Title: ATP-induced reversed thermal sensitivity of O₂ binding in both major hemoglobin polymorphs of the non-endothermic Atlantic cod, *Gadus morhua*.

Running title: Thermal adaptation of Atlantic cod hemoglobins.

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Summary statement: Phenotypic plasticity allows both major hemoglobin polymorphs of non-endothermic Atlantic cod to show ATP-induced reversed thermal sensitivity of oxygen binding at their warming southern distribution limit in the North East Atlantic.
Abstract

Atlantic cod is a species affected by climate change with a major polymorphic hemoglobin component (HbI) whose two polymorphs show an inverse change in frequency along a latitudinal temperature cline in the North East Atlantic, and that have been associated with differences in performance and behavioural traits. An earlier study at the northern distribution limit of the species reported differential temperature sensitivities of red blood cell oxygen ($O_2$) affinity between the northern cold-water HbI-2 polymorph and its southern, warm-water HbI-1 counter-part, which has since widely been held as adaptive for the species across its distributional range. The present study critically re-examined this hypothesis by comparing the thermal sensitivity of $O_2$ binding in both purified HbI polymorphs from the southern, high temperature distribution limit of the species under controlled conditions of allosteric modifiers of Hb function. Contrary to the prevailing view the $O_2$-affinity of the major HbI polymorphs did not differ from each other under any of the tested conditions. Depending on pH and ATP concentration, the temperature-sensitive and the temperature-insensitive Hb-$O_2$ affinity phenotypes -previously exclusively ascribed to the HbI-1 and HbI-2, respectively- could be induced in both HbI polymorphs. These results are the first to establish a molecular mechanism behind a reversed temperature-dependence of red blood cell $O_2$ affinity in an non-endotherm fish and lay the basis for future studies on alternative mechanisms behind the differences in distribution, performance, and behavioural traits associated with the different HbI polymorphs of Atlantic cod.
**Introduction**

Environmental temperature has been referred to as the ecological master factor that profoundly affects the life of all ectotherms, where high, stable body temperatures independent of the environment cannot be achieved by metabolic means (Brett, 1971; Brown et al., 2004; Ross et al., 2013). This particularly applies to aquatic ectotherms such as fishes, where the large volume and heat capacity of water ventilated across their gills tends to equilibrate body temperature with that of the environment (Fry, 1967). Thus, with the notable exception of some large-bodied partially endothermic teleosts and sharks with heat-conserving vascular counter-current exchangers that keep selected tissues above ambient temperatures (Carey and Teal, 1969a, 1969b; Block et al., 1993; Patterson, Sepulveda and Bernal, 2011; Wegner et al., 2015), all other groups of fishes typically experience body temperatures very similar to that of the ambient water (Simpson, 1908; Clausen, 1934; Gunn, 1942; Fry, 1967).

Increases in environmental water temperatures and consequently body temperatures of ectothermic fishes generally lead to exponential increases in the rates at which biochemical processes proceed and hence to exponentially increased overall metabolic rates and associated oxygen (O₂) demands (Brett, 1971; Fry, 1947, 1967; Fry and Hart, 1948; Tirsgaard et al., 2015). Yet the anatomical and physiological capacities of their O₂ supply systems for fuelling energy efficient aerobic metabolism are more finite and may even decrease at higher temperatures (Brett, 1971; Gollock et al., 2006; Eliason et al., 2011). Thus, the aerobic scope of activity (AS), the part of the maximal metabolic rate (MMR) that can be sustained above the maintenance or standard metabolic rate (SMR), eventually decreases at high enough environmental temperatures in many aquatic ectotherms and this is thought to limit the overall fitness, performance and abundance of these species at the higher end of their thermal niche (Fry, 1947, 1967; Claireaux et al., 2000; Gollock et al., 2006; Pörtner and Knust, 2007; Eliason et al., 2011). Conversely, at the lower temperature end of the thermal niche the exponentially reduced rates of biochemical processes, rather than the capacity of the cardio-respiratory O₂ supply system, can be expected to become limiting, such that very different selection pressures may be expected to operate on the steps of the O₂ supply cascade in populations at the warm and cold limits of a species’ geographical distribution.

Atlantic cod, *Gadus morhua*, is a commercially exploited aquatic ectotherm with a broad latitudinal distribution along the continental shelves of the western and eastern North Atlantic and up into the Arctic, occurring in waters between -1.5 and +20 °C (Drinkwater, 2005; Righton et al., 2010). Within this total thermal range of the species, stocks at different latitudes in the North East Atlantic show more limited thermal niches, encompassing, for example, -1.5 to 11.7 °C in the Barents Sea and 2.3 to 19.5 °C in the southern North Sea, the most northern and southern stocks, respectively, that have been investigated (Righton et al., 2010). The species is already considered to be impacted by climate change; based on the thermal sensitivities of life history parameters of Atlantic cod stocks across their current distributional range, it has been suggested that an average water temperature
increase of just 1 °C will lead to the collapse of several of the southernmost stocks, whereas the
abundance of the northernmost stocks is predicted to increase (Drinkwater, 2005). In the North Sea,
warming sea water temperatures as a result of climate change have been associated with distributional
shifts in this and other species to increasingly northern, cooler waters (Perry et al., 2005), an effect
that may have been exacerbated by fishing pressures (Engelhard, Righton and Pinnegar, 2014). The
projected shrinkage of Atlantic cod habitats due to warming at their southern distribution limits may
be further amplified by the increased occurrence of O₂ depleted zones (Deutsch et al., 2015) and by
the effects of simultaneous ocean acidification on the early life history stages of this species (Frommel
et al., 2011).

Concerns about the fate of aquatic ectotherms such as Atlantic cod in an age of climate
change has sparked renewed interest in the underlying mechanisms that allow some species or
populations to tolerate higher temperatures than others (Pörtner, 2001; Pörtner and Farrell, 2008;
Eliason et al., 2011; Anttila et al., 2013). One of the few known and most frequently discussed genetic
differences between southern, warm-water and northern, cold-water stocks of Atlantic cod that
involves a component of the cardio-respiratory O₂ supply cascade is the polymorphism of the major
hemoglobin (Hb) type (HbI) expressed in the red blood cells (RBCs) of juvenile and adult Atlantic
cod (reviewed by Andersen, 2012; Ross et al., 2013). Searching for selectively neutral genetic
markers of population structure of North East Atlantic fish species and using agar gel electrophoresis,
Sick (1961) described a minor (HbII) and a polymorphic major (Hbl) Hb component in blood of
Atlantic cod. The three major electrophoretic Hbl phenotypes were hypothesised to result from the
presence of two codominant alleles named Hbl₁ and Hbl₂, which in the homozygous genotypes
Hbl₁/Hbl₁ and Hbl₂/Hbl₂ yielded, respectively, the cathodic Hbl-1 type and the less cathodic Hbl-2
type, and resulted in double bands of type Hbl-1-2 in the heterozygous Hbl₁/Hbl₂ genotype (Sick,
1961). Subsequent studies revealed a strong inverse latitudinal cline in the frequencies of the Hbl₁ and
Hbl₂ alleles in the North East Atlantic, with Hbl₁ allele frequencies up to 0.73 in the southern North
Sea at long term (1960 - 2010) mean annual bottom temperatures around 12 °C, and Hbl₁ frequencies
below 0.20 in the White Sea at mean annual bottom temperatures of 2 °C (summarised in Ross et al.,
2013). The molecular basis of the Hbl polymorphism was eventually revealed (Andersen et al., 2009;
Borza et al., 2009), showing that Hbl-1and Hbl-2 proteins contained the same α-globin chain, but that
the Hbl-1 type contained a β₁-globin variant with methionine and lysine at positions 55 and 62 in the
polypeptide chain (β₁ Met55-Lys62), whereas the Hbl-2 type contained the β₁-globin variant with
valine and alanine at the respective positions (β₁ Val55-Ala62). These and subsequent studies
confirmed the strong coupling between the identities of respective amino acids in positions 55 and 62 of the two β1 globin variants across populations in the North Atlantic (Andersen et al., 2009;
Borza et al., 2009; Star et al., 2011; Wetten et al., 2012). The presence of the positively charged
Lys62 side chain instead of neutral Ala62 in the Hbl-1 type is consistent with the more cathodic (less
negatively charged) nature of the Hbl-1 compared to the Hbl-2 polymorph during agar gel
electrophoresis at alkaline pH that was first observed by Sick (1961). Due to the association between allele frequency and environmental temperature, Atlantic cod homozygous for Hbl with the Hbl-1 polymorph in their RBCs have been termed the warm-water type, and those homozygous for Hbl with the Hbl-2 polymorph in their RBCs the cold-water type (Ross et al., 2013).

Based on a preliminary study and apparently using single individuals, Karpov and Novikov, (1980) reported functional differences in the in vitro O2 binding properties of RBCs from White Sea Atlantic cod with the 3 Hbl phenotypes. They found that at a fixed buffer pH of 7.5, RBCs with the cold-water type Hbl-2 showed strong temperature dependence of Hb-O2 affinity, whereas Hb-O2 affinity was scarcely affected by temperatures between 0 and 20 °C in RBCs containing the warm-water type Hbl-1. RBCs with the heterozygous Hbl-1-2 type showed an intermediate thermal dependence of Hb-O2 affinity. This resulted in higher Hb-O2 affinities in RBCs of the cold-water type Hbl-2 in the colder temperature range (below ca. 12 °C), and higher Hb-O2 affinities of the warm-water type Hbl-1 in the warmer temperature range (above ca. 12 °C). The change in Hbl allele frequency across the distributional range of Atlantic cod was interpreted as an adaptation for efficient blood O2 transport at the warmer and cooler edges of the thermal range (Karpov and Novikov, 1980).

Significant associations between Atlantic cod Hbl-type and attributes of whole organism performance such as the thermal dependence of growth rates, hypoxia tolerance, temperature preference, and competitive behaviour have since been reported in several studies (for review see Andersen, 2012; Ross et al., 2013), yet it has proven difficult to link these associations mechanistically to the functional differences in Hb-O2 affinity ascribed to the polymorphic Hbl-types by Karpov and Novikov (1980) (e.g.: Colosimo et al., 2003; Gamperl et al., 2009). Moreover, attempts to repeat the findings on RBC-O2 affinity by Karpov and Novikov (1980) on the purified Hbl polymorphs from other cod stocks under defined levels of known intracellular allosteric modifiers of Hb function in fishes, such as pH, ATP and chloride, did not succeed in reproducing the clear-cut functional differences of the original study (Brix et al., 1998; Pörtner et al., 2001; Colosimo et al., 2003; Brix et al., 2004). More recently, Barlow et al. (2017) found statistically indistinguishable RBC O2-binding affinities and pH and temperature sensitivities between the Hbl polymorphs of Atlantic cod from the Irish Sea at the warm, southern edge of the species’ distribution range. Barlow et al. (2017) suggested that phenotypic plasticity rather than the genetic differences between the Hbl types may have caused the functional differences in RBC-O2 affinities reported by Karpov and Novikov (1980) and provided a number of alternative hypotheses for the significant associations between whole organism performance attributes and Hbl types. Phenotypic differences in RBC-O2 affinity of Atlantic cod may result from differences in the degree of catecholamine-stimulated intracellular pH regulation via the RBC sodium-hydrogen exchanger that has evolved in Atlantic cod and many other teleost fishes (Berenbrink and Bridges, 1994b; Berenbrink et al., 2005), or from ATP depletion during prolonged incubation of RBCs in the absence of glucose, an important substrate for Atlantic cod RBCs (Driedzic et al., 2014). It is well known that strong interactions between Hb and allosteric
modifiers of O$_2$-binding such as hydrogen ions, ATP, or chloride can compensate for the universal exothermic nature of haem-O$_2$ binding and result in temperature insensitive or even reverse temperature-sensitive, endothermic overall Hb-O$_2$ binding (for review: Powers, 1980; Weber and Campbell, 2011). This is best known for several of the active, large-bodied, partially endothermic teleost and sharks mentioned above, where the O$_2$ affinity of whole blood, purified hemolysates or Hb components (measured as $P_{50}$, the partial pressure of O$_2$ ($PO_2$) at half-saturation) may either be little affected or even increase with increasing temperatures [e.g. bluefin tuna (Rossi-Fanelli et al. 1960; Ikeda-Saito et al., 1983), lamnid sharks (Andersen et al., 1973; Larsen et al., 2003), and several species of billfishes (Weber et al., 2010). Partial endothermy is thought to have independently evolved in the three groups (Block et al., 1993) and it has been suggested that the reduced or reversed temperature sensitivity of Hb-O$_2$ affinity has each time convergently evolved as an adaptation against the problems associated with a pre-mature release of Hb-bound O$_2$ when cold arterial blood from the gills is rapidly warmed in the counter-current vascular heat exchangers of these animals (Larsen and Malte, 2003; Clark et al., 2008; Weber and Campbell, 2011). According to Weber and Campbell (2011) this is supported by apparent differences in the contributions of the heats of ATP-binding versus hydrogen ion-binding in the underlying molecular mechanisms of thermal insensitivity, with a major role ascribed to proton binding in bluefin tuna and to ATP binding in billfishes and the lamnid porbeagle shark. However, as also pointed out by these authors, the effect of natural organic phosphates on the thermal sensitivity of O$_2$ binding in tuna Hb never seems to have been assessed (Weber and Campbell, 2011). In addition, reversed thermal sensitivity of blood or RBC-O$_2$ affinity has now also been reported for two non-endothermic teleost species, the chub mackerel Scomber japonicus and Atlantic cod, suggesting that this phenomenon may be more widespread than previously assumed and be favoured by natural selection under conditions other than partial endothermy (Clark et al., 2010; Barlow et al., 2017), calling for closer investigations of the underlying mechanism(s).

Thus, the present study explored the hypothesis that differences in the temperature-dependence of RBC-O$_2$ affinity that were ascribed to the different Atlantic cod HbI phenotypes by Karpov and Novikov (1980), may equally be caused by differences in the levels of allosteric modifiers of Hb-O$_2$ binding as opposed to through the genetic differences ($\beta_1$ Met55-Lys62 versus $\beta_1$ Ala55-Val62) in HbI structure of Atlantic cod. We were further interested in characterising the molecular mechanism behind the unusual, reversed temperature dependence of RBC-O$_2$ affinity in the first non-endothermic fish. The overall aim was to provide a critical and comprehensive characterisation of the O$_2$-equilibrium curves of purified HbI-1 and HbI-2 polymorphs from Atlantic cod, including an assessment of their sensitivities to temperature at defined levels of the physiological allosteric modifiers; hydrogen ions, ATP and chloride. More specifically, we hypothesised that both HbI polymorphs would show identical Hb-O$_2$ equilibrium curves under identical conditions of temperature and allosteric modifier concentrations. We further predicted that the thermal sensitivity
of Hb-O₂ binding in both Hbl types would change from an overall exothermic oxygenation reaction under reduced levels of allosteric modifiers to thermo-neutral or even endothermic oxygenation reaction at higher levels of allosteric modifiers, mimicking quantitatively the alleged genetic differences in Hb-O₂ binding between RBCs of the warm- and cold-water type Hbl polymorphs of Atlantic cod.
**Materials and Methods**

**Animals**

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 between 13/01 and 01/03/2015 using hook and line aboard commercial fishing vessels from the mouth of the River Mersey in the Irish Sea (53° 25’ North, 3.02° 1’ East) as part of a previous study (Barlow et al., 2017). Animals experience sea surface temperatures between 6 and 8 °C at this time of year and, based on their lengths, consisted of a mix of immature and mature 2-year-olds (Fox et al., 2005). Animals were killed immediately on board according to a British Home Office approved Schedule 1 method, involving concussion and destruction of the brain, and 3 to 5 mL of blood was collected from caudal vessels using 1 mL heparinized syringes (ca. 20 μL 9.000 I. U. mL⁻¹ sodium heparin from porcine intestinal mucosa, Sigma-Aldrich). Samples were then kept on ice until landing (maximum 10 hours).

**Identification and purification of HbI polymorphs**

On arrival at the laboratory a portion of RBCs from each individual was isolated from plasma using centrifugation (3 000 rcf, 4°C, 4 minutes), before 20 μL of RBC pellet was lysed through addition of 64 μL cold, distilled water and vigorous mixing. Individual HbI polymorphs were identified in the resulting hemolysates using horizontal 1% agarose gel electrophoresis at pH 8.8, as previously described (Barlow et al., 2017; modified from: Sick, 1961; Fig. 1) and the remainder of the blood samples frozen at -80°C until further processing. We had previously established that the 3 different HbI phenotypes of the 16 individuals studied by Barlow et al. (2017) from the same study population were strictly associated with the expected linked 55Val-62Ala and 55Met-62Lys polymorphism of the β₁ gene established by Andersen et al. (2009) and Borza et al. (2009), by direct sequencing of the relevant portions of exon 2 in PCR products amplified from genomic DNA of these individuals (S. L. Barlow, unpublished information). Five individuals each of the HbI-1 and HbI-2 polymorphs with the inferred homozygous genotypes HbI¹/HbI¹ and HbI²/HbI², respectively, and matching average total lengths (45.8 ± 2.2 and 43.0 ± 1.8 cm, *p* = 0.366, *t*-Test) were selected for purification. The selected samples were thawed on ice and centrifuged to remove cell debris (21 000 rcf, 4 °C, 10 minutes). Supernatants were stripped of organic phosphate modulators and other small molecular weight components by repeated (3 times) gel filtration on Sephadex G-25 (PD10 desalting columns, GE Healthcare; Berenbrink et al., 2005) equilibrated with ice-cold 10 mM HEPES and 300 mM NaCl buffer solution (pH 8.0 at 15 °C; Brix et al., 1998) and then stored at -80 °C in 100 μL aliquots. Gel filtration was performed 3 times for maximum removal of organic phosphate modifiers as the desalting capacity of a single passage through a PD10 column may be only 98% according to the manufacturer’s application notes (GE Health Care). As visual inspection of Hb bands indicated the presence of equal to or greater than 90% HbI (Fig. 1), further due to the reported fragility of Atlantic cod Hbs (Sick, 1961; Brix et al., 1998) difficulties in their isolation (Verde et al., 2006), and in order to facilitate comparisons with
previous studies that were almost exclusively performed on un fractionated Hbs in blood, isolated red
blood cells, or hemolysates of this species, no further purification of HbI polymorphs was attempted.

O2 equilibrium curve analyses

For the analysis of O2 equilibrium curves, 50 to 100 μL of freshly thawed, purified hemolysate solution
was mixed with 1 mL of 100 mM HEPES/100 mM NaCl buffer (adjusted to pH 7.0, 7.5, or 8.0 at 15
°C), with the addition of 0 to 100 μL of ATP stock solution (100 mM in water, ATP di-sodium salt,
Sigma-Aldrich A2383), yielding final tetrameric Hb concentrations ([Hb]) between 2.6 and 8.7 μM
and final ATP concentrations between 0 and 9 mM. [Hb] was determined in fully oxygenated samples
using the extinction coefficient for oxygenated Hb at 540 nm of 15.3 mM⁻¹ cm⁻¹ (on a heme basis;
(Benesch et al., 1973). Chloride concentration was varied by mixing 50 to 100 μL of purified hemolysate
(containing 300 mM chloride) with 1 mL of 100 mM HEPES buffer pH 7.0 containing 0, 50 or 100 mM
chloride, yielding final chloride concentrations between 14 and 118 mM. No attempt was made to create
nominally chloride-free conditions because of the reported instability of Atlantic cod Hb at low chloride
concentrations (Brix et al., 1998). The pH of the final hemolysates was determined at each temperature
using a Lazar Model FTPH-2S pH electrode with a Jenco 6230N meter (Jenco Collaborative, CA,
USA). Within treatment variations of pH were below 0.01 pH units, presumably due to the high
concentration and volume of the HEPES buffer relative to the Hb samples in the diluted hemolysates,
and thus results for a given treatment are displayed for a single pH value to the nearest 0.01 pH unit.

The diluted hemolysates were equilibrated with fully humidified pre-determined gas mixtures
of O2 or air with N2 provided by Wösthoff gas mixing pumps (Wösthoff GmbH, Bochum, Germany) in
modified Eschweiler tonometers (50 mL capacity, Eschweiler GmbH, Engelsdorf, Germany) for at least
20 minutes at temperatures of 5.0, 12.5 or 20.0 °C. The tonometers were modified after the design of
Brix et al. (1998) that contained a gas flow-through system that could be shut off after equilibration and
incorporated a 1 cm-pathlength optical glass cuvette that allowed spectrophotometric Hb-O2 saturation
analysis with minimal disruption of the sample (Brix et al., 1998). O2 saturation of hemolysates was
determined by spectral deconvolution of hemolysate spectra between 500 and 700 nm (Unicam UV 500
spectrophotometer, Thermo Electron Corporation, OH, USA; with Vision 32 software) as described by
Völkel and Berenbrink, (2000) and modified by Barlow et al. (2017). Briefly, the relative contributions
of Hb derivatives (oxygenated, HbO2; deoxygenated, Hb; acid and alkaline metHb, Hb+; Fig. 2A) to the
optical signal at each wavelength was determined under all conditions tested using an iterative non-
linear curve-fit algorithm in SigmaPlot 13 (Systat Software Inc., San Jose, CA, USA). An overlay of
the predicted total sum of all Hb components and the original spectrum was plotted to enable visual
confirmation of the accuracy of the fit (Fig. 2B).

The fractional Hb-O2 saturation \( Y = [\text{HbO}_2] / ([\text{HbO}_2] + [\text{Hb}]) \) was calculated and Hill plots of
\( \log_{10}(Y / (1 - Y)) \) against \( \log_{10}P_{O2} \) were constructed, generally including data in the linear mid portion
of the Hill-plot between 10 – 90% O2 saturation (within the bracket of the reference lines in Fig. 3). Using linear regression of the Hill plot data, \( \log_{10}P_{50} \) was obtained as \( \log_{10}P_{O2} \) for \( \log_{10}(Y(1/Y)) = 0 \),
and the cooperativity of Hb-O₂ binding \( (n_H) \) was obtained as the slope of the regression line. MetHb was calculated as the percentage contribution of the sum of acid and alkaline metHb to the total sum of Hb derivatives and ranged from < 0.1% (Fig. 2B) up to 10% in some cases. Visual inspection of Hill-plots suggested that the varying levels of metHb did not affect the affinity and cooperativity of O₂ binding of functional Hb in this concentration range.

Bohr plots of \( \log_{10}P_{50} \) against pH were used to determine Bohr factors \( \varphi = -\Delta \log_{10}P_{50}/\Delta \mathrm{pH} \) and a curved, 2nd order polynomial was used to interpolate \( \log_{10}P_{50} \) values to fixed pH to allow the effect of temperature to be analysed (e.g. Weber et al., 2010; Barlow et al., 2017). The overall enthalpy change of Hb oxygenation (\( \Delta H' \)) was calculated separately for the temperature intervals 5.0-12.5 °C and 12.5-20.0 °C for a series of fixed pH values from the slope of Van ’t Hoff plots of \( \log_{10}P_{50} \) against the inverse of the absolute temperature (\( T \)): \( \Delta H' = 2.303 \, R \, [\Delta \log_{10}P_{50}/\Delta (1/1/T)] \), where \( R \) is the universal gas constant (8.314 J / (mol K)). All values for \( \Delta H' \) calculated in the present study or reported from the literature included the heat of O₂ solubilization (-14.0 kJ / mol, at 15 °C; Olofsson et al. (1984)). Literature values in units of kcal were converted to kJ using the factor 4.184 kJ / kcal.

**Statistics**

All data are displayed as raw data or mean values ± 1 standard error of the mean (s.e.m.). Statistical analyses were performed in SigmaPlot 13. Differences between two groups were evaluated using the \( t \)-test and between more than two groups using analysis of variance (ANOVA) or analysis of co-variance (ANCOVA) as indicated in the text. Prior to testing, data were checked for meeting the normality (Shapiro-Wilk test) and equal variance (Brown-Forsythe test) assumptions and where these tests failed, data were transformed using \( x' = \sqrt{x} \), \( x' = \sqrt{x + 2} \), or \( x' = 2^x \), as indicated. Where relevant, post-hoc multiple comparison tests (Holm-Sidak) were employed to investigate significant differences between groups. Significant effects were recognised at \( p < 0.05 \). Hyperbolic curve fits of the dose response curves relating the ATP:Hb₄ ratio \( (x) \) to \( \log_{10}P_{50} \ (y) \) of the different Hbl genotypes were of the form: \( y = a + b \, x / (c + x) \), where \( a \) is \( \log_{10}P_{50} \) in the absence of ATP, \( b \) is the maximal increase in \( \log_{10}P_{50} \), and \( c \) is the ATP concentration at half-maximal increase of \( \log_{10}P_{50} \).
### Results

Purified hemolysates from individual Atlantic cod with either the HbI-1 or HbI-2 polymorph showed generally very similar O₂-binding characteristics, as revealed by the analysis of 108 linearized O₂ equilibrium curves in Hill-plots (Fig. 3). This was observed across three levels of pH, at three different temperatures and in the presence and absence of an excess of ATP (Fig. 3A-F). Decreases in pH caused consistent decreases in Hb-O₂ affinity, that is: the Bohr effect, at all temperatures and in the presence and absence of ATP, as indicated by the rightward shifts of the linearized O₂-equilibrium curves towards higher PO₂ values (Fig. 3). In the presence of ATP, the Root effect was observed at the lowest value of pH, as indicated by a decrease of the slope of the Hill-plots to values below unity and by Hb-O₂ saturations below 50% at the PO₂ of air (log₁₀([HbO₂])/[Hb]) values below zero and log₁₀PO₂ values of ca. 2.2, respectively; Fig. 3A-C). Figure 4 shows as a function of pH the individual values for log₁₀P₅₀ and Hill’s cooperativity constant (n_H) that were extracted from these Hill-plots as measures for the affinity and cooperativity of Hb-O₂ binding, respectively (Fig. 4A, B). Comparison of grouped log₁₀P₅₀ values, according to treatment and Hb-type, initially revealed highly significant differences in Hb-O₂ affinity (p < 0.001, one-way ANOVA on square-root transformed data to meet the normality test assumption). However, post-hoc all-pairwise comparisons revealed that none of the significant differences in log₁₀P₅₀ occurred between the two HbI polymorphs under any experimental conditions (p > 0.05, Holm-Sidak multiple comparison method). A similar analysis of n_H values revealed highly significant differences between groups (p < 0.001, one-way ANOVA on untransformed data), yet again, all-pairwise post-hoc comparisons failed to demonstrate any significant differences in the cooperativity of Hb-O₂ binding between the two HbI polymorphs across all experimental conditions (p > 0.05, Holm-Sidak multiple comparison method). Given the statistically indistinguishable Hb-O₂ binding characteristics under the complete set of experimental conditions, data from the two HbI polymorphs were combined for all further statistical analyses.

The pH values of the buffered hemolysates varied with temperature and the presence of ATP (Fig. 3A-F). To account for this variation during the assessment of the thermal sensitivity of Hb-O₂ binding, a series of covariance analyses was carried out on the combined data set in the presence and absence of ATP, with temperature as a factor, log₁₀P₅₀ or n_H as dependent variables, and pH as a covariate.

In the absence of ATP, there was no significant interaction between the effects of temperature and pH on log₁₀P₅₀ (p = 0.817, ANCOVA), with both parameters exerting highly significant effects on Hb-O₂ affinity (p < 0.001 in both cases). The lack of significant interaction indicated statistically indistinguishable slopes of the linear regression lines relating changes in log₁₀P₅₀ to changes in the covariate pH at each temperature, which is equivalent to a temperature-independent magnitude of the Bohr effect $\varphi = -\Delta$log₁₀P₅₀ (ΔpH)^{-1} of 0.607 (Fig. 4A, -ATP). All pairwise comparisons revealed highly significant increases of the elevations of these linear regression lines with each step increase in temperature according to the following regression equations:
\[ \log_{10}P_{50} (5.0 \, ^\circ C) = 5.721 - 0.607 \, \text{pH} \]  
(Eq. 1)

\[ \log_{10}P_{50} (12.5 \, ^\circ C) = 5.851 - 0.607 \, \text{pH} \]  
(Eq. 2)

\[ \log_{10}P_{50} (20.0 \, ^\circ C) = 5.952 - 0.607 \, \text{pH} \]  
(Eq. 3)

A similar analysis of \( m_1 \) values revealed that in the absence of ATP there was no interaction between the factor temperature and the covariate pH in their effects on Hb-O\( _2 \) binding cooperativity (\( p = 0.500, \) ANCOVA). Neither changes in temperature or pH significantly affected \( m_1 \) (\( p = 0.715 \) and 0.532, respectively), which showed an overall average value of 1.34 ± 0.02 (Fig. 4B, -ATP).

However, in the presence of ATP, there was a highly significant interaction between the factor temperature and the covariate pH in their effects on \( \log_{10}P_{50} \) (\( p < 0.001, \) ANCOVA on \( x^\prime = 2^\prime \)-transformed data to meet test assumptions of normality and equal variance). This was equivalent to a highly significant difference in the slopes of the linear regression lines relating the transformed \( \log_{10}P_{50} \) values to changes in pH at the different temperatures. Tab. 1 gives the magnitude of the Bohr effect in the presence of ATP as a function of temperature as calculated from paired observations at neighbouring pH values in Fig. 3A.

The Bohr effect magnitude under these conditions was significantly affected by both temperature and pH range, with a significant interaction between these two parameters (\( p < 0.001 \) in all three cases, ANOVA on \( x^\prime = \text{square-root} (x + 2) \)-transformed data). The influence of pH on \( \log_{10}P_{50} \) was strongest at 20 \( ^\circ \)C with statistically indistinguishable Bohr effect magnitudes (\( \phi \)) of 1.24 and 1.17 over the upper and lower pH interval, respectively (Tab. 1). At lower temperatures \( \phi \) generally decreased, but more so over the lower pH range, where the values at 12.5 and 5.0 \( ^\circ \)C were both significantly lower than at 20 \( ^\circ \)C and did not significantly differ from each other. Over the upper pH range mean values for \( \phi \) decreased more gradually with decreasing temperature and became only significantly different from the value at 20 \( ^\circ \)C at 5 \( ^\circ \)C (Tab. 1). As a result of the interaction between temperature and pH in their effects on \( \log_{10}P_{50} \), Hb-O\( _2 \) affinity below about pH 7.0 showed the classical decrease with increasing temperature, as also observed in the absence of ATP; however, in the presence of ATP and above about pH 7.2, Hb-O\( _2 \) affinity showed a reversed temperature sensitivity, with increases in temperature causing an increase in Hb-O\( _2 \) affinity (Fig. 4A).

These relations caused a pH-independent overall exothermic nature of Hb oxygenation with an enthalpy of ca. -25 kJ mol\(^{-1}\) over both temperature intervals in the absence of ATP, whereas in the presence of ATP there was a pH-sensitive transition from an overall exothermic Hb oxygenation at pH 6.5 (\( \Delta H^\circ \) of -31 and -45 kJ mol\(^{-1}\) between 5.0 - 12.5 \( ^\circ \)C and 12.5 - 20.0 \( ^\circ \)C, respectively) to temperature-independent Hb oxygenation around about pH 7.0 and 7.2 (\( \Delta H^\circ \) close to 0 kJ mol\(^{-1}\)) and endothermic Hb oxygenation at pH 7.6 (\( \Delta H^\circ \) of +18 and +13 kJ mol\(^{-1}\) between 5.0 - 12.5 \( ^\circ \)C and 12.5 - 20.0 \( ^\circ \)C, respectively; Fig. 4C).

In the presence of ATP there was further a highly significant interaction between the factor temperature and the covariate pH in their effects on the cooperativity of Hb-O\( _2 \) binding (\( p < 0.001, \) ANCOVA).
ANCOVA). This was equivalent to highly significant differences between the slopes of the three regression equations that relate changes in pH to changes in \( n_H \) (Fig. 2B, +ATP):

\[
\begin{align*}
\text{Eq. 4} & : n_H (5.0 \degree C) = -3.369 + 0.597 \text{pH} \\
\text{Eq. 5} & : n_H (12.5 \degree C) = -3.625 + 0.661 \text{pH} \\
\text{Eq. 6} & : n_H (20.0 \degree C) = -5.690 + 0.977 \text{pH}
\end{align*}
\]

Thus, the decrease in Hb\( \cdot \)O\(_2\) binding cooperativity upon lowering pH was steepest at 20.0 °C, followed by 12.5 °C and then 5.0 °C (Eqs. 4-6). Values for \( n_H \) in the presence of ATP were generally between 1.0 and 2.0 over the higher ranges of pH and decreased below unity at the lowest pH value for each temperature (Fig. 2B, +ATP), indicating the presence of the Root effect.

A dose response curve confirmed that ATP was exerting its maximal effect on \( \log_{10} P_{50} \) at the ATP:Hb\(_4\) ratios used in the experiments in Figs. 1 and 2, (9 mM ATP and 2.6 - 8.7 \( \mu \)M Hb\(_4\), yielding ratios of 1 000 to 3 500 mol/mol; Fig. 5). This was the case for both major HbI polymorphs, which also required similar ATP:Hb\(_4\) ratios of around 20 mol/mol for eliciting half-maximal effect on \( \log_{10} P_{50} \) (Fig. 5), corresponding to ATP concentrations of 0.1 mM for both Hbl polymorphs (data not shown). Changes in chloride concentration between 14 and 118 mM and in the absence of ATP had little effect on Hb\( \cdot \)O\(_2\) affinity (Fig. 6). An analysis of covariance with Hb-type as factor, \( \log_{10} P_{50} \) as the dependent variable, and chloride concentration as a co-variate did not reveal any interaction between the effects of Hb-type and chloride (ANCOVA, \( p > 0.05 \)) and both parameters did not significantly affect \( \log_{10} P_{50} \) (\( p > 0.05 \) in both cases).
Discussion

The results of the present study show that the strong functional differences in RBC-O\textsubscript{2} affinity between Atlantic cod HBI polymorphs described for the White Sea (Karpov and Novikov, 1980) and that have been widely held to be of genetic origin and to reflect environmental adaptation across the entire range of the species (reviewed by Andersen, 2012; Ross et al., 2013), can be produced by merely adjusting the concentrations of intracellular allosteric modifiers in purified solutions of Hb from Irish Sea Atlantic cod of both HBI polymorphs. Moreover, the affinity and cooperativity of Hb-O\textsubscript{2} binding was statistically indistinguishable between the two HBI polymorphs from the Irish Sea under the entire set of experimental conditions employed in the present study. Both HBI polymorphs showed O\textsubscript{2} binding characteristics that were very similar to those found in a previous study on Atlantic cod Hb that comprehensively investigated the effects of organic phosphate modulators and pH on O\textsubscript{2} binding equilibria in the purified major Hb of adult animals of unidentified HBI genotype, but off the coast of Greenland or Norway (Verde et al., 2006). With a P\textsubscript{50} value of ca. 100 mmHg, a cooperativity constant of nH = 1.3 and a value of Δlog\textsubscript{10}P\textsubscript{50} \textDelta pH\textsuperscript{-1} of -0.86 at pH 7.5, 12.5 °C and in the presence of saturating ATP concentrations, the present study confirms the unusually low affinity and cooperativity of O\textsubscript{2} binding and the strong Bohr effect of Atlantic cod Hb that have been established previously on purified hemolysates or RBC suspensions of this species (Karpov and Novikov, 1980; Brix et al., 2004; Verde et al., 2006; Barlow et al., 2017). The low Hb-O\textsubscript{2} affinity was not due to a strong effect of physiological chloride concentrations on the P\textsubscript{50} value (Fig. 6), indicating that a lack of chloride sensitivity of P\textsubscript{50} in teleost Hbs is not necessarily associated with a higher O\textsubscript{2} affinity as recently proposed for the high O\textsubscript{2}-affinity Hb of another teleost (Damsgaard et al., 2015). Hb-O\textsubscript{2} saturations of ≤ 50% in the presence of ATP and at pH ≤ 6.5, even in the presence of 1 atmosphere of pure O\textsubscript{2} (760 mmHg; Fig. 3A, B, C), further confirm the strong Root effect found in this species (Krogh and Leitch, 1919; Berenbrink et al., 2005, 2011; Verde et al., 2006, Barlow et al., 2017). Some of these features have also been reported previously for purified hemolysates of the HBI polymorphs of Atlantic cod, yet under more limited experimental conditions, e.g. a smaller pH range, or only in either the presence or the absence of ATP (Brix et al., 1998; Pörtner et al., 2001; Colosimo et al., 2003; Brix et al., 2004). However, none of the above studies was able to fully replicate the strongly divergent temperature dependence of RBC-O\textsubscript{2} affinity of the different HBI polymorphs reported by Karpov and Novikov (1980). The comprehensive study by Verde et al. (2006) reported an overall exothermic Hb oxygenation, with O\textsubscript{2}-affinity decreasing at higher temperature in both the presence and absence of ATP (ΔH\textsuperscript{T} values of ca. -50 to -70 kJ/mol; including the heat of O\textsubscript{2} solubilization), which is in apparent contrast to both the present study (Fig. 4C) and that of Brix et al. (2004), which reported a reduced or even reversed thermal sensitivity of Hb-O\textsubscript{2} affinity in the presence of ATP. Comparing the globin amino acid sequences of the major Atlantic cod Hb component studied by Verde et al. (2006) with those of the HBI polymorphs determined by Andersen et al. (2009) and Borza et al. (2009) suggests that Verde et al. (2006) investigated the HBI-2
polymorph of Atlantic cod, with B1 Val55-Ala62. This is, however, the cold-water type, whose RBCs display an essentially temperature-independent O₂ affinity according to Karpov and Novikov (1980).

How can the apparent discrepancies in the thermal sensitivities of the O₂ affinity of HbI polymorphs between all these studies be resolved?

The present study reveals that in the presence of ATP there is a significant interaction between pH and temperature in their effects on Hb-O₂ affinity, which disappears in the absence of ATP, when the effects of temperature and pH on Hb-O₂ affinity become merely additive. More specifically, in both HbI polymorphs the presence of saturating ATP levels at a physiological RBC pH of around 7.3 caused an unusual overall temperature-independent Hb oxygenation, whereby Hb-O₂ affinity was constant between 5.0 and 20 °C, compared to the classic pattern of a decrease of Hb-O₂ affinity with increasing temperature that was observed in the absence of ATP (Fig. 4A). Thus, depending on the level of ATP, the temperature-sensitive and the temperature-insensitive Hb-O₂ affinity phenotypes that were previously exclusively ascribed to the HbI-1 and HbI-2 polymorphs, respectively, could be induced in both HbI polymorphs. The effect of ATP on the overall exothermic, thermoneutral, or endothermic nature of Hb oxygenation (with a positive, zero, or negative change in oxygenation enthalpy ΔH°, respectively; Fig. 4C) was critically dependent on pH, as previously observed in Hbs of ectothermic rainbow trout and carp, and partially endothermic billfishes (Greaney et al., 1979; Weber et al., 1976, 2010). The strong biphasic effect of pH on the interaction between ATP and Hb can be rationalised as follows: at the pH of its maximal effect, 4-fold negatively charged ATP binds to the cluster of positively charged amino acid residues between the two β chains of the Hb tetramer that becomes accessible upon deoxygenation when the conformational equilibrium of Hb tetramers shifts from the high O₂-affinity relaxed (R) state to the low O₂-affinity tensed (T) state of the Hb tetramer (Powers, 1980; Weber and Campbell, 2011). The heat released upon binding of ATP to T-state Hb at this pH counters the heat that would be released upon oxygenation of the 4 heme groups in R-state Hb and contributes to making the overall oxygenation reaction less ectothermic or even endothermic. However, as pH decreases, 4-fold negatively charged ATP is increasingly titrated to 3-fold negatively charged ATP, reducing its binding affinity to Hb and its effect on the overall enthalpy of oxygenation (Greaney et al., 1979). Equally, at higher pH values, the effect of ATP on the overall enthalpy change of oxygenation also diminishes (Greaney et al., 1979; Weber et al., 2010), which can be ascribed to a reduced binding affinity of ATP to the partially neutralised positive charge cluster of the ATP-Hb binding site (Greaney et al., 1979). This phenomenon explains why a reduced or even reversed thermal dependency of Atlantic cod Hb oxygenation was not observed by Verde et al. (2006), despite the presence of ATP, because these authors reported oxygenation enthalpy changes at pH 6.5 and 8.7, which is ≥ 0.7 pH-units away from the optimal pH of ca. 7.3 for ATP’s maximal effect on the enthalpy change of Hb-oxygenation established for several teleost fishes (Greaney et al., 1979; Weber et al., 2010).
The present results on the thermal sensitivity of the O$_2$ affinity of purified HbI-polymorphs confirms and expands our previous study of the same population of Atlantic cod that was performed at the RBC level (Barlow et al., 2017). Using procedures to ensure the absence of adrenergically-induced changes in intracellular RBC pH (Berenbrink and Bridges, 1994b) and the presence of the same, naturally occurring levels of ATP in all HbI polymorphs, Barlow et al. (2017) found statistically indistinguishable affinities and cooperativities of RBC-O$_2$ binding of the different HbI polymorphs under all conditions of temperature and pH. Moreover, at an extracellular RBC pH of 7.65 (corresponding to an intracellular RBC pH of 7.21 (Berenbrink and Bridges, 1994a)), RBC-O$_2$ affinity of all HbI polymorphs was independent of temperature between 5.0 and 20.0 °C. This thermoneutral RBC oxygenation pattern changed to an overall exothermic RBC oxygenation reaction at more alkaline pH values (Barlow et al., 2017), as predicted from a progressive neutralisation of the positive-charge cluster of the ATP binding site and an associated reduction of ATP-binding affinity at higher pH (Greaney, Hobish and Powers, 1979). These results suggest that the thermal dependence of RBC oxygenation in Atlantic cod depends more strongly on the concentration of ATP and the intracellular pH inside their RBCs than on any genetic differences between HbI polymorphs.

ATP is the major natural organic phosphate modulator of Hb function in Atlantic cod RBCs, occurring at concentrations of 2.5 to 3.9 mmol/L RBCs and ATP:Hb$_4$ molar ratios of 1.20 to 1.57 (Leray, 1982; Barlow et al., 2017). Thus, given its low concentration of half-maximal effect of about 0.1 mM in dilute Hb solutions, it is likely that ATP will exert close to maximal effects on Hb-O$_2$ affinity under physiological conditions, which is in line with the above mentioned close similarity of Hb-O$_2$ binding characteristics between RBCs and Hb in the presence of saturating ATP, once the pH difference between intra and extracellular pH of RBCs has been considered.

The in vivo concentration of ATP in teleost RBCs has been shown to vary with season and during thermal or hypoxic acclimation (e.g.: Wood and Johansen, 1973; Powers, 1980; Tetens and Lykkeboe, 1981; Albers et al., 1983; Andersen et al., 1985). Acute reductions of RBC ATP concentrations in teleost fishes can further be achieved in vitro by incubating RBCs under anoxia, in the presence of adrenalin, or at elevated temperatures in glucose-free saline (e.g.: Powers, 1980; Tetens and Lykkeboe, 1981; Nikinmaa, 1983; Vorger, 1985). Thus, depletion of RBC ATP in one of the Hbl polymorphs, for example due to differences in acclimation history or RBC storage duration or conditions, may not only lead to higher RBC-O$_2$ affinities through its diminished interaction with the low O$_2$-affinity T-state of Hb, but also to an overall more exothermic Hb oxygenation reaction. A reduction of ATP levels will also reduce the number of negatively-charged membrane-impermeable poly-anions inside the RBCs, which will increase the intracellular RBC pH due to changes in the Donnan equilibrium and lead to an additional increase in Hb-O$_2$ affinity via the Bohr effect (Wood and Johansen, 1973). Thus, it is entirely possible that the different O$_2$-binding characteristics reported for the different Atlantic cod Hbl-polymorphs by Karpov and Novikov (1980) may have been caused
simply by variations in intracellular pH and/or ATP concentration, rather than by the $\beta_1$ Met55-

Due to its position at an intradimer $\alpha_i\beta_1$ contact site, the Met/Val polymorphism at position 55
in the $\beta$ chains of HbI polymorphs was previously hypothesized to affect Hb-$O_2$ affinity, analogous to
substitutions at this contact site in Hbs of high-altitude geese, whereas the Lys/Ala polymorphism at
position 62 was suggested to influence the thermal sensitivity of Hb-$O_2$ affinity by the differential
interaction of amino acids at that site with the heme cavity (Andersen et al., 2009). However, the
absence of any significant differences in the oxygenation characteristics of HbI polymorphs under
carefully controlled conditions of allosteric modifiers in the present study casts doubts on such a
molecular mechanism. It should be mentioned though, that despite the significant linkage between
Val-Ala and Met-Lys, respectively in positions 55 and 62 of the $\beta_1$ polymorphs (see introduction), the
recombinant $\beta_1$ haplotypes Met55-Val55-Ala62 and Val55-Lys62 do occasionally occur and may
theoretically show functional differences in Hb-$O_2$ binding (Wetten et al., 2012). However, the
recombinant Met-Ala haplotype is considered extremely rare and even the Val-Lys haplotype only
occurs at frequencies below 2% in the northern and southern populations of North East Atlantic cod
(Wetten et al., 2012). Thus, even if the recombinant haplotypes were causing functional differences in
Hb-$O_2$ affinity (which is unproven), it is extremely unlikely that they occur at high enough
frequencies in either the southern Irish Sea or the northern White Sea populations to cause consistent
functional differences in Hb-$O_2$ binding within and between populations.

However, the possibility remains that the observed structural differences between HbI
polymorphs affect RBC-$O_2$ transport properties in another, more indirect way. The concentration of
Hb inside RBCs is considered close to its solubility limit and charge-changing amino acid
replacements on the surface of the Hb tetramer may affect the solubility of the protein, such as in the
classic example of the human sickle Hb variant (HbS) that causes the aggregation of deoxygenated
HbS and sickle cell disease (for review: Bunn, 1997). Furthermore, the Hbs of several teleost fish
species, including of Atlantic cod, have been shown to be prone to in vivo polymerisation (Hárosi et
al., 1998; Koldkjær and Berenbrink, 2007; Koldkjær et al., 2013) and it is conceivable that the more
negatively-charged Hbl-2 cold-water polymorph may permit higher RBC Hb concentrations than the
warm-water Hbl-1 polymorph and/or that the two different Hbl polymorphs differentially affect the
threshold or degree of Hb polymerisation.

Lastly, it is also possible that the Hbl polymorphism in Atlantic cod is selectively neutral and
that the inverse latitudinal clines in allele frequencies along the coast of the North East Atlantic are
due to a significant association between these alleles and other genetic differences that are the target
of natural selection. Support for such a hitchhiking effect has been provided by the Atlantic cod whole
genome sequencing project (Star et al., 2011). The authors of that study have shown that the Hbl
structural polymorphism is genetically linked to a regulatory polymorphism that involves the common
promotor region of the $\alpha_1$ and $\beta_1$ globin genes that encode the Hbl hetero-tetramer, illustrating the
distinct possibility of hitch-hiking effects in determining the distribution of HbI polymorphs (Star et al., 2011; Andersen, 2012).

To conclude, contrary to the prevailing view the present study shows that there are no significant, genetically-based differences in the O$_2$-affinity of the major HbI polymorphs of Atlantic cod at the warm, southern range of its distribution in the North East Atlantic. Karpov and Novikov’s (1980) report of such strong differences in a population at the cold, northern range of the species’ distribution may have been due to phenotypic plasticity and differing levels of intracellular allosteric modifiers of Hb function, and/or as yet undocumented additional, electrophoretically silent, genetic differences between HbI polymorphs in Atlantic cod from the White Sea. However, the present study fully confirms at the level of Hb in solution the existence of a temperature independent Hb-O$_2$ affinity in the non-endothermic Atlantic cod that was first postulated on the RBC level by Karpov and Novikov (1980) for individuals with the HbI-1 polymorph and then expanded to include reversed thermal dependence of Hb-O$_2$ affinity and RBCs of the HbI-2 polymorph of Atlantic cod in our previous study (Barlow et al., 2017). The current study reveals the dominant role of ATP and its interaction with pH behind this observation, thereby providing the first demonstration of the molecular mechanism of such a reversed temperature dependent Hb-O$_2$ affinity in a non-endothermic fish. These results support our previous findings at the RBC level, which concluded that above a temperature of 20 °C there was no further scope for increased Hb-O$_2$ delivery through adjustments of the Hb-O$_2$ equilibrium curve in any of the major HbI polymorphs of Atlantic cod at their current southern, warming limit of distribution in the North East Atlantic (Barlow et al., 2017). While the structural polymorphism of HbI in Atlantic cod has served as a useful genetic marker for a number of fitness-related traits on the whole organism level for several decades, we show that this association is not due to differing Hb-O$_2$ affinities between HbI polymorphs in our southern population of Atlantic cod in the Irish Sea that is potentially among the ones most vulnerable to warming sea water temperatures (Drinkwater, 2005; Deutsch et al., 2015). Thus, the search must go on for the mechanistic cause(s) behind the documented differences in the distribution, thermal preference, hypoxia tolerance, growth rate, and competitive behaviour of Atlantic cod carrying the different HbI polymorphs. Integration of physiological, genetic and behavioural studies of Atlantic cod populations, including in their natural environment and across life history stages, appears an essential tool towards that goal.
Competing Interests Statement:
No competing interests declared.

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References


Table 1: Effect of pH range and temperature on the Bohr effect $\varphi$ of Atlantic cod Hb in the presence of saturating ATP concentrations.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>5.0 °C</th>
<th>12.5 °C</th>
<th>20.0 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper pH range</td>
<td>7.30 - 7.90</td>
<td>7.20 - 7.70</td>
<td>7.10 - 7.60</td>
</tr>
<tr>
<td>$-\log_{10} P_{50}/\Delta pH$</td>
<td>0.97$^a$</td>
<td>1.11$^{ab}$</td>
<td>1.24$^b$</td>
</tr>
<tr>
<td>± S. E.</td>
<td>±0.03</td>
<td>±0.02</td>
<td>±0.01</td>
</tr>
<tr>
<td>(N)</td>
<td>(3)</td>
<td>(3)</td>
<td>(4)</td>
</tr>
<tr>
<td>Lower pH range</td>
<td>6.50 - 7.30</td>
<td>6.30 - 7.20</td>
<td>6.40 - 7.10</td>
</tr>
<tr>
<td>$-\log_{10} P_{50}/\Delta pH$</td>
<td>0.63$^c$</td>
<td>0.75$^c$</td>
<td>1.17$^b$</td>
</tr>
<tr>
<td>± S. E.</td>
<td>±0.07</td>
<td>±0.04</td>
<td>±0.05</td>
</tr>
<tr>
<td>(N)</td>
<td>(6)</td>
<td>(6)</td>
<td>(6)</td>
</tr>
</tbody>
</table>

Dissimilar superscript letters within rows and columns indicate mean values significantly different from each other ($p < 0.05$, Holm-Sidak multiple comparison method after a two-way ANOVA of $\Delta \log_{10} P_{50}/\Delta pH$ values, transformed according to $x' = \text{square-root} (x + 2)$ to meet the normality and equal variance assumptions, and with temperature and pH as factors).
Figure legends

Figure 1: Separation of Atlantic cod HbI polymorphs by agarose gel electrophoresis. Hb components were separated in a 1% agarose gel at pH 8.8 and the major HbI-1, HbI-2 and HbI-1,2 polymorphs were identified by the indicated banding patterns. Anode (+), cathode (-) and origin of electrophoresis (arrow) are indicated. The minor HbII component resolves into two bands of approximately equal size under these conditions.

Figure 2: Example of spectrophotometric determination of Hb-O₂ saturation by spectral deconvolution. A, Template spectra of oxygenated Hb (HbO₂, red), deoxygenated Hb (Hb, blue) and acid and alkaline metHb (Hb⁺_acid, purple; Hb⁺_alkaline, green) used for spectral deconvolution. The black dashed line corresponds to the spectrum of diluted milk, which was included to account for differences in the turbidity of the samples. B, Example of measured (solid coloured lines) and predicted (black dotted lines) optical spectra of Hb solutions using spectral deconvolution. The samples were equilibrated at the indicated PO₂ values, and the percentage Hb-O₂ saturation (S) and the percentage metHb (Hb⁺) were determined by estimating the fractional contributions of the template spectra in A to each measured spectrum as explained in Materials and Methods. The predicted spectra present the summed contributions of each Hb derivate and the turbidity component shown in A under each condition.

Figure 3: Hill-plots showing the effect of pH, temperature, and the presence or absence of 9 mM ATP (A-C and D-F, respectively) on the linearized Hb-O₂ equilibrium curves of purified hemolysates of Atlantic cod with either the HbI-1 or the HbI-2 polymorph as major Hb component (filled symbols with solid regression lines, and open symbols with dashed regression lines, respectively). Squares, triangles and circles indicate the three buffers used to vary pH, with final measured pH values indicated in each panel. For both columns, the top, middle and lower panels show data recorded at 5.0 °C, 12.5 °C and 20.0 °C respectively. For clarity, a single linear regression line is shown for the combined data from the three individuals per HbI polymorph at each pH value. Grey dotted lines indicate 50% Hb-O₂ saturation. Final hemolysate concentrations were 83 - 95 mM HEPES, 100 - 118 mM chloride, 2.6 – 8.7 μM tetrameric Hb.

Figure 4: Effects of pH on the affinity (A), cooperativity (B), and overall enthalpy (C) of Hb-O₂ binding in the major HbI polymorphs of Atlantic cod, measured at the indicated temperatures and in the absence and presence of 9 mM ATP (± ATP). In A and B, blue, black, and red symbols and lines indicate values and curve-fits, respectively, for 5.0, 12.5 and 20.0 °C. Triangles pointing up and down indicate HbI-1 and HbI-2 polymorphs, respectively, in the presence and absence of 9 mM ATP (filled symbols and solid lines, and open symbols and dashed lines, respectively). Other conditions as in Fig. 1. Hb-O₂ affinity and cooperativity are expressed as log₁₀P₅₀ and nH values, respectively. In C, the average overall enthalpy change during Hb-O₂ binding (ΔH') is shown in the presence and absence of ATP, as calculated by applying the van ’t Hoff equation to the respective
values interpolated between the mean log$_{10} P_{50}$ values in $A$ over the lower (blue lines) and the higher (red lines) temperature interval and across the range of experimental pH. Note the reversal of the $y$-axis in $C$, with negative $\Delta H'$ values that signify an overall exothermic Hb oxygenation on top. See text for further information.

**Figure 5:** Dose-response curves showing the effect of increasing ATP:Hb$_4$ ratios on the Hb-O$_2$ affinity of the major HbI polymorphs in Atlantic cod. Filled and open triangles indicate HbI-1 and HbI-2 polymorphs, respectively, with solid and dashed lines indicating the respective hyperbolic curve fits (which appear sigmoidal on a log scale of ATP:Hb$_4$ ratios). Hb-O$_2$ affinities are expressed as log$_{10} P_{50}$ values and refer to a temperature of 12.5 °C, pH 7.0, other conditions as indicated in Fig. 1. Values at ATP:Hb$_4$ ratios of zero and above 1 000 (0 and 9 mM ATP) are based on Figure 2A, with the latter values having been interpolated to pH 7.0 from values at higher and lower pH values.

**Figure 6:** Effect of chloride on Hb-O$_2$ affinity in the two major HbI polymorphs of Atlantic cod. Filled and open triangles indicate HbI-1 and HbI-2 polymorphs, respectively, with solid and dashed lines indicating the respective linear curve fits. At a given chloride concentration, each symbol indicates a different individual. Hb-O$_2$ affinities are expressed as log$_{10} P_{50}$ values and refer to a temperature of 12.5 °C, pH 7.0 and the absence of ATP; other conditions as in Fig. 1. Values at 118 mM chloride are from Figure 2A.
Figure 1

HbI Polymorphs

Origin

HbI

HbII

HbI-2 HbI-1 HbI-1,2

1 2

+ +
Figure 3

A) 5.0°C +ATP

B) 12.5°C +ATP

C) 20.0°C +ATP

D) 5.0°C -ATP

E) 12.5°C -ATP

F) 20.0°C -ATP
Figure 4

Bohr-plot

\[ \log_{10} [P_{50} \text{ (mmHg)}] \]

\[ n_H \]

\[ \Delta H (kJ \text{ mol}^{-1}) \]

A: Exothermic Hb oxygenation
B: Endothermic Hb oxygenation

\( + \text{ATP} \)
\( - \text{ATP} \)

\( 5.0^\circ \)
\( 12.5^\circ \)
\( 20.0^\circ \)
Figure 5

$\frac{[\text{ATP}]}{[\text{Hb}_4]} \text{ (mol/mol)}$

$\log_{10} (P_{50} \text{ [mmHg]})$

$HbI-1$

$HbI-2$
Figure 6

The graph shows the relationship between $\log_{10}(P_{50})$ (in mmHg) and $[\text{Cl}^-]$ (in mM) for two different hemoglobins, HbI-1 and HbI-2. The data points indicate a relatively constant value of $\log_{10}(P_{50})$ across different concentrations of $[\text{Cl}^-]$. The graph suggests that HbI-1 and HbI-2 have similar $P_{50}$ values under these conditions.