**Life on the edge: Temperature and Hb genotype in-sensitive O2 binding in Atlantic cod red blood cells near their southern distribution limit**

Running title: Red cell O2 binding in Atlantic cod

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Summary statement:

Analysis of red blood cell O2 binding in Atlantic cod near their warming, equatorward limit of distribution suggests that, contrary to expectations, it does not depend on functional differences in haemoglobin genotypes, and is as important as cardiac output in setting upper limits to circulatory O2 supply under environmental warming.

**Abstract**

Atlantic cod are a commercially important species believed to be threatened by warming seas near their southern, equatorward upper thermal edge of distribution. Limitations to circulatory O2 transport, in particular cardiac output, and the geographic distribution of functionally different haemoglobin genotypes have separately been suggested to play a role in setting thermal tolerance in this species. The present study assessed the thermal sensitivity of O2 binding in Atlantic cod red blood cells with different Hb genotypes near their upper thermal distribution limit and modelled its consequences for the arterial-venous O2 saturation difference, *S*a-v, another major determinant of circulatory O2 supply rate. Results showed statistically indistinguishable red blood cell O2 binding between the three HbI genotypes in wild-caught Atlantic cod from the Irish Sea (53°North). Red blood cells had an unusually low O2 affinity, with reduced or even reversed thermal sensitivity between pH 7.4 and 7.9 and 5.0 and 20.0°C. This was paired with strongly pH-dependent affinity and cooperativity of red blood cell O2 binding (Bohr and Root effects). Modelling of *S*a-v at physiological pH, temperature and O2 partial pressures revealed a substantial capacity for increases in *S*a-v to meet rising tissue O2 demands at 5.0 and 12.5°C, but not at 20°C. There was further no evidence for an increase of maximal *S*a-v with temperature. It is suggested that Atlantic cod at such high temperatures may solely depend on increases in cardiac output and blood O2 capacity, or thermal acclimatisation of metabolic rate, for matching circulatory O2 supply to tissue demand.

**Introduction**

The 5th assessment report of the Intergovernmental Panel on Climate Change documents an increase in average global sea surface temperatures over the last century and predicts their continued rise (Field *et al*., 2014). The body temperature of marine ectothermic organisms is directly affected by warming seas, which makes an understanding of their physiological capabilities to withstand elevated temperatures vital for predicting future redistributions of species and influencing management regimes (e.g., Deutsch *et al*., 2015).

Atlantic cod (*Gadus morhua*) are widely distributed in coastal and shelf seas throughout the North Atlantic, but stocks near the southern, equatorward upper thermal margin of their historic distribution limit in the Irish and Southern North Sea have declined over the past decades, which has in part been ascribed to warming seas (Brander, 2005; Drinkwater, 2005; Perry *et al*., 2005; Beggs *et al*., 2014; Deutsch *et al*., 2015). Given in addition its high commercial importance and resulting fishing pressures, this has led to extensive research into thermal effects on Atlantic cod life history traits, physiology, behaviour, abundance and distribution (Mork *et al*., 1984; Petersen and Steffensen, 2003; Gamperl *et al*., 2009; Righton *et al.,* 2010; Behrens *et al*., 2012; Engelhard *et al*., 2014; Kreiss *et al*., 2015; Rutterford *et al*., 2015). Based on the thermal sensitivity of life history traits and projected future temperature changes, Atlantic cod stocks near their current upper thermal distribution limit in the North East Atlantic have been predicted to disappear entirely from the Celtic and Irish Seas by the end of this century (Drinkwater, 2005). Likewise, alternative mechanistic models based on a metabolic index of the O2 supply to demand ratio and projected future temperature and O2 partial pressure (*P*O2) changes predict reductions in the current habitat volume (occupied area x depth range) by 12-32% at the equatorward upper thermal margin of Atlantic cod by the end of the present century (Deutsch *et al*., 2015).

The oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis attempts to provide a general mechanistic explanation for the thermal distribution limits of aquatic organisms, suggesting that the capacity of O2 supply mechanisms in aquatic ectotherms, such as the circulatory and ventilatory systems, becomes insufficient to meet rising O2 demands at thermal extremes, thus affecting their ability to maintain an adequate aerobic scope for activities such as feeding, digestion, growth, migration, reproduction and predator evasion (Pörtner, 2001; Pörtner and Knust, 2007).

Studies on the acute thermal tolerance of Atlantic cod have identified the circulatory system as a primary limiting factor in the O2 supply cascade from the environment to the tissues, with cardiac function suggested to become compromised close to the critical thermal maximum (Sartoris *et al*., 2003; Lannig *et al.*, 2004; Gollock *et al*., 2006). According to the Fick equation, cardiac output, *Q̇* (the product of heart rate, *f*H, and stroke volume, *V*S) and the arterial-venous O2 difference, *C*aO2 - *C*vO2, together determine the rate of circulatory O2 delivery (*Ṁ*O2) between respiratory organs and tissues (Fick, 1870):

*Ṁ*O2 = *Q̇* (*C*aO2 - *C*vO2) Equation (1)

The contribution of changes in *C*aO2 - *C*vO2 in the assessment of maximal O2 supply capacities during warming of marine ectotherms is largely unknown, although it has long been recognised that, e.g. in humans, the increase in *C*aO2 - *C*vO2 may surpass the increase in *Q̇* in its contribution to meeting elevated *Ṁ*O2 during heavy exercise (factorial increases of 3.45 and 2.51, respectively; Ekelund and Holmgren, 1964; Dejours, 1975). *C*aO2 - *C*vO2 essentially equals the maximal blood O2 binding capacity multiplied by the arterio-venous O2 saturation difference, *S*a-v (ignoring the relative small contribution of physically dissolved O2 in blood with average haemoglobin (Hb) concentration). *S*a-v is in turn determined by the arterial and mixed venous *P*O2 values (*P*aO2 and *P*O2, respectively) and the shape and properties of the blood O2 equilibrium curve (OEC; e.g. Weber and Campbell, 2011). In fact, left-shifts of the OEC with increasing temperature or decreasing pH have classically been linked to improved rates of tissue O2 supply (Bohr *et al*., 1904; Barcroft and King, 1909). Yet the contribution of such OEC changes to meeting increased O2 demands in marine ectotherms at elevated temperatures is poorly known.

Atlantic cod are of particular interest in this context because the different Hb phenotypes of their polymorphic major HbI component (Sick, 1961) have been associated with differences in the thermal sensitivity of O2 binding in their red blood cells (RBCs) (Karpov and Novikov, 1980; Andersen *et al*., 2009). The frequencies of the two co-dominant alleles underpinning the HbI polymorphism vary inversely along a latitudinal cline in the North East Atlantic, from the Barents Sea with frequencies of the HbI 1 allele as low as 0-0.1, to the Southern North Sea, where HbI 1 frequency rises as high as 0.6-0.7 (Sick, 1965; Jamieson and Birley, 1989; Andersen *et al*., 2009; Ross *et al.* 2013). These clines have been attributed to natural selection acting on divergent temperature sensitivities of Atlantic cod harbouring the different HbI genotypes regarding growth, physiology and behaviour (reviewed by Andersen, 2012; Ross *et al*.,2013). However, the brief but influential report by Karpov and Novikov (1980) that first suggested functional differences in RBC-O2 affinity between the HbI genotypes, was based on RBC OECs of White Sea Atlantic cod (67° North) near their northern, lower thermal distribution limit and measured at a single, physiologically rather low pH value (7.5; Karpov and Novikov, 1980). Its findings and extrapolations for the efficiency of RBC O2 transport in Atlantic cod HbI genotypes near their southern, upper thermal limit of distribution have to our knowledge never been experimentally verified.

The present study was undertaken to assess the thermal sensitivity of RBC O2 binding, and its consequences for *S*a-v under *in vivo*-relevant conditions in Atlantic cod HbI genotypes near their upper thermal distribution limit in the North East Atlantic. The results showed statistically indistinguishable RBC O2 affinities and their pH and temperature sensitivities between all three HbI genotypes in wild-caught Atlantic cod from the Irish Sea (53°North). All animals showed an unusually low RBC O2 affinity, with no -or even reversed- thermal sensitivity over much of the physiological pH and temperature range. This was paired with strongly pH-dependent affinity and cooperativity of RBC O2 binding. Modelling of *S*a-v at physiological values for pH, temperature and *P*O2 revealed a substantial capacity for increases in this factor to meet rising tissue O2 demands at 5.0 and 12.5°C, but not at 20°C, where further increases in the maximal rate of O2 delivery by the circulatory system are predicted to solely rely on increases in cardiac output and O2 capacity.

**Materials and Methods**

Wild Atlantic cod, *Gadus morhua* (Linnaeus, 1758), with a total length of 46.4 ± 0.45 cm (here and elsewhere: mean ± S.E.M; *N* = 106 animals) were caught by hook and line on board of commercial fishing boats in the Mersey Estuary adjoining the Irish Sea near Liverpool, U.K., (53°25’ North, 3.02°1’ East) between mid-January and end of February 2015 at sea surface temperatures between 6.8 and 7.9°C. Animals were killed by a British Home Office approved Schedule 1 method, involving concussion and destruction of the brain. Blood was removed from caudal vessels using heparinized 1 ml syringes, whose dead space had been filled with 9.000 units ml-1 sodium heparin solution (from porcine intestinal mucosa, Sigma-Aldrich). Up to 8 animals of undetermined sex were bled on the day before each experiment and samples were kept on ice for maximally 10 h before landing and genotyping. Immediately after, blood of a single individual was selected for experiments the next day in accordance with a pre-determined random selection of genotype order.

*Genotype determination*

RBCs were isolated from plasma and buffy coat by centrifugation (3000 rcf, 4°C, 4 mins) and 20 µl of RBC pellet were lysed by adding 64 µl cold distilled water. Hbs in the haemolysate were separated by horizontal agarose gel electrophoresis, modified from Sick (1961). A 1% agar gel was prepared in diluted (1:1, with water) Smithies buffer (45 mM Tris, 25 mM boric acid and 1 mM EDTA, adjusted to pH 8.8 at room temperature). Undiluted Smithies buffer was used as an electrode buffer and samples were run towards the positive pole at 120 volts for 40 minutes at 4°C in a cold room, whereupon Hb bands were viewed immediately without staining.

*Preparation of RBC suspensions*

Remaining RBC pellets of selected samples were resuspended in physiological saline composed of (in mmol l-1) NaCl (125.5), KCl (3), MgCl2 (1.5), CaCl2 (1.5), D-glucose (5) and Hepes (20), adjusted to pH 7.97 at 15°C (Koldkjaer and Berenbrink, 2007). The above washing procedure of centrifugation and resuspension in fresh saline was repeated twice and during the last step RBCs were re-suspended at an approximate haematocrit (Hct) of 5-10 % and stored overnight at 4°C in a 15 ml falcon tube with a large air reservoir, placed on the side to maximise exchange surface area between saline and sedimented cells. Following the overnight rest and immediately prior to establishing RBC O2 equilibrium curves (OECs), RBCs were washed again, re-suspended in fresh saline at 8-13% Hct, and the concentrations of tetrameric Hb [Hb4], mean corpuscular Hb (MCHC), ATP, and GTP were determined.

*Analytical procedures*

[Hb4] was determined by the cyan-methaemoglobin method using modified Drabkin’s solution (11.9 mmol l−1 NaHCO3, 0.61 mmol l−1 K3[Fe(CN)6] and 0.77 mmol l−1 KCN) and a haem-based extinction coefficient of 11.0 l mmol-1 cm-1 at a wavelength of 540 nm, as described earlier (Völkel and Berenbrink, 2000). Hct was measured in micro-haematocrit tubes using a SpinCrit Micro-Hematocrit centrifuge and MCHC was calculated as [Hb4]/(Hct/100). For ATP and GTP determinations, equal volumes of washed RBC suspension and 0.6 M perchloric acid (PCA) were mixed before freezing at -80°C for later analysis. Samples were defrosted and centrifuged at 4°C and 13,000 rcf. The PCA extract was neutralised to an approximate pH of 7 by the addition of concentrated potassium carbonate to the supernatant and the resultant precipitate was removed by centrifugation. ATP and GTP concentrations in the supernatant were then determined enzymatically via the two step process outlined by Albers *et al*. (1983), with the following modifications: The enzymes hexokinase **with glucose-6 phosphate dehydrogenase (H8629, Sigma-Aldrich) and** Nucleoside 5′-diphosphate kinase **(N0379, Sigma-Aldrich) were used at concentrations of** 13 units/mL and 5000 units/mL, respectively. The accuracy of the test and potential losses of nucleotide triphosphates (NTPs) during PCA extractions were examined using ATP and GTP standard solutions (Sigma-Aldrich, A2383 and G8877). Recovery was 96.4 ± 0.9% and 80.4 ± 0.64% (*n* = 18) for ATP and GTP respectively, and all measurements were corrected accordingly. Concentrations were converted to mmol l-1 RBCs using equation presented by Albers *et al*., (1983), then standardised using MCHC and presented as ATP/Hb4 and GTP/Hb4 molar ratios.

*Oxygen equilibrium curve determinations*

After the above measurements were taken, RBC suspensions were further diluted 10-fold in pH 7.97 saline and then pH was varied by final 10-fold dilutions in salines of pH 7.45, 7.70 and 7.97 (all adjusted at 15°C). Thermally-induced saline pH changes were assessed in air equilibrated RBC suspensions using a Lazar Model FTPH-2S pH electrode with a Jenco 6230N meter (Jenco Collaborative, California, USA). Given the buffering properties of the saline (20 mM Hepes) and small quantity of cells (0.08 – 0.13% Hct) oxygenation-linked changes in pH of RBC suspensions during OEC measurements were deemed negligible. For each individual, 1.2 ml aliquots of final RBC suspension were incubated, at the three pH values in parallel, in 50 ml capacity Eschweiler glass tonometers (Eschweiler GmbH, Engelsdorf, Germany) with custom attached 1 cm path length optical glass cuvettes (following a design by Brix *et al*., 1998). This was performed at temperatures of 5.0, 12.5 and 20.0°C and a minimum of five *P*O2 values covering the range between of 20 to 80% RBC O2 saturation. *P*O2 was varied by mixing air and N2 in pre-determined ratios using a Wösthoff gas mixing pump (Wösthoff GmbH, Bochum, Germany) and the final gas mixture was fully humidified at the experimental temperature. RBC suspensions were equilibrated for at least 20 minutes with each gas mixture. Solutions remained sealed within the tonometer to ensure *P*O2 remained constant while an optical spectrum was taken between 500-700 nm (Unicam UV 500 spectrophotometer, Thermo Electron Corporation, Ohio, USA; with Vision 32 Software) and O2 saturation of RBC suspensions was determined by spectral deconvolution (Völkel and Berenbrink, 2000).

*Data analysis and statistics*

Spectral deconvolution of the optical spectra (see Völkel and Berenbrink, 2000) was used to determine the concentrations of haemoglobin derivatives within RBC suspensions (oxyhaemoglobin, HbO2; deoxyhaemoglobin, deoxyHb; and the two forms of methaemoglobin, acid Hb+ and alkaline Hb+) at each temperature, pH and *P*O2 value using SigmaPlot 12.5 (Systat Software Inc., San Jose, California). The unknown concentrations (mmol l-1) of the different tetrameric Hb derivatives, were calculated using

*f* = *au* + *bv* + *cw* + *dx* Equation (2) where *a, b, c,* and *d,* represent [HbO2], [deoxyHb], [acid Hb+], and [alkaline Hb+], respectively and were restricted to values greater than or equal to zero.  *f* is the predicted dependent variable to be fitted to the measured absorption data for each nm step between 500 and 700 nm. *u, y, w* and *x* represent the respective experimentally determined absorption coefficients for each Hb derivative at each wavelength between 500 and 700 nm, respectively. Absorption coefficients for HbO2 and deoxyHb were created with RBC suspensions in pH 8.05 saline at 5.0°C, exposed to 100% oxygen or 100% nitrogen. Acid Hb+ and alkaline Hb+ absorption coefficients were constructed using Hb suspensions oxidised with tri-potassium hexacyanoferrat at pH 6.5 and 8.05 respectively, although the analysis showed that no methaemoglobin formation had occurred in any of our samples. In all cases, the predicted values by the curve fitting procedure were plotted for each wavelength between 500 and 700 nm together with the measured spectra for visual inspection of the accuracy of the prediction.

The level of RBC O2 saturation (*S*) was calculated as [HbO2]/ ([HbO2] + [deoxyHb]). Hill plots on data between 20 and 80% saturation were created using log (*S*/(1-*S*)) versus log *P*O2. Log *P*50 was calculated by linear regression as the log *P*O2 when log (*S*/(1-*S*)) equalled 0. The slope of the regression line indicated the apparent cooperativity of RBC O2 binding or Hill number (*n*H). The Bohr coefficient was calculated by *Φ* = Δlog *P*50/ ΔpH for each pH interval. Because of nonlinearity, at each temperature, log *P*50 and *n*H were plotted against measured saline pH and 2nd order polynomials were used to standardise themto pH 7.40, 7.65 and 7.9, removing the effect of temperature-induced pH shifts on these variables. Once standardised to fixed pH, thermal sensitivities of OECs were expressed as apparent heat of oxygenation, Δ*H*’. These were calculated using the van’t Hoff equation Δ*H*’ = 2.303 *R* ((Δlog *P*50)/(Δ1/*T*)), where *R* = universal gas constant (0.008314 kJ K-1 mol-1), and *T* = temperature in K.

OECs for a series of fixed pH values were produced using values for *n*H and *P5*0 predicted at a given pH for each individual from the same 2nd order polynomial equations used above for standardising log *P*50 and *n*H. RBC O2 saturation *S* was then calculated as a function of *P*O2 using:

*S* = *P*O2 exp(*n*H) [*P*O2 exp(*n*H) + *P*50 exp(*n*H)]-1 Equation (3) *S*a-v during acute temperature and/or pH changes was modelled as the differences between *S*aO2 and *S*vO2 at physiologically relevant pH and arterial and venous *P*O2 values read from RBC OECs. An arterial pH of 7.86 and average values of 85 and 30 mmHg for *P*aO2 and *P*O2 were assumed for resting normoxic Atlantic cod at 12.5°, based on literature values for this species close to this temperature (Kinkead *et al*., 1991; Perry *et al*., 1991; Claireaux and Dutil, 1992; Nelson *et al*., 1996; Larson *et al*., 1997; Karlsson *et al*., 2011; Petersen and Gamperl, 2011). *P*aO2 was assumed constant during acute thermal change (Sartoris *et al*., 2003), whereas values for *P*O2 at 5.0 and 20.0°C of 60 and 15 mmHg, respectively, were based on the percentage changes observed by Lannig *et al*. (2004). Changes in arterial pH were assumed to follow the relationship with temperature established for marine teleosts and elasmobranchs by Ultsch and Jackson (1996). Owing to the generally larger deoxygenation-linked proton uptake in teleosts Hbs compared to other vertebrates (Berenbrink *et al*., 2005), venous pH was assumed to be similar to arterial pH, as previously recorded in normoxic Atlantic cod (Perry *et al*., 1991).

Maximal *S*a-v at each temperature was taken as the maximally observed *S*a-v at any pH and *P*aO2 and *P*O2 equalling 85 and 15 mmHg, the lowest average *P*O2 reported for Atlantic cod in the literature under any condition.

All values are reported as means ± standard error of the mean (SEM). Sigmaplot 12.5 (Systat Software Inc., San Jose, California) was used for all statistical analysis and significance was accepted at *p* < 0.05. Differences between mean values were generally assessed by one-way ANOVA, followed by a post-hocTukey test, if relevant. Other test statistics (two-way, three way ANOVA, χ2 and one-sample *t*-tests) were used as indicated directly in the text.

**Results**

In 106 Atlantic cod caught between mid-January and end of February 2015 in the River Mersey Estuary near Liverpool, the HbI 1/1 genotype dominated with 45% of individuals, followed by 41% HbI 1/2 heterozygotes and just 14% HbI 2/2 homozygotes (Table 1). These genotype frequencies did not significantly deviate from the expectations according to the Hardy-Weinberg equilibrium, (χ2 = 1.09, d.f. = 2, *p* > 0.5) or from the averaged values recorded for the Irish Sea between 1971 and 1977 (χ2= 5.73, d.f. = 2, *p* > 0.05; Jamieson and Birley, 1989). HbI 1 allele frequency was 0.66 and thus among the highest values recorded for Atlanitc cod stocks across their geographical range (Ross *et al*., 2013), and similar to values reported in recent years for the Southern North Sea (0.66, Pörtner *et al*., 2001; 0.64, Andersen *et al*. 2009). There was no difference in total length between HbI genotypes in 84 animals that were available for length measurement, nor in the subset of 16 animals selected for OECs (*p* = 0.073 and 0.226, respectively; Table 1). In the latter group there were also no significant HbI genotype-related differences in haematocrit (*p* = 0.834), haemoglobin concentration (*p* = 0.697), MCHC (*p* = 0.371) and ATP/Hb4 (*p* = 0.284) or GTP/Hb4 (*p* = 0.620) ratios of washed RBC suspensions immediately prior to experiments (Table 1). The ATP/Hb4 and GTP/Hb4 ratios further were similar to values previously reported on whole blood (Leray, 1982).

OECs of Atlantic cod RBCs at all three temperatures and for all three HbI genotypes revealed strong Bohr and Root effects, as shown by strong pH-induced reductions in RBC O2 affinity and O2 saturation at atmospheric *P*O2, respectively (Figure 1A-C). At each nominal saline pH, increasing temperatures appeared to reduce O2 affinity, shifting OECs to the right and increasing *P*50 (Figure 1A-C). However, this effect will have been partially due to the temperature-induced shifts in the pH of the HEPES buffer. Thus, for example, the actual pH values experienced by RBCs suspended in saline with a nominal pH of 7.90 were 7.99, 7.89 and 7.81 at 5.0, 12.5 and 20.0°C, respectively, with SEM values for pH below 0.005.

In the Bohr plot (Figure 2) the stepwise reductions of pH from nominal pH 7.90 to 7.65 and then 7.40 resulted in significant increases in log *P*50 within all genotypes and all temperatures (*p* < 0.001). Thus, the southern HbI 1/1 genotype at 5.0°C and pH 7.99 had a log *P*50 of 1.52 ± 0.02 (corresponding to a *P*50 of 33 mmHg). As pH decreased, O2 affinity showed a corresponding decrease, with a log *P*50 of 1.79 ± 0.03 (*P*50 62 mmHg) at pH 7.75 and a further decrease at pH 7.51 to 2.20 ± 0.06 (158 mmHg). Similar effects of pH were also observed at 12.5 and 20.0°C, although increasing temperatures caused a general shift of curves towards higher log *P*50 values and lower pH values (Figure 2).

Surprisingly, log *P*50 values were not affected by HbI genotype at any tested pH or temperature (*p* = 0.161 – 0.421), although there was a tendency of values in the northern HbI 2/2 type to be consistently lower than the other two genotypes.

The relationship between log *P*50 and pH appeared distinctly curvilinear and a three-way ANOVA with pH-range, temperature and genotype as factors, revealed that the Bohr coefficient *Φ*, Δ log *P*50 (Δ pH)-1, significantly increased in magnitude from around -1.08 in the higher pH range, to about -1.65 in the lower pH range (*p* < 0.001). This increased pH dependence of RBC O2 affinity at lower pH is likely due to the more pronounced Root effect at the lowest pH values. Both genotype and temperature had no significant effect on the Bohr coefficient (*p* = 0.183 and 0.840, respectively).

Hill’s cooperativity constant *n*H did not vary significantly between the upper two saline pH values at any temperature, attaining values between 1.5 and 2.0. At the lowest saline pH however, *n*H was significantly reduced down to values between 1.0 and 0.7 compared to the highest saline pH (*p* < 0.001) indicating the onset of the Root effect. Similar to log *P*50 above, *n*H also remained unaffected by HbI genotype at all pH values and temperatures (*p* = 0.161 – 0.421). Given the lack of significant Hb genotype differences in all analyses above, data for all animals were pooled for the following analyses.

After standardising log *P*50 values of the combined HbI genotypes to constant pH values (Table 2), log *P*50 at pH 7.65 was completely independent of temperature over the entire range from 5.0 to 20.0⁰C (Table 3). At pH 7.90, log *P*50 was also statistically indistinguishable between 5.0°C and 12.5°C, and only increased significantly at 20.0°C compared to these values (*p* = 0.002 and *p* < 0.001, respectively; Table 3). At pH 7.40, log *P*50 was unaffected by temperature between 20.0 and 12.5⁰C, and only significantly increased at 5⁰C compared to these values (*p* < 0.001), revealing a reversed temperature sensitivity at the lower temperature range.

The pH-adjusted cooperativity coefficient *n*H (Table 2)was unaffected by temperature at pH 7.9 ((*p* = 0.412; Table 3), but at pH 7.65 it was significantly reduced at 5.0°C when compared to 12.5 and 20.0°C (*p* < 0.001), although values at 12.5 and 20°C did not differ significantly. At pH 7.4, *n*H significantly increased with temperature over the whole range (*p* < 0.001; Table 3).

Δ*H*’ for the oxygenation reaction of Atlantic cod RBCs was significantly affected by both pH (*p* < 0.001) and temperature range (*p* < 0.001), with no significant interaction (*p* = 0.574) between factors (two-way ANOVA, with temperature range and pH as factors; Figure 3). Between 12.5 and 20.0°C and at pH 7.90, Atlantic cod RBCs showed a typical exothermic oxygenation reaction, with a negative Δ*H*’ value of -15.7 ± 2.9 kJ mol-1. However, in the same thermal range, thermal sensitivity was significantly reduced at pH 7.65 and 7.40, where Δ*H*’ values amounted to -2.5 ± 1.9 kJ mol-1 and +5.8 ± 3.9 kJ mol-1, respectively. These values were not significantly different from each other and One-sample *t*-tests showed that they also did not significantly differ from zero (*p* = 0.208 and 0.158, respectively; Figure 3). At all pH values, the magnitude of Δ*H*’ was significantly higher between 5.0-12.5°C than between 12.5-20.0°C. In the lower temperature range at pH 7.9 this resulted in a Δ*H*’ value of -3.8 ± 2.3 kJ mol-1, which was not significantly different from zero (One-sample *t*-test, *p* = 0.119). Stepwise, significantly more endothermic RBC oxygenation was observed at pH 7.65 (+8.9 ± 2.4 kJ mol-1) and then pH 7.40 (+23.2 ± 4.4 kJ mol-1).

Using 2nd order polynomials (Table 2), log *P*50 and *n*H values from Figure 2 were standardized for a series of fixed pH values and the corresponding OECs shown for three temperatures (Figure 4). At each temperature, literature values for *in vivo* *P*aO2 and *P*O2 and the resulting *S*a-v are indicated for each pH. The curves suggest *in vivo* arterial O2 saturations, across temperature, at resting arterial pH (7.91 to 7.81 between 5.0°C and 20.0°C, respectively) and constant arterial *P*O2 (85 mmHg) of no more than 80% (Figure 4A-C). Increasing temperatures are associated with greater use of the venous reserve, as shown by decreases in *P*O2, and consequent increases in *S*a-v from 0.11 at 5.0° to 0.44 and 0.58 at 12.5 and 20.0°C, respectively. Further, at each temperature and fixed *P*aO2 and *P*O2 values, acidification-induced decreases in *S*vO2 were accompanied by similar, or even greater decreases in *S*aO2 (Figure 4A-C). This suggests that in Atlantic cod RBCs the benefits of the Bohr effect under general acidosis in facilitating O2 offloading to tissues at a given *P*O2, are minimised by parallel or even greater decreases in arterial O2 loading.

Estimates of maximal *S*a-v values at the three temperatures (Figure 4 D-F) show a substantial potential for increasing *S*a-v above routine values at 5.0 and 12.5°C, where *S*a-v rises, by factors of 4-5 and 1.5-2.0, respectively, when *P*O2 is allowed to drop to the minimally observed value of 15 mmHg (Figure 4E, F vs. A, B). However, there was no additional capacity for *S*a-v increases above routine values at 20.0°C (Figure 4C, F). Similarly, across pH values, maximal *S*a-v values tended to decrease, rather than increase with temperature, such that even taking into account a temperature-associated decrease in *in vivo* arterial pH from 7.91 at 5.0°C to 7.81 at 20.0°C did not increase *S*a-v (Figure 4D-F).

**Discussion**

The results of the present study suggest that the O2 binding properties of Atlantic cod RBCs near their southern, upper thermal distribution limit in North East Atlantic are contrary to common expectation independent of HbI genotype, characterized by an unusually low O2 affinity that is strongly affected by pH and remarkably temperature in-sensitive over much of the physiological pH range. These factors combine to create a blood O2 transport system in which maximal *S*a-v under *in vivo* conditions does not increase with temperature or general blood acidosis, which universally accompanies elevated temperature across ectothermic vertebrates (Ultsch and Jackson, 1996). This is surprising in light of the fact that increased temperature and general blood acidification are the classic, textbook examples of how the rate of O2 supply to tissues can be increased by right-shifts of the OEC and increased *S*a-v (Barcroft and King, 1909; Bohr *et al.* 1904; Dejours, 1975; Berenbrink 2006, 2011a). Similarly, temperature-dependent differences in O2 affinity between the HbI genotypes of Atlantic cod have been held crucial in the adaptation of this species to environmental temperature for more than 35 years (Karpov and Novikov, 1980; Andersen, 2012; Ross *et al*., 2013). The clear lack of both a temperature and HbI genotype effect on RBC O2 affinity demonstrated in the present study, together with results from carefully controlled whole animal studies (Gamperl *et al*., 2009), points to an emerging paradigm shift in our understanding of thermal adaptation of O2 supply mechanisms and the roles of HbI genotypes differences in Atlantic cod. In the following the results are critically evaluated and the underlying mechanisms and consequences for maximal circulatory O2 supply rates of Atlantic cod at elevated temperatures are discussed.

*Low O2 binding affinity of Atlantic cod RBCs*

The average *P*50 of Atlantic cod RBCs across the three genotypes was 40 mmHg (calculated from log *P*50 values at pH 7.90 between 5.0 and 12.5°C in Table 4). This value is among the lowest O2-affinities that have been reported for blood or RBCs of any fish under the standardised conditions given above (e.g. Herbert *et al*., 2006). Such a low *P*50 results in arterial blood in gills lying on the edge of the steep part of the OEC, with modelled RBC O2 saturations of no more than 80% at typical *P*O2 and pH values and at any temperature between 5.0 and 20.0°C (Figure 4). This guarantees that across all temperatures, small decreases in venous *P*O2 enable large increases in O2 unloading in the tissues at a relatively high venous *P*O2, which will safeguard a sufficiently large diffusion gradient from the blood plasma to tissue mitochondria. Blood O2 tissue extraction [*S*a-v x *S*aO2-1] was accordingly as high as 53% for normoxic resting animals at pH 7.90 and 12.5°C (calculated from Figure 4A), which compares well with estimates in Atlantic cod *in vivo* under similar conditions (57%, Perry *et al*., 1991; 51%, Petersen and Gamperl, 2011). The high venous unloading *P*O2 may be particularly important for cardiac O2 supply in species like Atlantic cod, where the ventricle lacks a coronary blood supply and consists entirely of spongey myocardium that relies exclusively on the O2 remaining in luminal blood that is returned from the other tissues (Santer and Walker, 1980; Farrell *et al*., 2012). However, too low a blood O2 affinity comes at the cost of potentially reducing the efficiency of a further right-shift of the OEC for increasing *S*a-v under, e.g., warming or general acidosis.

*(In)efficiency of the Bohr effect in enhancing O2 supply under general acidosis*

The low O2 affinity of Atlantic cod RBCs was paired with one of the largest Bohr effects reported for blood or RBCs under controlled standard conditions (Δ log*P*50 (Δ pH)-1 = -1.08 ± 0.05, pH 7.9 to 7.65 and 5.0 to 20.0° ). At still lower pH values the pH-induced decline in RBC O2 affinity was associated with a reduced cooperativity of RBC O2 binding and with O2 saturations below 60% in air-equilibrated RBCs. This indicated a strong Root effect and confirmed the positive correlation between the magnitudes of the Bohr and Root effects that has been found across a wide range of diverse ray-finned fishes (Berenbrink *et al*., 2005). Low O2 affinity and a strong Bohr effect were both previously reported for Atlantic cod haemolysates in the presence of saturating ATP concentrations (Pörtner *et al*., 2001; Brix *et al*., 2004; Verde *et al*., 2006). Importantly, these findings on Hb solutions in artificial buffers also closely reflect results for Atlantic cod whole blood in the presence of a physiological CO2/bicarbonate buffer system (Herbert *et al*., 2006). Bohr *et al*. (1904) first emphasized the biological importance of elevated blood carbon dioxide partial pressures (*P*CO2s) and thereby blood acidification for enhancing blood O2 utilisation in the tissues, without affecting O2 uptake at the higher *P*O2 values in the respiratory organ. The present study surprisingly suggests that these generally accepted benefits of the Bohr effect are partially cancelled in Atlantic cod due to their low blood O2 affinity, whereby any decrease in *S*vO2 during general acidosis is accompanied by a similar, or even larger decrease in *S*aO2, such that Sa-v remains the same or even decreases upon acidification (Figure 4). Thus, the unusually large effect of elevated CO2 or low pH on Atlantic cod RBC O2 binding affinity and capacity (Krogh & Leitch, 1919; Herbert *et al*., 2006; Berenbrink *et al*., 2011) will be mainly useful during localized tissue acidification, such as at the tissue poles of the vascular counter-current exchangers (*retia mirabilia*) in the eye and swim bladder of Atlantic cod, where they are crucial for generating super-atmospheric *P*O2 values that support the high metabolic demands of the poorly vascularized retina, and for swim bladder gas filling against increasing hydrostatic pressures at depth (Bohr, 1894; Wittenberg and Wittenberg, 1962; Berenbrink *et al.*, 2005; Berenbrink, 2007).

These considerations do not negate the benefits of the Bohr effect in increasing Sa-v due to arterio-venous pH differences that are caused by the differences in arterial and venous *P*CO2 or by selective short-circuiting of catecholamine-activated RBC intracellular pH regulation in tissues with plasma-accessible carbonic anhydrase, as recently suggested for rainbow trout (Rummer *et al*., 2013). Instead they emphasize that parallel pH shifts in arterial and venous blood, such as during exercise-induced lactacidosis or environmental warming, are unlikely to increase Sa-v in Atlantic cod at physiological *P*aO2 and minimal *P*O2. Any increases in circulatory blood O2 supply under these conditions must come from increases in cardiac output, blood O2 capacity, or alternative mechanism that may increase *S*a-v.

*Reduced and reversed thermal sensitivity of O2 binding in Atlantic cod RBCs*

Whole body or local increases in temperature, such as in working muscle, are classically thought to increase blood O2 transport by increasing Sa-v (Barcroft and King, 1909). In many animals the intrinsically exothermic nature of haem O2 binding determines the overall heat of Hb oxygenation, resulting in a lowered Hb O2 affinity at elevated temperature (Weber and Campbell, 2011). However, binding of allosteric effectors such as protons and ATP or GTP preferentially to deoxy Hb requires their endothermic release during oxygenation and this can compensate for the heat released by exothermic haem oxygenation, leading to a reduced or even reversed temperature sensitivity of Hb O2 affinity. This is best known for heterothermic tuna, billfishes, and lamnid sharks, where exothermic Hb O2 binding may cause problems in heat-conserving vascular counter-current exchangers (Weber & Campbell, 2011). The finding of largely thermally insensitive RBC O2 affinity in Atlantic cod in this study, together with the study by Clark *et al.* (2010) on Pacific mackerel, suggests that low thermal sensitivity of RBC O2 affinity may be more widespread among ectotherm fishes than previously thought.

Normally, with an overall exothermic reaction of Hb O2 binding, increased temperatures decrease Hb O2 affinity and cause a right-shift of the OEC. This will generally allow an increased *S*a-v in any organism with *S*aO2 and *P*aO2 in the flat upper part of the OEC because a decrease in *S*vO2 allows of a greater exploitation of the venous reserve. However, for a species with a RBC O2 affinity as low as reported for Atlantic cod in the present study, any gain in O2 offloading by a decrease in *S*vO2 will be obliterated by a parallel decrease in *S*aO2 at typical *P*aO2. This may be the ultimate, evolutionary driving cause behind the reduced thermal sensitivity of O2 binding in Atlantic cod RBCs.

The proximate, mechanistic explanation for the phenomenon may involve at least two not necessarily exclusive factors. First, the large Bohr effect suggests an above average increase in the number of proton binding sites in deoxyHb compared to oxyHb (for review see Berenbrink 2006, 2011a),. The release of these protons during oxygenation may compensate for exothermic haem O2 binding. This is supported by the strong effect of pH on the overall enthalpy of RBC oxygenation over the whole temperature range (Figure 3). Second, the increase in cooperativity of RBC O2 binding with temperature at low pH (Table 3) suggests that the over-stabilisation of deoxy Hb by the Root-effect (with *n*H ≤ 1; see Berenbrink 2011b) is weakened at higher temperatures, where increasing values of *n*H indicate an endothermic transition to the oxy conformation of Hb. This is consistent with previous work demonstrating the large endothermic nature of the deoxy to oxy Hb conformational transition in teleosts (Saffran and Gibson, 1979). In addition, the endothermic release of the organic phosphate modulators ATP and GTP from deoxy Hb upon oxygenation may contribute to the overall heat of oxygenation of Atlantic cod RBCs, a mechanism that has previously been shown to contribute to the reduced and reversed oxygenation enthalpy of several species of billfish (Weber *et al*., 2010). However, elucidation of the detailed molecular mechanism(s) behind reduced or even reversed thermal sensitivity of Atlantic cod RBC O2 affinity awaits detailed studies on purified Hbs under tightly controlled conditions of allosteric modifiers.

*Lack of HbI genotype effects on O2 binding in Atlantic cod RBCs*

The increased frequency of the HbI 1 allele towards the Southern range of Atlantic cod has been widely related to a parallel cline in environmental temperature and to a presumed advantage of HbI 1/1 cod in having a higher RBC O2 affinity at temperatures above 15°C compared to HbI 2/2 cod where this is higher below 15°C (e.g., Karpov & Novikov, 1980; Andersen *et al*., 2009; reviewed by Andersen, 2012, and Ross *et al*., 2013). The current study establishes the absence of any statistically supported differences in the RBC O2 binding characteristics between Atlantic cod of all three HbI genotypes near their southern upper thermal distribution limit. This result has been consistently obtained over a range of pH values at each of three physiologically relevant temperatures and is considered robust, because factors well known to modify the genetically determined, intrinsic O2 binding affinity of Hb inside RBCs have carefully been controlled. To ensure environmental relevance but at the same time minimise differences in prior thermal or hypoxic acclimatization of individuals, RBCs were obtained immediately after capture from wild Atlantic cod at a single location and over a 6 week period in winter where long term annual water temperature changes were minimal and stratification was absent (Neat *et al*. 2014; O’Boyle & Nolan, 2010). In contrast to earlier studies (Karpov and Novikov, 1980; Gollock *et al*., 2006; Petersen and Gamperl, 2011) RBCs were washed in glucose-containing physiological saline and incubated overnight before experimentation. This removes any catecholamine hormones, which are known to be released into plasma during blood sampling stress and modify the concentration of intracellular allosteric modifiers of Hb O2 binding, and allows any catecholamine-initiated effects to wear off during pre-incubation in standardised physiological saline (Berenbrink and Bridges, 1994a, b). This ensures equilibration of extra and intracellular ion concentrations and well defined RBC extracellular and intracellular pH values (Berenbrink and Bridges, 1994a, b) and resulted in comparable RBC intracellular Hb and nucleotide triphosphate concentrations between HbI genotypes that were similar to values in fresh whole blood (Table 1; Leray, 1982). Extreme dilution of RBCs (Hct 0.08-0.13%) in buffered physiological saline ensured full control of RBC extracellular pH and ion composition during the actual OEC measurements and avoided the need for correction of points on the OECs to constant pH, which may otherwise vary by more than 0.1 pH units with oxygenation status in Atlantic cod whole blood *in vitro* (Herbert *et al*., 2006). Extreme dilution also avoided potential problems with RBC O2 consumption that may have been behind O2 contents of zero at *P*O2 values of 15 mmHg in OECs obtained at high Hct with a gasometric method (Gollock *et al*., 2006; Petersen and Gamperl, 2011). Full spectrophotometric assessment of RBC O2 saturation between 500 and 700 nm in the present study also avoided having to assume full RBC O2 saturation at some arbitrary high *P*O2 which may have led to a systematic overestimation of O2 saturation and affinity in some previous studies (Karpov and Novikov 1980; Gollock *et al*., 2006; Herbert *et al*., 2006; Petersen and Gamperl, 2011). Finally, 5-6 specimens per HbI genotype were used to reduce outlier effects in the interpretation of results. Together this makes the present study the most comprehensive test yet for HbI genotype differences in RBC O2 binding properties. The negative finding in this study raises the question what other characteristic(s), if any, of the different HbI alleles is behind the documented differences in geographical distribution, growth rates, hypoxia tolerance, and preference temperature (reviewed by Andersen, 2012; Ross *et al*., 2013)?

*Possible reasons for the variability of HbI genotype effects*

In theory, any potentially existing genetic differences in the intrinsic O2 binding characteristic between the Hb genotypes, or in their interactions with allosteric modulators such as organic phosphates, could have been masked in the present study by the large phenotypic plasticity in Hb O2 binding properties of ectotherms (Weber and Jensen, 1988). However, despite several attempts, the alleged large genotype effects reported for RBCs by Karpov and Novikov (1980) have been difficult to reproduce in haemolysates of the different genotypes in the presence of controlled levels of allosteric modifiers (e.g. in both the presence and the absence of ATP; Brix *et al*. 1998; Colosimo *et al*., 2003; Brix *et al.*, 2004) . This rather suggests that the differences found by Karpov and Novikov (1980) on the RBC level may have been due to phenotypic plasticity rather than Hb genotype, such as different levels of intracellular organic phosphates or different degrees of catecholamine stimulation. Unfortunately we do not have any information on RBC organic phosphate levels or treatments aimed at controlling catecholamine effects from the latter study. Thus, while there is evidence for effects of Hb genotype on Atlantic cod behaviour in thermal choice experiments (Petersen & Steffensen, 2003; Behrens et al., 2012), the present study shows that they are not necessarily due to differences in RBC oxygen affinity. These considerations are in line with , who have suggested that the adaptive value of the different Atlantic cod Hb genotypes on O2 supply rates in different environments may have been overemphasized.

As an alternative explanation, natural selection of HbI genotypes may act on different life history stages than the juveniles or adults that are most commonly studied. For example, unfertilised eggs of Atlantic cod have been shown to contain transcripts of all four major adult expressed globins, including the β1 globin responsible for the HbI polymorphism (Wetten *et al*., 2010). The functional relevance of these gene products, by necessity of maternal origin, is unclear and transcripts disappear upon fertilisation in the embryonic stages until expression is switched on again later in juveniles and adults (Wetten *et al*., 2010). However, if the maternal HbI genotype in eggs affects their fertilisation success, then this may explain the significantly skewed HbI genotype ratios in offspring of heterozygote parents that was observed by Gamperl *et al*. (2009) and was later in life balanced by significantly higher growth rates of the underrepresented genotype. Thus, differing costs and benefits during different life history stages and/or in different micro environments may lead to balanced HbI polymorphisms that differ in HbI 1 frequencies across the distributional range.

In addition, the HbI polymorphism may be genetically linked to other traits that are under selection, such as the regulatory polymorphism of the HbI promoter in Atlantic cod (Star *et al*., 2011; Andersen, 2012) that may be responsible for HbI genotype-associated differences in Hct and Hb concentration observed in some studies (Mork and Sundnes, 1984). Clearly our understanding of molecular mechanisms enabling adaptation of marine ectotherms to environmental temperature change is just at the beginning and more studies linking the genetics, physiology, ecology and evolution of these organisms are required.

*Concluding remarks on physiological consequence of Atlantic cod RBC O2 binding characteristics*

Atlantic cod are regularly exposed to acute temperature shifts in their natural environments, similar to those employed in the present study, e.g. during upwelling and turbulent mixing events of water bodies with different temperatures (Freitas *et al.* 2015), or when crossing the thermocline (Righton *et al.*, 2010). The latter is particularly relevant for Irish Sea cod that continue actively changing depth during the warmer summer months, compared to North Sea cod that remain confined in bottom waters throughout June to September (Righton *et al*., 2001; Righton and Metcalfe, 2002). Our modelling approach suggests that during acute warming the O2 binding characteristics of Atlantic cod RBC will enable uncompromised gill O2 loading at *in vivo* arterial *P*O2 values and at the same time permit increased O2 offloading at falling venous *P*O2. However, the theoretical maximal *S*a-v at physiological pH and arterial and venous *P*O2 does not increase with temperature (Figure 4 D-F), and is already reached under conditions of acute gradual warming to 20⁰C (Figure 4 C, F). Under these conditions Atlantic cod can only further increase the capacity of their circulatory O2 transport system by increasing blood O2 capacity and/or cardiac output. However, in a complex network of feedback systems an increase in cardiac output may itself be limited, firstly by low *P*O2 of cardiac luminal blood returning from systemic tissues, secondly by an increased cardiac workload and thus O2 demand imposed by the higher viscosity of blood with an increased RBC number, and lastly by O2-supply-independent physiological and anatomical limits to cardiac performance such as maximal heart rate and ventricle size, respectively. Ultimately, when all these avenues to increase blood O2 transport rate are exhausted, long term preservations of aerobic scope for activity at elevated temperature may rely on the extent to which standard metabolic rate can be reduced by thermal acclimatisation.

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**Competing interests**

The authors do not declare competing interests.

**Author contributions**

SLB, JM, DAR and MB conceived the project and interpreted the findings. SLB and MB designed the study. SLB executed the experiments and drafted the manuscript. SLB, DAR and MB revised the manuscript.

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**Table 1:** Summary of all captured and experimental animals. Number of Atlantic cod of each genotype captured and selected for further experiments, total lengths, and values for haematocrit (Hct), haemoglobin concentration ([Hb4]), mean cellular haemoglobin concentration (MCHC), and RBC ATP/Hb4 and GTP/Hb4 molar ratios in washed red blood cells (RBCs) immediately before experiments (mean values ± SEM., differing numbers of experimental individuals are indicated in brackets).

|  |  |  |  |
| --- | --- | --- | --- |
|  | **HbI 1/1** | **HbI 1/2** | **HbI 2/2** |
|  |  |  |  |
| Number of captured individuals | 48 | 43 | 15 |
| Total Length (cm) | 46.1 ± 0.7 (38) | 47.5 ± 0.8 (36) | 43.9 ± 1.2 (10) |
|  |  |  |  |
| Number of experimental individuals | 6 | 5 | 5 |
| Total Length (cm) | 43.8 ± 1.7 | 49.5 ± 3.3 | 46.0 ± 2.6 (4) |
| Properties of washed RBC cell suspensions |  |  |  |
| Hct (%) | 11.2 ± 0.63 | 10.8 ± 0.48 | 10.9 ± 0.64 |
| [Hb4] (mmol per litre RBC suspension) | 0.27 ± 0.01 | 0.27 ± 0.02 | 0.28 ± 0.01 |
| MCHC (mmol per litre RBC) | 2.43 ± 0.11 | 2.48 ± 0.08 | 2.62 ± 0.09 |
| ATP/Hb4 (mol/mol) | 1.39 ± 0.11 | 1.57 ± 0.18 | 1.27 ± 0.09 |
| GTP/Hb4 (mol/mol) | 0.80 ± 0.10 | 0.81 ± 0.08 | 0.76 ± 0.06 |

**Table 2:** Parameters ( ± SE) for 2nd order polynomial fits of log *P*50 or *n*H (*y*) as a functions of pH (*x*), according to *y* = *ax*2 + *bx* + *c*, of the individual data in Figures 1 and 2 with all genotypes pooled together (*N* = 16)

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Log [*P*50 (mmHg)]** | | |  |  | ***n*H** |  |
| ***t* (⁰C)** | *a* | *b* | *c* |  | *a* | *b* | *c* |
| 5.0 | 1.2 ± 0.2 | -20.2 ± 3.5 | 85.4 ± 13.4 |  | -2. 8 ± 0.8 | 45.6 ± 12.7 | -185.1 ± 49.3 |
| 12.5 | 1.1 ± 0.2 | -18.6 ± 3.4 | 78.2 ± 9.2 |  | -4.4 ± 0.7 | 69.8 ± 10.5 | -273. 5 ± 40.2 |
| 20.0 | 1.3 ± 0.1 | -21.5 ± 1.7 | 88.7 ± 6.6 |  | -3.8 ± 0.6 | 59.6 ± 8.8 | -230.4 ± 33.4 |

**Table 3:** Oxygen equilibrium curve properties, corrected for pH change with temperature, of Atlantic cod red blood cells, with all haemoglobin genotypes combined, when exposed to a range of temperatures and pH values; log *P*50, with *P*50 in mmHg and *n*H (co-operativity at 50 % saturation); mean ± SEM, *N* = 16. For each parameter different subscript letters within a row indicate significant differences (One-way ANOVA for log *P*50 and One-way ANOVA on ranks for *n*H).

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Log [*P*50 (mmHg)]** | | |  | ***n*H** | | |
| **pH** | 5.0°C | 12.5°C | 20.0°C |  | 5.0°C | 12.5°C | 20.0°C |
| 7.90 | 1.60 ± 0.01a | 1.61 ± 0.02a | 1.69 ± 0.01b |  | 1.79 ± 0.06a | 1.86 ± 0.09a | 1.71 ± 0.05a |
|  |  |  |  |  |  |  |  |
| 7.65 | 1.93 ± 0.02a | 1.89 ± 0.02a | 1.90 ± 0.01a |  | 1.19 ± 0.04a | 1.61± 0.05b | 1.68± 0.03b |
|  |  |  |  |  |  |  |  |
| 7.40 | 2.42 ± 0.04a | 2.31± 0.03b | 2.28 ± 0.02b |  | 0.25 ± 0.08a | 0.80± 0.04b | 1.18 ± 0.03c |



pH ‘7.40’

pH ‘7.65’

pH ‘7.90’

**Figure 1: Oxygen equilibrium curves of Atlantic cod RBCs with different HbI genotypes**. Data points are for 5.0, 12.5 and 20.0°C (blue, purple, red symbols and lines, respectively) and at nominal saline pH values of A) 7.90, B) 7.65 and C) 7.40. Circles indicate measured values while lines are based on sigmoidal curve fits for each temperature and HbI genotype (HbI 1/1, solid lines, filled symbols, *N* = 6; HbI 1/2, long-dashed lines, half-filled symbols, *N* = 5; HbI 2/2, short-dashed lines, open symbols, *N* = 5). For each individual 5 data points were obtained at each pH and temperature.



**Figure 2: Effect of pH, HbI genotype, and temperature on the affinity and cooperativity of O2 binding in Atlantic cod red blood cells.** Mean values ± SEM for A) log*P*50 versus pH for HbI 1/1 (closed symbols, solid lines *N* = 6), HbI 1/2 (semi-open symbols, long-dashed lines, *N* = 5) and HbI 2/2 (open symbols, short-dashed lines, *N* = 5), at 5.0, 12.5 and 20.0°C (blue, purple and red symbols and lines, respectively), and B) nH, Hill’s cooperativity coefficient at 50% RBC O2 saturation, for the same data as in *A*.



exotherm

endotherm

a

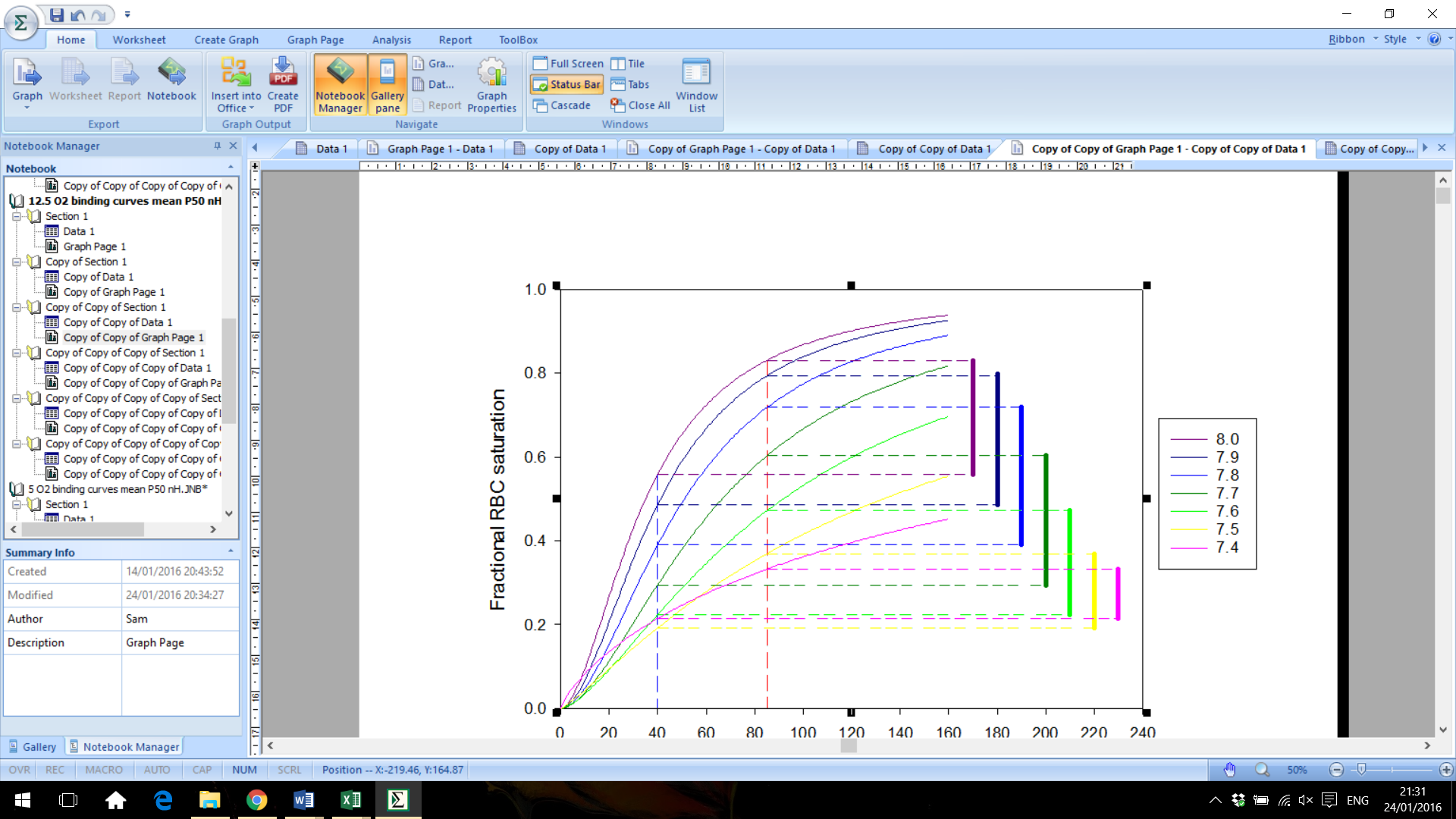
d

a

e

**Figure 3:** **Apparent heat of oxygenation, Δ*H*’, for Atlantic cod red blood cells**. Values between 5.0 and 12.5°C (blue lines and symbols) and 12.5 and 20.0°C (red lines and symbols) are shown at each reference pH (mean ± SEM, *N* = 16). Note reversal of y axis, with negative values denoting an exothermic reaction at top. Differing letters within a temperature interval or at constant pH indicate significantly different Δ*H*’ values (two-way ANOVA), Ψ indicates values not significantly different from zero (one-sample *t*-test).



pH

**Figure 4:** **Modelled RBC O2 equilibrium curves and arterial-venous O2 saturation differences in Atlantic cod at different values for pH and temperature**. OECs are shown for series of standardised pH values (see inset) and temperatures of 5.0 (A, D), 12.5 (B, E), and 20.0°C (C, F). Red dashed vertical line indicates routine arterial *P*O2 values, *P*aO2. Blue dashed vertical line indicates either resting mixed venous *P*O2 values (*P*O2, A-C), or minimally observed mixed venous *P*O2 values (*P*O2min, D-F), respectively (see Methods). Corresponding arterial and venous O2 saturations, *S*aO2 and *S*vO2, and their difference, *S*a-v, are indicated for each pH by colour-matched horizontal dashed lines and vertical bars, respectively. Due to pH shifts with temperature in the underlying data set (Figure 2) OECs have not been modelled for pH 8.0 at 20.0° and pH 7.4 at 5.0°C.