Electronic Supplementary Materials (ESM) for

Functional support for a novel mechanism that enhances tissue oxygen extraction in a teleost fish

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Supplementary Materials and Methods

Animals and holding conditions

Atlantic salmon (*Salmo salar*, Linnaeus 1758) were obtained from Northern Harvest Sea Farms, PIT-tagged, and kept at the Ocean Science Centre (Memorial University of Newfoundland, MUN) for 6 months before experiments. Seventy animals were weighed (average initial mass 654 ± 10 g) before being transferred into one of two 1.2 m^3 tanks supplied with flow-through seawater at 12°C. All animals were held for two weeks at a water PO_2 corresponding to 100% air saturation (~21 kPa O_2) and were fed commercial trout pellets to satiation. Thereafter, the PO_2 in one of the two tanks was decreased to 40% air saturation over the course of three weeks. Water PO_2 was adjusted by decreasing the water flow into the hypoxic tank (from 10 to 6 L min^{-1}) and by automatically bubbling N_2 into the tank when PO_2 was over a pre-determined threshold. Despite the lower water flow, there were no differences in water quality between the normoxia and hypoxia tanks (assessed as water [ammonia], [nitrite] and pH). Once the target PO_2 of 40% air saturation was reached, animals were acclimated at this PO_2 for six weeks before experiments began; therefore, depending on when a fish was removed, acclimation time ranged from 6-10 weeks. Over this period, average water PO_2 and temperature in the tanks housing the hypoxia- and normoxia-acclimated groups were 41.6±0.2 and 102.8±0.3% air saturation, and 11.5±0.1 and 11.6±0.1°C, respectively. Food consumption in both groups was matched by feeding animals in the hypoxic group first, recording their feed intake, and then feeding the same amount to the normoxic group.

Fish holding and all experimental procedures were in strict compliance with the guidelines of the Canadian Council on Animal Care (CCAC) and were approved by the MUN Institutional Animal Care Committee (Protocol 17-94-KG).

Surgery and recovery

The salmon were anaesthetised in seawater containing 0.10 g L^{-1} tricaine methanesulfonate (MS-222). Once unresponsive, animals were measured for final weight and length, and transferred to a surgery table where their gills were continuously irrigated with oxygenated and chilled water (4°C) containing a maintenance dose of 0.05 g L^{-1} MS-222. A catheter (PE 50; BD Intramedic, Franklin Lakes, NJ) was chronically implanted into the dorsal aorta according to Soivio et al. [1]. Thereafter, a Doppler flow probe (Cuff-type transducer model ES, ID = 1.3-1.8 mm, frequency = 20 MHz; Iowa Doppler Products, Iowa City, IA, USA) was fitted around the ventral aorta just outside of the pericardium [2], and the DA cannula and Doppler flow-probe lead were sutured to the fish just anterior to the dorsal fin.

After surgery, fish were quickly recovered in aerated seawater without anaesthetic. Once equilibrium was re-gained, they were placed individually in respirometry chambers that were connected to a recirculation system that was continuously flushed with aerated seawater at 12°C. During recovery from surgery all catheters were flushed twice per day with heparinised saline (0.9% NaCl with 50 I.U. per mL of Na-Heparin, Sigma H3149) to prevent clotting. Animals in the present study were allowed to recover from surgery for 48 h in normoxia. This timeframe represented a trade-off between a full recovery and the risk of reversing the effects of hypoxic acclimation. Nevertheless, in Atlantic salmon that had undergone a similarly invasive hepatic portal vein cannulation, blood parameters had already recovered 24 h after surgery [3]. Furthermore, previous work on Atlantic cod [4,

5] and steelhead trout [2] that used a similar protocol of acclimation, surgery and recovery, detected large effects of hypoxic acclimation, indicating that normoxic recovery from surgery for 48 h did not fully reverse acclimation effects in these fish.

In the evening before the swim trials, fish were lightly anaesthetised (in 0.05 g L^{-1} MS-222) and transferred to a 81 L Blazka-type swim tunnel (University of Waterloo, Biotelemetry Institute, Waterloo, ON, CA) that is described in detail by [4]. The tunnel was supplied with recirculating seawater from a temperature-controlled and aerated reservoir at 10 L min^{-1}, which kept water in the tunnel at ~95 - 100% air saturation and at 12°C. After a short recovery period, water flow in the swim tunnel was gradually increased to 1 body length per second (bl s^{-1}) and the fish were "conditioned" to swim in the mid-front section of the tunnel for 10 min; if the salmon rested against the back end of the tunnel, an electrified grid was engaged very briefly (5 V, 0.2 A) to entice the fish to continue swimming. In addition, the front section of the tunnel was shaded and provided a refuge that the salmon generally preferred over the exposed back half of the tunnel. Preliminary experiments showed that "conditioned" fish swam more readily during the experiments on the next day. After this short episode of mild exercise, water velocity in the swim tunnel was decreased to 10 cm s^{-1}, which allowed the salmon to rest overnight at the bottom of the tunnel without swimming. The surgeries, recovery procedures, and all experiments were carried out in normoxia for both acclimation groups.

Experimental design

To examine the effect of paCA inhibition on cardiovascular O_2 transport, Atlantic salmon were swum at a constant swimming speed of 1 bl s^{-1}, injected with a solvent control (saline: 0.9% NaCl, 0.1% DMSO), and then injected with saline containing C18 (injections were at 10 µmol kg^{-1} for a final concentration in the blood of 200 µM C18; based on animal mass, assuming a density equal to water and a blood volume of 5%), a CA inhibitor that is effectively membrane-impermeable [6]. The sequential injections of sham and C18 (~15 min apart) significantly reduced the amount of experimental animals required. This protocol was applied to salmon that had been acclimated to normoxia or hypoxia and this resulted in a mixed-model design, that compared the effects of C18 injection against the injection of the solvent control (sham), and the effect of hypoxia against normoxia acclimation.

In preliminary trials it was determined that U_{crit} of these Atlantic salmon was ~2 bl s^{-1}, which is in line with previous measurements [7], and that the optimal swimming speed for these experiments was 1 bl s^{-1}; when swimming at 1.5 bl s^{-1} the fish would collapse and stop swimming after the injection of C18 ($N = 2$). Measurements of oxygen consumption rate ($\dot{M}O_2$) and cardiovascular parameters (cardiac output, $\dot{Q}$; stroke volume, V_s; and heart rate, f_h) were performed at five points during the experimental protocol: i) at rest (water velocity of 10 cm s^{-1}); ii) after gradually increasing swimming speed to 1 bl s^{-1} and 10 min of steady swimming (swim); iii) 5 min after the saline injection (sham); iv) 5 min after C18 injection (C18); and v) after decreasing water velocity to 10 cm s^{-1} and allowing the fish to recover from exercise for 10 minutes (recov). Each step in the protocol lasted ~30 min, with a total duration of ~3 h, during which the fish swam for ~2 h.

In a separate trial the effect of paCA inhibition was assessed in resting Atlantic salmon. After surgery these animals were transferred into individual respirometry

chambers that were continuously supplied with aerated seawater from a reservoir at 12°C; described in detail by [2]. Measurements of $\dot{M}O_2$ and cardiovascular parameters after 48 h of recovery were: i) initial (rest); ii) 5 min after a saline injection (sham); and iii) 5 min after C18 injection.

A previous study on rainbow trout found no inhibitory effect of 200 µM C18 on RBC CA within 30 min of exposure, but that it significantly inhibited RBC CA by ~30% after 1 h of injection, by slowly diffusing across the RBC membrane [8]. To avoid a confounding effect of RBC CA inhibition, all fish were swum for only 30 min after the injection of C18 and the final measurements during recovery were performed < 50 min of C18 injection, when the trial was terminated. Over this time-period C18 has been shown to be effectively membrane impermeable in rainbow trout [8] and in humans [6, 9]. In addition, a separate control experiment was performed to validate that $\dot{M}O_2$ and cardiovascular parameters were not confounded by the duration of the swimming protocol, the injections of saline, or the repeated sampling of blood. Therefore, normoxia-acclimated fish (N = 3) were subjected to the above swimming protocol, however, the C18 injection was replaced with a second saline injection.

Measurements of $\dot{M}O_2$ and cardiovascular parameters

The Doppler lead from the animal was connected to a pulsed Doppler flow meter (Model 545C-4; Department of Bioengineering, University of Iowa, Ames, IA, USA), and the crystal's focus was adjusted so that a clean signal of the greatest magnitude was obtained before each trial. The analogue signal from the Doppler flow meter was then recorded continuously using a MP100 data acquisition system and Acqknowledge software v3.7.3 (BIOPAC Systems, Santa Barbara, CA, USA). $\dot{Q}$ was assessed as the average voltage reading over a selected period, and all values were expressed relative to the average voltage measured at rest. Average f_h was measured using the Acqknowledge heart rate function, and these measurements were validated by manually measuring the time required for 20 heart beats; if there was a discrepancy between the two measurements of f_h, the manually calculated values were used. Stroke volume (V_s) was calculated as $\dot{Q}/f_h$, and thus, was also relative to average values at rest [2].

PO_2 in the swim tunnel was measured continuously using a fiber-optic sensor (Dipping probe mini sensor, Loligo Systems, Viborg, Dk) connected to a Witrox 1 O_2 meter (Loligo Systems). $\dot{M}O_2$ was measured using an intermittent respirometry approach, and by manually stopping the flow of water into the swim tunnel. The duration of stop-flow at rest and during recovery was ~15 min, over which time PO_2 decreased by ~3% air saturation. During swimming, stop-flow periods were shorter (~5 min) due to the higher $\dot{M}O_2$ of the animal and PO_2 was prevented from decreasing by more than ~ 5% air saturation. Therefore, all experiments were well within a range of PO_2 values that can be considered normoxic. $\dot{M}O_2$ was calculated in LabChart v8.1.5 (ADInstruments, Dunedin, New Zealand) from the slope of the decrease in PO_2 over the final 8 min of the stop-flow period at rest, and over the final 3 min of the stop-flow period during exercise, according to:

$$\dot{M}O_2 = -\frac{\delta PO_2}{\delta t} \times (V_c - V_a) \times \alpha_{O_2} \times \frac{3600}{m_a}$$

where $-\frac{\delta PO_2}{\delta t}$ (% air saturation s^{-1}) is the slope of the PO_2 curve during stop-flow, V_c and V_a (L) are the volumes of the swim tunnel and the animal (based on animal wet mass and assuming a density equal to water), α_{O_2} (mg O_2 L^{-1} % air saturation^{-1}) is the solubility of O_2 in water [10] and m_a is the mass of the animal (kg).

$\dot{M}O_2$ in the resting trial was measured using an automated intermittent respirometry system that controlled the flush pumps to individual 20 L respirometry chambers as described previously [2]. PO_2 was measured with fiber-optic sensors (Dipping probe mini sensor, Loligo systems) connected to an Oxy-4 mini meter (PreSens, Regensburg, Germany) that was interfaced with a DAQ-4 module (Loligo systems). $\dot{M}O_2$ was calculated automatically by the AutoResp software v2.2.2 (Loligo systems) and the average values over two respirometry cycles are reported for each measurement period.

Blood sampling and analyses

To account for potentially confounding changes in arterial O_2 transport due to the experimental treatments [11] or hypoxia acclimation [4, 12], blood was sampled from the catheter at four time-points during the experiment: i) at rest; ii) during swimming and after the injection of the sham; iii) during swimming and after the injection of C18; and iv) during recovery, ~15 min after exercise. A blood volume of 600 µL was removed from the animal at each sampling time-point, and this volume was replaced by saline. To assess the stress response of Atlantic salmon during the swim trials, 400 µL of blood was immediately centrifuged for 20 s using a Mini-Centrifuge 05-090-128 (Fisher Scientific, Hampton, NH, USA) and 200 µL of plasma was then pipetted into an Eppendorf® tube containing 10 µL of 0.2 M EDTA and glutathione, and rapidly frozen in liquid N_2 (within ~1 min from sampling). Plasma adrenaline and noradrenaline concentrations were measured in these samples using an ELISA kit (ABNova KA1877, Taipei City, Taiwan), following the manufacturer's instructions. The remainder of the plasma was frozen in liquid N_2 and plasma lactate concentration was measured spectrophotometrically using a kit (Sigma 826) according to the manufacturer's instructions.

Haematocrit (Hct) was measured in triplicate on 15 µL of blood in capillary tubes that were centrifuged at 10,000 x g for 3 min. Blood pH was measured with a thermostatted (12°C) microelectrode (16-705 and 16-702; Microelectrodes Inc., Bedford, NH). Arterial O_2 content in the blood (C_aO_2) was measured on 10 µL of blood, in triplicate, using a Tucker chamber [13] and by lysing the RBCs and converting all haemoglobin (Hb) into methaemoglobin in a ferricyanide solution at 40°C. The change in PO_2 in the Tucker chamber was measured with a Clark-type electrode (E101, Radiometer, Copenhagen, Denmark) and a OM200 meter (Cameron Instruments, Guelph, ON, Canada). Hb concentration was measured in triplicate on 10 µL of blood using the cyanomethaemoglobin method (Drabkin's reagent, Sigma D5941) and measuring absorbance at 540 nm using a plate reader (SpectraMax 5, Molecular Devices, Sunnyvale, USA). Hb concentration was calculated in reference to standard curves with bovine Hb (Sigma, H-2500) that were run on the same plates. Hb-O_2 saturation (sO_2) was calculated from C_aO_2 and Hb concentration after subtracting physically dissolved O_2 according to [10, 13]. At the end of the experiment, the salmon were removed from the

swim tunnel and euthanised in 0.15 g L^{-1} MS-222. Finally, the ventricle was dissected out of the animal, blotted dry and weighed on a scale (to 0.001 g). The relative ventricular mass (RVM) was then calculated as percentage of animal mass.

Data analysis and statistics

All data were analysed in R studio v1.0.153 (R v3.4.1) and figures were generated with the ggplot2 v.2.2.1 package [14]. The mixed design model (lme function in R) included a random effect of individual, a fixed effect of treatment that was nested within individual, and a fixed effect of acclimation that was independent of individual. This model tested the main effects of treatment (treat) and acclimation (accl), and their interaction (treat × accl), and these results are reported in all figures. In addition, differences between treatments were analysed with Tukey's post-hoc tests ($N = 9$ for the swimming trial, $N = 5$ for the resting trial and $N = 3$ for control fish, unless indicated otherwise; $P < 0.05$). If the main model indicated a significant effect of acclimation or an interaction effect (treat × accl), the post-hoc analysis was carried out separately for both acclimation groups. All data are shown as means ± SEM.

Supplementary Results

During the entire 10-week acclimation period, only one mortality was observed in the hypoxia-acclimated group, and there was no significant effect of acclimation on final animal mass (774 ± 194 g; $P = 0.618$; $N = 69$) or condition factor (Fulton's K; 1.04 ± 0.01 g cm^{-3}; $P = 0.111$). However, in those Atlantic salmon that were used in swim trials, statistical analysis indicated that normoxia-acclimated fish were larger (868 ± 41 g; $N = 12$) compared to hypoxia-acclimated fish (768 ± 27 g; $N = 14$; $P = 0.046$) and had a significantly higher RVM (0.075 ± 0.003 vs. 0.068 ± 0.002%: $P = 0.042$).

A significant treatment effect, but no acclimation or interaction effects were detected for blood Hct (Fig. S5A). In both acclimation groups, Hct decreased throughout the experiment, from 26.6 ± 1.0% at rest to 23.5 ± 0.8% during recovery, but the injection of C18 had no significant effect on Hct ($P = 0.621$). In addition, no effects of treatment, acclimation or their interaction were detected on Hb concentration (Fig. S5B), MCHC or Hb-O_2 saturation (Fig. S5C), and average values for these parameters were 1.0 ± 0.0 mM, 4.1 ± 0.2 mM and 95.2 ± 2.8%, respectively. However, a treatment effect was detected on C_aO_2, which decreased from 3.8 ± 0.1 mM at rest to 3.2 ± 0.2 mM during recovery (Fig. S5D). Injection of C18 into swimming Atlantic salmon significantly ($P = 0.020$) decreased C_aO_2 compared to sham injections (from 3.8 ± 0.1 to 3.5 ± 0.2 mM). In control fish that were injected with saline twice (Fig. S6A-D), there were no significant treatment effects or differences between the first and second sham injection on Hct ($P = 0.402$), Hb concentration ($P = 0.745$), Hb-O_2 saturation ($P = 0.207$) or C_aO_2 ($P = 0.465$), but Hb concentration increased transiently due to exercise and the first sham injection ($P = 0.035$).

Supplementary Figures

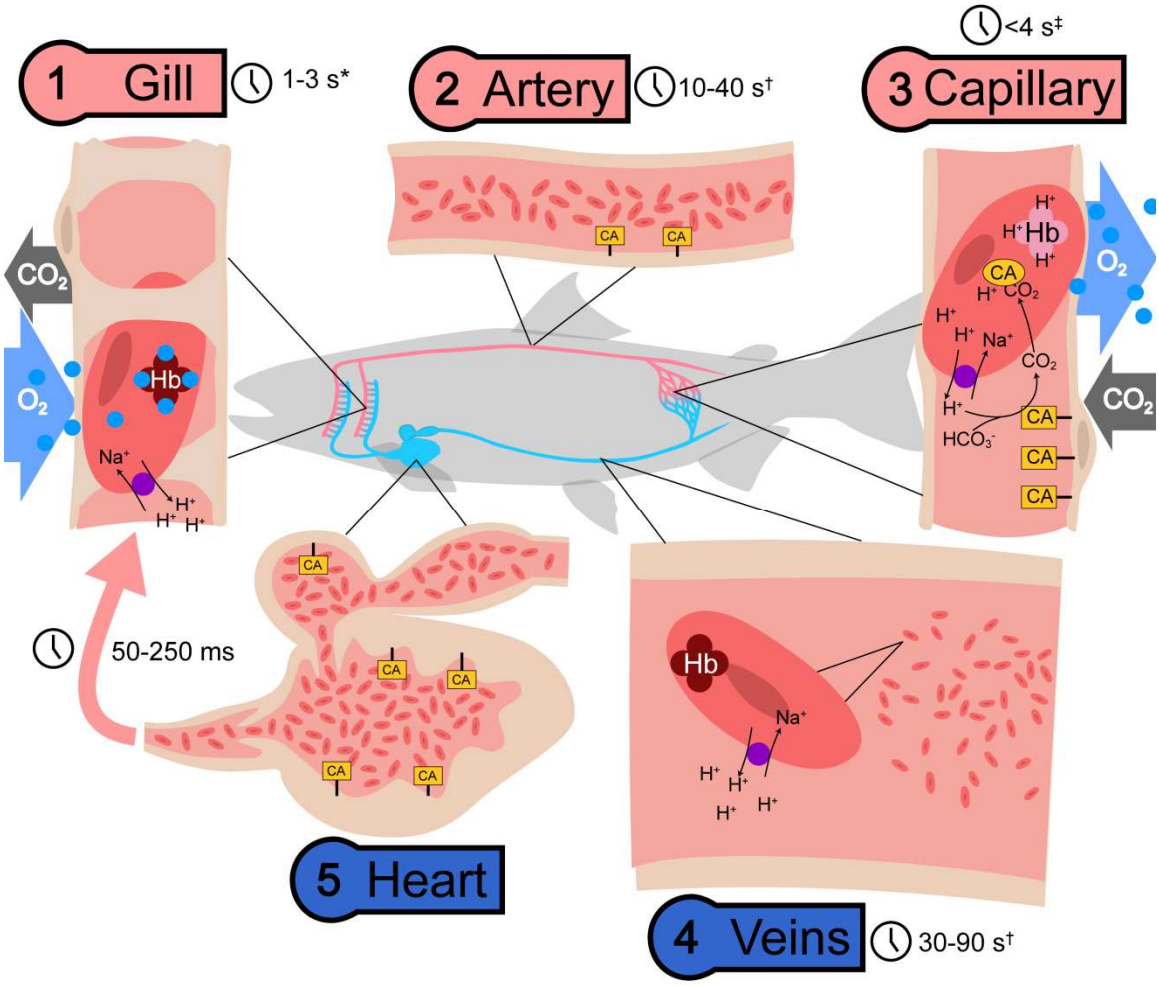

Fig. S1. Summary of the putative mechanism that enhances tissue oxygen (O_2) extraction in teleosts. 1) During a blood acidosis, catecholamines are released into the bloodstream and activate β-adrenergic sodium-proton exchangers (β-NHE) on the red blood cell (RBC) membrane. By actively extruding protons (H^+) into the plasma, β-NHEs protect RBC intracellular pH (pH_i) and O_2 loading from the water to pH-sensitive haemoglobin (Hb). 2-3) At the tissue capillaries (and perhaps in the arterial system in general), plasma-accessible carbonic anhydrase (paCA) is anchored to the endothelium and catalyses CO_2-HCO_3^--reactions in the plasma [15-17]. Thus, H^+ that are extruded by β-NHE, immediately combine with HCO_3^- to form CO_2, a small lipid soluble molecule that quickly diffuses across RBC membranes ($t_{1/2}$ ~1 ms; [18]). Within the RBCs, CO_2 is re-hydrated to form H^+ and HCO_3^-, a reaction that is catalysed by the abundant CA pool within the RBC [19]; thus, plasma pH (pH_e) and pH_i become coupled. The result is a rapid transfer of H^+ into the RBC that effectively short-circuits β-NHE activity, enhancing Hb-O_2 unloading via the Bohr effect. 4) In the venous system paCA is absent and the transfer of H^+ across the RBC membrane is rate-limited by the uncatalysed production of CO_2 in the plasma ($t_{1/2}$ ~90 s at 10°C [20]). Thus, when RBCs leave the capillaries, β-NHE activity will recover pH_i during venous transit and protect renewed O_2

uptake at the gills [21]. 5) In the teleost heart paCA is anchored to walls and trabeculae of the atrium and ventricle [22], and β-NHE short-circuiting may secure the O_2 supply to the avascular spongy myocardium [23]. The large volume of the lumen precludes a significant exposure of RBCs to paCA, and thus, the bulk of blood flow (~90%, based on ventricular ejected fraction [24]) will retain its high O_2 affinity; this is critical as transit times in the ventral aorta are too short to allow for a recovery of RBC pH_i before the gill. Additional references: * [25, 26]; † [15, 21]; and ‡ [27, 28].

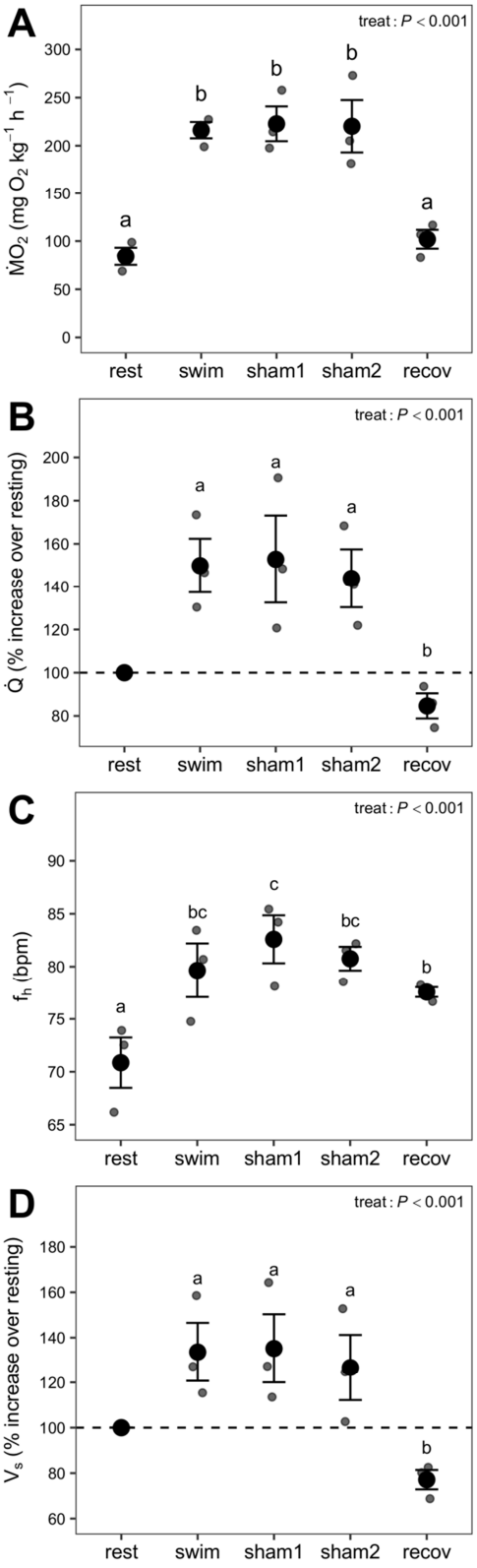
A
treat : P < 0.001
ṀO2 (mg O2 kg−1 h−1)
300
250
200
150
100
50
0
a
b
b
b
a
rest
swim
sham1
sham2
recov
B
treat : P < 0.001
Q̇ (% increase over resting)
200
180
160
140
120
100
80
a
a
a
b
rest
swim
sham1
sham2
recov
C
treat : P < 0.001
fh (bpm)
90
85
80
75
70
65
a
bc
c
bc
b
rest
swim
sham1
sham2
recov
D
treat : P < 0.001
Vs (% increase over resting)
180
160
140
120
100
80
60
a
a
a
b
rest
swim
sham1
sham2
recov

Fig. S2. Rate of oxygen consumption ($\dot{M}O_2$; mg O_2 kg^{-1} h^{-1}), B) cardiac output ($\dot{Q}$; % increase over resting), C) heart rate (f_h; bpm) and D) stroke volume (V_s; % increase over resting) in normoxia-acclimated Atlantic salmon. Measurements were taken at rest, during moderate exercise at 1 bl s^{-1} (swim), after successive saline injections (sham1 and sham2) that corresponded to the time-points of saline and C18 injection in the main experiment, and 10 min after the swim trial ended (recov). Data were analysed with a linear mixed model and the main effect of treatment (treat) is shown for the full model. Differences between treatments were analysed with Tukey's post-hoc tests ($N = 3$; $P < 0.05$) and are indicated by different superscript letters. Raw values are plotted as small grey points and means ± SEM are shown as large black symbols with error bars.

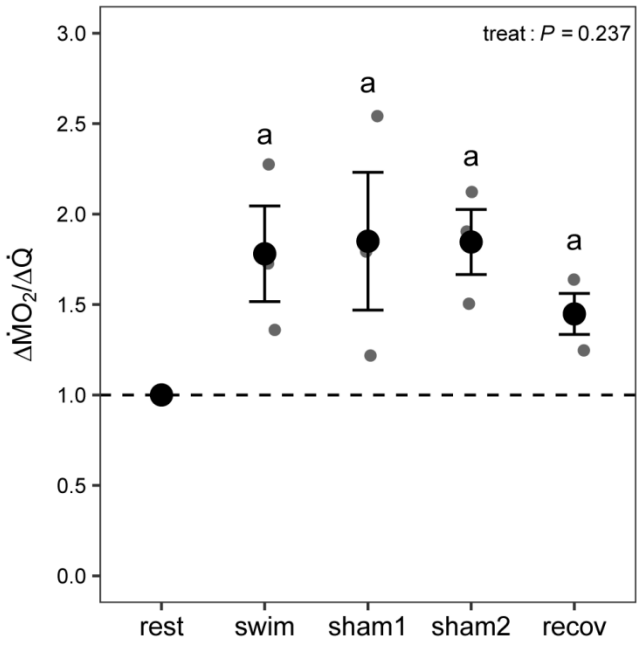

Fig. S3. Ratio between the change in oxygen consumption and the change in cardiac output ($\Delta\dot{M}O_2/\Delta\dot{Q}$), relative to resting values, for normoxia-acclimated Atlantic salmon. Measurements were taken at rest, during moderate exercise at 1 bl s^{-1} (swim), after successive saline injections (sham1 and sham2) that corresponded to the time-points of saline and C18 injection in the main experiment, and 10 min after the swim trial ended (recov). Data were analysed with a linear mixed model and the main effect of treatment (treat) is shown for the full model. Differences between treatments were analysed with Tukey's post-hoc tests ($N = 3$; $P < 0.05$) and are indicated by different superscript letters. Raw values are plotted as small grey points and means ± SEM are shown as large black symbols with error bars.

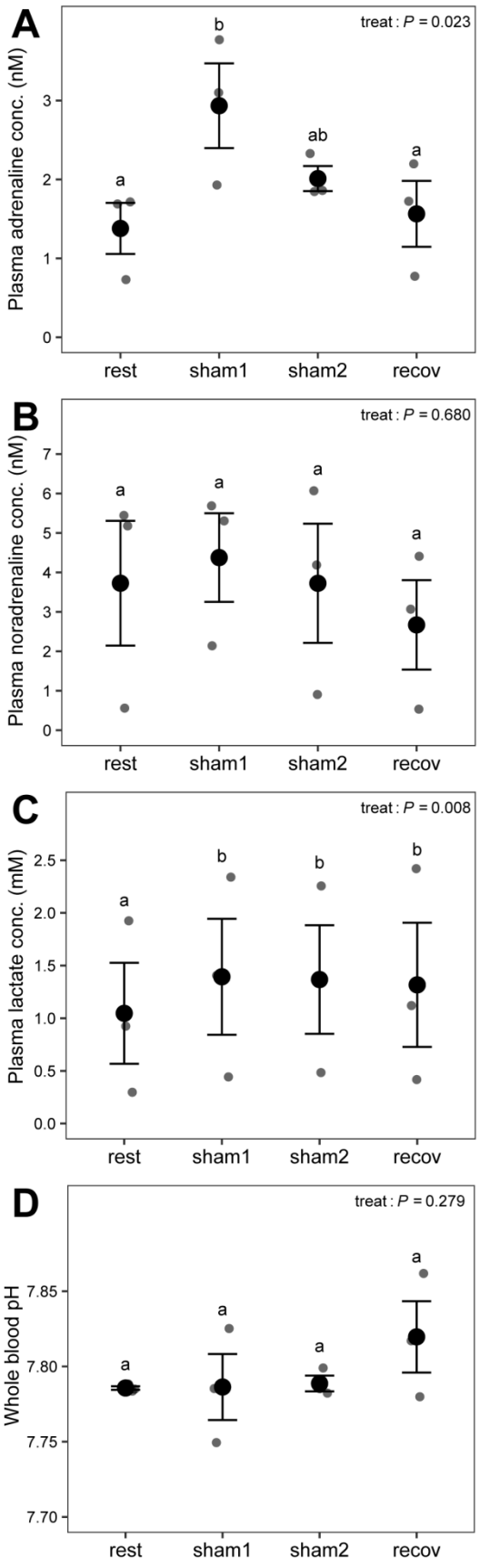
A
treat : P = 0.023
Plasma adrenaline conc. (nM)
0
1
2
3
a
b
ab
a
rest
sham1
sham2
recov
B
treat : P = 0.680
Plasma noradrenaline conc. (nM)
0
1
2
3
4
5
6
7
a
a
a
a
rest
sham1
sham2
recov
C
treat : P = 0.008
Plasma lactate conc. (mM)
0.0
0.5
1.0
1.5
2.0
2.5
a
b
b
b
rest
sham1
sham2
recov
D
treat : P = 0.279
Whole blood pH
7.70
7.75
7.80
7.85
a
a
a
a
rest
sham1
sham2
recov

Fig. S4. Plasma adrenaline, B) noradrenaline (nM) and C) lactate (mM) concentrations and D) whole blood pH, in normoxia-acclimated Atlantic salmon during control swim trials. Measurements were taken at rest, during moderate exercise at 1 bl s^{-1} (swim), after successive saline injections (sham1 and sham2) that corresponded to the time-points of saline and C18 injection in the main experiment, and 10 min after the swim trial ended (recov). Data were analysed using a linear mixed model and the main effect of treatment (treat) is shown for the full model. Differences between treatments were analysed with Tukey's post-hoc tests ($N = 3$; $P < 0.05$) and are indicated by different superscript letters. Raw values are plotted as small grey points and means ± SEM are shown as large black symbols with error bars.

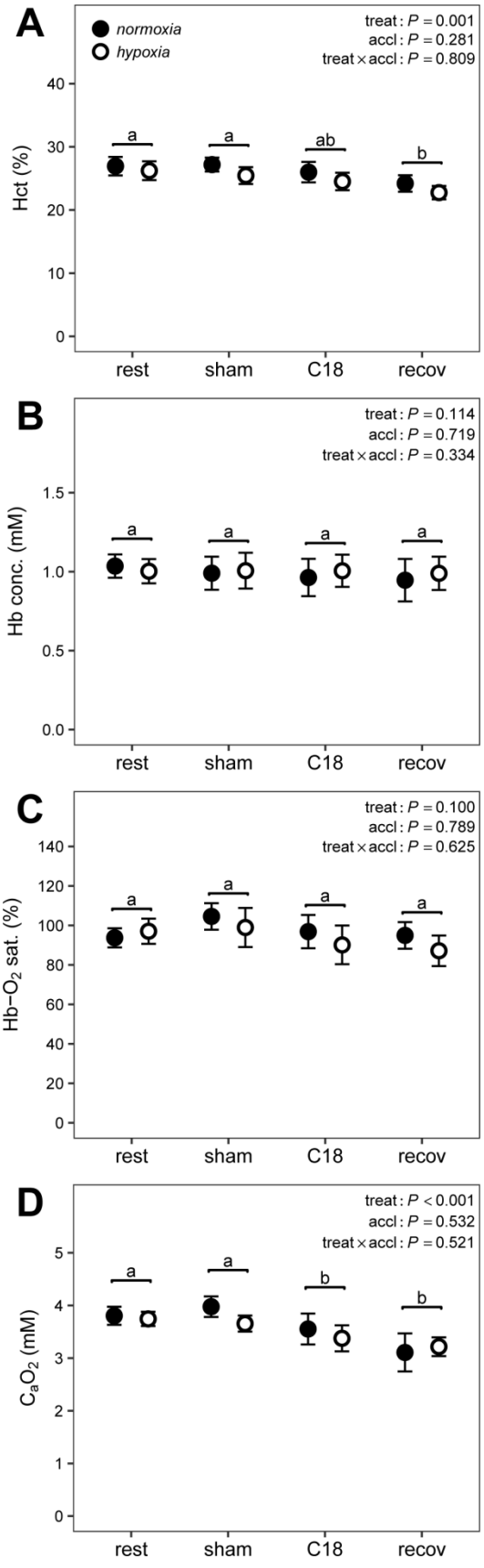
A
normoxia
hypoxia
treat : P = 0.001
accl : P = 0.281
treat × accl : P = 0.809
Hct (%)
0
10
20
30
40
a
a
ab
b
rest
sham
C18
recov
B
treat : P = 0.114
accl : P = 0.719
treat × accl : P = 0.334
Hb conc. (mM)
0.0
0.5
1.0
1.5
a
a
a
a
rest
sham
C18
recov
C
treat : P = 0.100
accl : P = 0.789
treat × accl : P = 0.625
Hb–O2 sat. (%)
0
20
40
60
80
100
120
140
a
a
a
a
rest
sham
C18
recov
D
treat : P < 0.001
accl : P = 0.532
treat × accl : P = 0.521
CaO2 (mM)
0
1
2
3
4
5
a
a
b
b
rest
sham
C18
recov

Fig. S5. A) Haematocrit (Hct; %), B) haemoglobin concentration (mM), C) haemoglobin oxygen saturation (Hb-O_2 sat.; %) and D) arterial O_2 content of the blood (C_aO_2; mM) of normoxia- (black symbols) and hypoxia- (white symbols) acclimated Atlantic salmon. Measurements were taken at rest, during moderate exercise at 1 bl s^{-1} (swim), after injection with saline (sham), after injection with a CA inhibitor that is effectively membrane-impermeable (C18; 200 µM), and 10 min after the swimming trial ended (recov). All trials were performed in normoxia for both acclimation groups. Data were analysed with a linear mixed model and the main effects of treatment (treat), acclimation (accl) and their interaction (treat × accl) are shown for the full model. Differences between treatments were analysed with Tukey's post-hoc tests ($N = 9$; $P < 0.05$) and are indicated by different superscript letters. Mean cell Hb concentration (MCHC) was also calculated, but there were no significant main effects or interactions for this parameter. All data are means ± SEM.

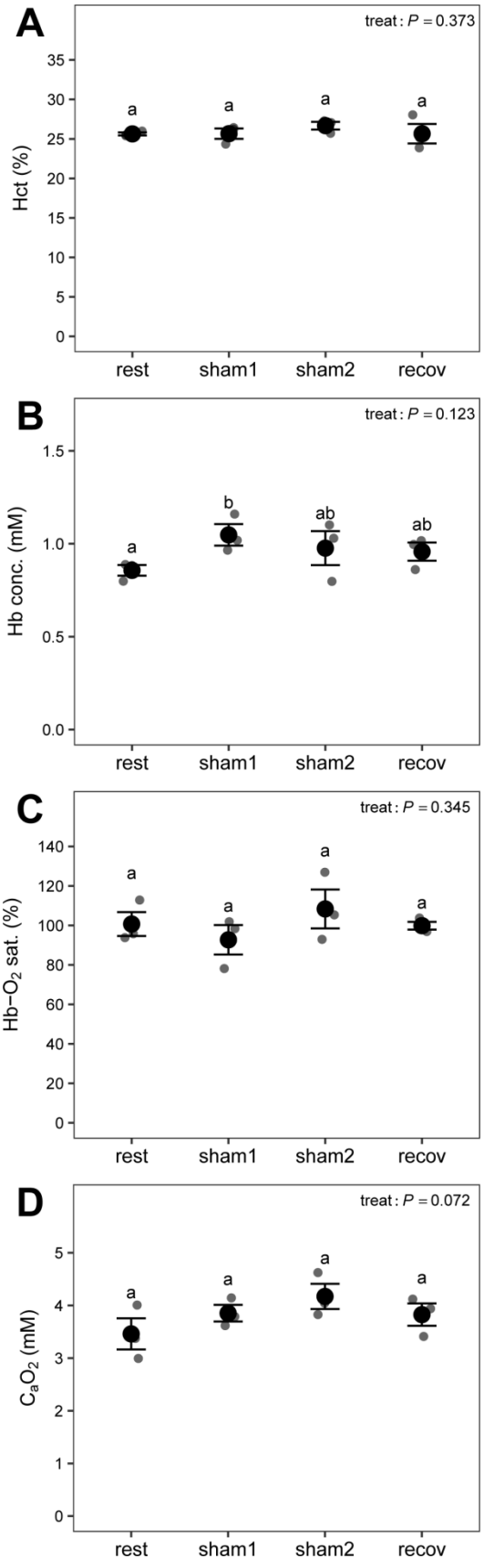
A
treat : P = 0.373
Hct (%)
35
30
25
20
15
10
5
0
a
a
a
a
rest
sham1
sham2
recov
B
treat : P = 0.123
Hb conc. (mM)
1.5
1.0
0.5
0.0
a
b
ab
ab
rest
sham1
sham2
recov
C
treat : P = 0.345
Hb–O2 sat. (%)
140
120
100
80
60
40
20
0
a
a
a
a
rest
sham1
sham2
recov
D
treat : P = 0.072
CaO2 (mM)
5
4
3
2
1
0
a
a
a
a
rest
sham1
sham2
recov

Fig. S6. A) Haematocrit (Hct; %), B) haemoglobin concentration (mM), C) haemoglobin oxygen saturation (Hb-O_2 sat.; %) and D) arterial O_2 content of the blood (C_aO_2; mM) of normoxia-acclimated Atlantic salmon. Measurements were taken at rest, during moderate exercise at 1 bl s^{-1} (swim), after successive saline injections (sham1 and sham2) that corresponded to the time-points of saline and C18 injection in the main experiment, and 10 min after the swim trial ended (recov). Data were analysed with a linear mixed model and the main effect of treatment (treat) is shown for the full model. Differences between treatments were analysed with Tukey's post-hoc tests ($N = 3$; $P < 0.05$) and are indicated by different superscript letters. Raw values are plotted as small grey points and means ± SEM are shown as large black symbols with error bars. Mean cell Hb concentration (MCHC) was also calculated and there was no significant treatment effect for this parameter.

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