry, E. R. Swenson, The distribution and physiological significance of carbonic anhydrase in vertebrate gas exchange organs. *Respir. Physiol.* **121**, 1 (2000). doi:10.1016/S0034-5687(00)00110-9 Medline

15. Y. Wang, R. P. Henry, P. M. Wright, G. J. F. Heigenhauser, C. M. Wood, Respiratory and metabolic functions of carbonic anhydrase in exercised white muscle of trout. *Am. J. Physiol.* **275**, R1766 (1998). Medline

16. C. T. Supuran, Carbonic anhydrases: Novel therapeutic applications for inhibitors and activators. *Natl. Rev.* **7**, 168 (2008). doi:10.1038/nrd2467 Medline

17. D. J. McKenzie *et al.*, The effects of sustained exercise and hypoxia upon oxygen tensions in the red muscle of rainbow trout. *J. Exp. Biol.* **207**, 3629 (2004). doi:10.1242/jeb.01199 Medline

18. M. Nikinmaa, Membrane transport and control of hemoglobin-oxygen affinity in nucleated erythrocytes. *Physiol. Rev.* **72**, 301 (1992). Medline

19. Y. R. Weaver, K. Kiessling, A. R. Cossins, Responses of the Na^+/H^+ exchanger of European flounder red blood cells to hypertonic, beta-adrenergic and acidotic stimuli. *J. Exp. Biol.* **202**, 21 (1999). Medline

20. M. G. Romero, H. Guizouarn, B. Pellissier, F. Garcia-Romeu, R. Motais, The erythrocyte Na^+/H^+ exchangers of eel (*Anguilla anguilla*) and rainbow trout (*Oncorhynchus mykiss*): A comparative study. *J. Exp. Biol.* **199**, 415 (1996). Medline

21. M. Berenbrink, Historical reconstructions of evolving physiological complexity: O_2 secretion in the eye and swimbladder of fishes. *J. Exp. Biol.* **210**, 1641 (2007). doi:10.1242/jeb.003319 Medline

22. M. Berenbrink, P. Koldkjaer, O. Kepp, A. R. Cossins, Evolution of oxygen secretion in fishes and the emergence of a complex physiological system. *Science* **307**, 1752 (2005). doi:10.1126/science.1107793 Medline

23. G. N. Lapennas, The magnitude of the Bohr coefficient: Optimal for oxygen delivery. *Respir. Physiol.* **54**, 161 (1983). doi:10.1016/0034-5687(83)90054-3 Medline

24. B. P. North *et al.*, The impact of stocking density on the welfare of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **255**, 466 (2006). doi:10.1016/j.aquaculture.2006.01.004

25. M. Malapert *et al.*, Regulation of Na^+/H^+ antiporter in trout red blood cells. *J. Exp. Biol.* **200**, 353 (1997). Medline

26. M. Berenbrink, Haemoglobin function and the evolution of adrenoresponses in lower vertebrates. *Comp. Biochem. Physiol. B* **126**, S10 (2000). doi:10.1016/S0305-0491(00)80019-X

27. S. Caldwell, J. L. Rummer, C. J. Brauner, Blood sampling techniques and storage duration: Effects on the presence and magnitude of the red blood cell beta-adrenergic response in rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. A* **144**, 188 (2006). doi:10.1016/j.cbpa.2006.02.029 Medline

28. J. G. Nickerson, S. G. Dugan, G. Drouin, S. F. Perry, T. W. Moon, Activity of the unique ß-adrenergic Na^+/H^+ exchanger in trout erythrocytes is controlled by a novel ß3-AR subtype. *Am. J. Physiol.* **285**, R526 (2003).

29. J. B. Thomas, K. M. Gilmour, The impact of social status on the erythrocyte beta-adrenergic response in rainbow trout, *Oncorhynchus mykiss*. *Comp. Biochem. Physiol. A* **143**, 162 (2006). doi:10.1016/j.cbpa.2005.11.008 Medline

30. R. M. Effros, M. L. Weissman, Carbonic anhydrase activity of the cat hind leg. *J. Appl. Physiol.* **47**, 1090 (1979). Medline

31. C. Geers, G. Gros, Inhibition properties and inhibition kinetics of an extracellular carbonic anhydrase in perfused skeletal muscle. *Respir. Physiol.* **56**, 269 (1984). doi:10.1016/0034-5687(84)90064-1 Medline

32. C. Geers, G. Gros, Carbon dioxide transport and carbonic anhydrase in blood and muscle. *Physiol. Rev.* **80**, 681 (2000). Medline

33. A. Soivio, K. Nynolm, K. Westman, A technique for repeated sampling of the blood of individual resting fish. *J. Exp. Biol.* **63**, 207 (1975). Medline

34. G. Campbell, B. J. Gannon, The splanchnic nerve supply to the stomach of the trout, *Salmo trutta* and *S. gairdneri*. *Comp. Biochem. Physiol. C* **55**, 51 (1976). doi:10.1016/0306-4492(76)90011-3 Medline

35. T. P. Johnson, J. D. Altringham, I. A. Johnston, The effects of Ca^{2+} and neuromuscular blockers on the activation of fish muscle fibres. *J. Fish Biol.* **38**, 789 (1991). doi:10.1111/j.1095-8649.1991.tb03165.x

36. R. L. Oswald, Injection anaesthesia for experimental studies in fish. *Comp. Biochem. Physiol. C* **60**, 19 (1978). doi:10.1016/0306-4492(78)90021-7 Medline

37. C. R. Reilly, S. H. Thompson, Temperature effects on low-light vision in juvenile rockfish (genus *Sebastes*) and consequences for habitat utilization. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* **193**, 943 (2007). doi:10.1007/s00359-007-0247-5 Medline

38. Y. M. Le Bras, Effects of anaesthesia and surgery on levels of adrenaline and noradrenaline in blood plasma of the eel (*Anguilla anguilla* L.). *Comp. Biochem. Physiol. C* **72**, 141 (1982). doi:10.1016/0306-4492(82)90221-0 Medline

39. M. Hilvo *et al*., Biochemical characterization of CA IX, one of the most active carbonic anhydrase isozymes. *J. Biol. Chem.* **283**, 27799 (2008). doi:10.1074/jbc.M800938200 Medline

40. O. Hisar, S. Beydemir, I. Gülçin, O. I. Küfrevioğlu, C. T. Supuran, Effects of low molecular weight plasma inhibitors of rainbow trout (*Oncorhynchus mykiss*) on human erythrocyte carbonic anhydrase-II isozyme activity *in vitro* and rat erythrocytes *in vivo*. *J. Enzyme Inhib. Med.* **20**, 35 (2005). doi:10.1080/14756360400017044461 Medline

41. V. A. Tucker, Method for oxygen content and dissociation curves on microliter blood samples. *J. Appl. Physiol.* **23**, 410 (1967). Medline

42. J. J. Woodward, Plasma catecholamines in resting rainbow trout, *Salmo gairdneri* Richardson, by high pressure liquid chromatography. *J. Fish Biol.* **21**, 429 (1982). doi:10.1111/j.1095-8649.1982.tb02848.x

43. R. Zeidler, H. D. Kim, Preferential hemolysis of postnatal calf red cells induced by internal alkalinization. *J. Gen. Physiol.* **70**, 385 (1977). doi:10.1085/jgp.70.3.385 Medline

44. M. R. Gervais, B. L. Tufts, Evidence for membrane-bound carbonic anhydrase in the air bladder of bowfin (*Amia calva*), a primitive air-breathing fish. *J. Exp. Biol.* **201**, 2205 (1998). Medline

45. R. P. Henry, in *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics*, S. J. Dodgson, R. E. Tashian, G. Gros, N. D. Carter, Eds. (Plenum, New York, 1991), pp. 119–126.

46. T. H. Maren, A simplified micromethod for the determination of carbonic anhydrase and its inhibitors. *J. Pharmacol. Exp. Ther.* **130**, 26 (1960). Medline

47. T. H. Maren, E. O. Couto, The nature of anion inhibition of human red cell carbonic anhydrases. *Arch. Biochem. Biophys.* **196**, 501 (1979). doi:10.1016/0003-9861(79)90302-3 Medline

48. V. Sundaram, P. Rumbolo, J. Grubb, P. Strisciuglio, W. S. Sly, Carbonic anhydrase II deficiency: Diagnosis and carrier detection using differential enzyme inhibition and inactivation. *Am. J. Hum. Genet.* **38**, 125 (1986). Medline

49. M. Bülbül, O. Hisar, S. Beydemir, M. Ciftçi, O. I. Küfrevioğlu, The *in vitro* and *in vivo* inhibitory effects of some sulfonamide derivatives on rainbow trout (*Oncorhynchus mykiss*) erythrocyte carbonic anhydrase activity. *J. Enzyme Inhib. Med.* **18**, 371 (2003). doi:10.1080/1475636031000138769 Medline

50. R. G. Khalifah, The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. *J. Biol. Chem.* **246**, 2561 (1971). Medline

51. P. K. Bourne, A. R. Cossins, On the instability of K^+ influx in erythrocytes of the rainbow trout, *Salmo gairdneri*, and the role of catecholamine hormones in maintaining *in vivo* influx activity. *J. Exp. Biol.* **101**, 93 (1982). Medline

52. V. Tetens, G. Lykkeboe, N. J. Christensen, Potency of adrenaline and noradrenaline for β-adrenergic proton extrusion from red cells of rainbow trout, *Salmo gairdneri*. *J. Exp. Biol.* **134**, 267 (1988). Medline

53. D. J. Randall, S. F. Perry, in *Fish Physiology*, W. S. Hoar, D. J. Randall, A. P. Farrell, Eds. (Academic Press, New York, 1992), vol. 12B, pp. 255–300.

54. R. G. Boutilier, T. A. Heming, G. K. Iwama, Appendix —Physiochemical parameters for use in fish respiratory physiology. *Fish Physiol.* **10**, 403 (1984). doi:10.1016/S1546-5098(08)60323-4

55. C. J. Brauner *et al*., Limited extracellular but complete intracellular acid-base regulation during short-term environmental hypercapnia in the armoured catfish, *Liposarcus pardalis*. *J. Exp. Biol.* **207**, 3381 (2004). doi:10.1242/jeb.01144 Medline

56. F. M. Smith, D. R. Jones, The effect of changes in blood oxygen-carrying capacity on ventilation volume in the rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* **97**, 325 (1982). Medline

57. J. E. McKendry, S. F. Perry, Cardiovascular effects of hypercarbia in rainbow trout (*Oncorhynchus mykiss*): A role for externally oriented chemoreceptors. *J. Exp. Biol.* **204**, 115 (2001). Medline

58. C. J. Brauner, thesis, University of British Columbia, British Columbia, Canada (1995).

59. S. F. Perry, C. M. Wood, Control and coordination of gas transfer in fishes. *Can. J. Zool.* **67**, 2961 (1989). doi:10.1139/z89-419

60. S. F. Perry, K. M. Gilmour, Consequences of catecholamine release on ventilation and blood oxygen transport during hypoxia and hypercapnia in an elasmobranch Squalus acanthias and a teleost Oncorhynchus mykiss. *J. Exp. Biol.* **199**, 2105 (1996). Medline

61. C. M. Wood, R. S. Munger, Carbonic-anhydrase injection provides evidence for the role of blood acid-base status in stimulating ventilation after exhaustive exercise in rainbow-trout. *J. Exp. Biol.* **194**, 225 (1994). Medline

62. M. S. Graham, J. D. Turner, C. M. Wood, Control of ventilation in the hypercapnic skate *Raja ocellata*: I. Blood and extradural fluid. *Respir. Physiol.* **80**, 259 (1990). doi:10.1016/0034-5687(90)90088-G Medline

63. N. Heisler, D. P. Toews, G. F. Holeton, Regulation of ventilation and acid-base status in the elasmobranch *Scyliorhinus stellaris* during hyperoxia-induced hypercapnia. *Respir. Physiol.* **71**, 227 (1988). doi:10.1016/0034-5687(88)90018-7 Medline

64. R. Kinkead, S. F. Perry, The effects of catecholamines on ventilation in rainbow trout during hypoxia or hypercapnia. *Respir. Physiol.* **84**, 77 (1991). doi:10.1016/0034-5687(91)90020-J Medline

65. D. W. Baker, C. J. Brauner, Metabolic changes associated with acid-base regulation during hypercarbia in the CO_2-tolerant chondrostean, white sturgeon (*Acipenser transmontanus*). *Comp. Biochem. Physiol. A* **161**, 61 (2012). doi:10.1016/j.cbpa.2011.09.002 Medline

66. A. Scozzafava, F. Briganti, M. A. Ilies, C. T. Supuran, Carbonic anhydrase inhibitors: synthesis of membrane-impermeant low molecular weight sulfonamides possessing in vivo selectivity for the membrane-bound versus cytosolic isozymes. *J. Med. Chem.* **43**, 292 (2000). doi:10.1021/jm990479+ Medline

67. K. M. Gilmour, S. F. Perry, Carbonic anhydrase and acid-base regulation in fish. *J. Exp. Biol.* **212**, 1647 (2009). doi:10.1242/jeb.029181 Medline

68. M. Nikinmaa, *Vertebrate Red Cells: Adaptations of Function to Respiratory Requirements* (Springer Verlag, Berlin, ed. 1, 1990), vol. Zoophysiology 28, pp. 262.

69. F. Jung, H. Kessler, G. Pindur, R. Sternitzky, R. P. Franke, Intramuscular oxygen partial pressure in the healthy during exercise. *Clin. Hemorheol. Microcirc.* **21**, 25 (1999). Medline

70. S. W. Suttner *et al*., The influence of hyperoxic ventilation during sodium nitroprusside-induced hypotension on skeletal muscle tissue oxygen tension. *Anesthesiology* **96**, 1103 (2002). doi:10.1097/00000542-200205000-00012 Medline

71. G. Gutierrez, N. Lund, A. L. Acero, C. Marini, Relationship of venous PO_2 to muscle PO2 during hypoxemia. *J. Appl. Physiol.* **67**, 1093 (1989). Medline

72. I. A. Johnston, Respiratory characteristics of muscle fibres in a fish (*Chaenocephalus aceratus*) that lacks haem pigments. *J. Exp. Biol.* **133**, 415 (1987). Medline

73. A. P. Farrell, S. M. Clutterham, On-line venous oxygen tensions in rainbow trout during graded exercise at two acclimation temperatures. *J. Exp. Biol.* **206**, 487 (2003). doi:10.1242/jeb.00100 Medline

74. G. F. Holeton, D. J. Randall, The effect of hypoxia upon the partial pressure of gases in the blood and water afferent and efferent to the gills of rainbow trout. *J. Exp. Biol.* **46**, 317 (1967). Medline

75. S. Egginton, C. Skilbeck, L. Hoofd, J. Calvo, I. A. Johnston, Peripheral oxygen transport in skeletal muscle of Antarctic and sub-Antarctic notothenioid fish. *J. Exp. Biol.* **205**, 769 (2002). Medline

76. S. E. Curtis, T. A. Walker, W. E. Bradley, S. M. Cain, Raising P50 increases tissue PO2 in canine skeletal muscle but does not affect critical O_2 extraction ratio. *J. Appl. Physiol.* **83**, 1681 (1997). Medline

77. S. F. Perry, K. M. Gilmour, N. J. Bernier, C. M. Wood, Does gill boundary layer carbonic anhydrase contribute to carbon dioxide excretion: a comparison between dogfish (*Squalus acanthias*) and rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **202**, 749 (1999). Medline