Characterization of the oxygen binding properties of hemoglobin from the ruby-throated hummingbird (*Archilochus colubris*)

by

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Abstract

Weighing only between 2.5 and 4.8 g, the ruby-throated hummingbird (*A. colubris*) has one of the highest known mass-specific metabolic rates among birds. Despite this, there is a lack of available data on the oxygen binding properties of their hemoglobin (Hb). Here I measured the effect of allosteric effectors, pH and temperature on the O_2-affinity (defined as the O_2 partial pressure required for 50% Hb O_2 saturation; P_{50}) of ruby-throated hummingbird Hb, while also estimating their Hb buffering capacity. A comparison of Hb-O_2 affinity (i.e. P_{50} values) between *A. colubris* and its sole congener, the black-chinned hummingbird (*A. alexandri*), revealed differences likely attributed to two β-chain amino acid substitutions. Under treatment conditions resembling the natural physiological state of hummingbird blood, *A. colubris* exhibited a similar Bohr effect (-0.401) and buffering capacity (4.88 mol H^+ mol Hb_4^{-1} pH^{-1}) to those of other hummingbird species, though both variables were among the lowest values previously reported for birds. Nonetheless, the low buffering capacity is modelled to enhance the efficiency of their (relatively low) Bohr effect and may enable a high blood [Hb], which together with a relatively low blood O_2 affinity is shown to markedly increase tissue O_2 delivery. I also found that O_2 binding of *A. colubris* Hb was thermally insensitive under natural physiological conditions. This thermal insensitivity could help ensure adequate O_2 delivery both during and upon arising from torpor, while minimize heat loss at the lungs. These findings highlight the various O_2 binding properties of *A. colubris* that together facilitate the efficient delivery of O_2 to the tissues irrespective of temperature.

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Introduction

Belonging to the family Trochilidae, hummingbirds are comprised of some of the smallest endotherms in the world (Johansen et al. 1987; Suarez 1992). Owing to their diminutive size, they exhibit the highest recorded mass-specific (i.e. per gram body mass) metabolic rates of all vertebrates (Johansen et al. 1987; Projecto-Garcia et al. 2013). Accordingly, per gram of cells, a ~3 g ruby-throated hummingbird (*Archilochus colubris*) consumes >40 times more oxygen (O_2) at rest than does a ~100 kg ostrich (Lasiewski 1963; Withers 1983), and a remarkable ~400 times higher during hovering flight (Chai and Dudley 1996). To sustain these exceptionally high metabolic needs, gas exchange and O_2 transportation to the tissues must be highly efficient.

There are a number of physiological and morphological adaptations that have evolved in hummingbirds to enhance O_2 delivery. These include high blood hemoglobin (Hb) concentrations, high capillary densities (Johansen et al. 1987), and a relatively large heart that can beat over 1,000 times per minute (Lasiewski 1964). Molecular adaptations of Hb presumably also play a key role in O_2 delivery, though surprisingly few studies have examined this aspect of hummingbird physiology. Hemoglobin is a tetrameric protein composed of two α-type and two β-type globin subunits that binds to and transports O_2 (Perutz 1983). Most avian species, including all hummingbirds examined to date, express two distinct Hb isoforms within the erythrocytes: hemoglobin A (HbA: $\alpha^A{}_2\beta_2$), the major isoform, in which the α-chain subunits are encoded by the α^A-globin gene, and hemoglobin D (HbD: $\alpha^D{}_2\beta_2$), a high O_2 affinity minor isoform, whereby the α-chain subunits are encoded by the α^D-globin gene (Grispo et al. 2012; Natarajan et al. 2016). The same β-chain subunits are shared by both Hb isoforms (Natarajan et al. 2016). Among hummingbirds, the HbD isoform constitutes from 1.6% to 24.2% of the blood Hb (Projecto-Garcia et al. 2013); hence

changes in the HbA/HbD ratio could potentially contribute to adaptive changes in Hb-O_2 affinity and O_2 transportation within this clade (Storz 2018).

Blood O_2 affinity, which is usually expressed as the partial pressure of O_2 (PO_2) at which half of the Hb is saturated (i.e. the P_{50} value), varies fairly widely among avian species (Baumann and Baumann 1977). For small endothermic species, efficient offloading of O_2 at the tissues has been suggested to be largely accomplished by reductions in blood O_2 affinity (Schmidt-Nielsen and Larimer 1958; Johansen et al. 1987). In small mammals, it has been suggested that this trait is often accompanied by a relatively high Bohr effect (i.e. the reduction in blood O_2 affinity arising from a decrease in pH) (Riggs 1960). However, Northam (2022) found no significant relationship between body mass and Bohr effect in either birds or mammals. Briefly, as pH decreases, the Hb-O_2 affinity decreases (P_{50} increases) due to the binding of protons (H^+), which stabilizes the low O_2-affinity (*T*- or tense-state; see below) conformation of the protein (Jensen 2004). Therefore, a high Bohr effect and high P_{50} (low blood O_2 affinity) both increase O_2 offloading at the tissues.

Cooperativity refers to how the binding/release of one O_2 molecule to Hb can influence the binding affinity of other O_2 molecules to the same Hb tetramer (Perutz 1983). Cooperativity increases the efficiency of Hb as an O_2 carrier by permitting larger changes in blood O_2 saturation over a given (lung to tissue) PO_2 range (Perutz 1983). Allosteric effectors are non-O_2 ligands that reversibly bind to specific amino acid residues of Hb and alter blood O_2 affinity (Perutz 1983). These effectors include H^+, chloride ions (Cl^-), carbon dioxide (CO_2) and organic phosphates such as inositol pentaphosphate (IPP) that are found in bird erythrocytes (Storz 2018). Hemoglobin occurs in two states: the high O_2 affinity, predominately oxygenated, *R*-state and the low O_2 affinity, predominately deoxygenated, *T*-state (Perutz 1983). Most allosteric

effectors bind to the *T*-state and stabilize it, which consequently decreases Hb-O_2 affinity, enhancing O_2 delivery to the tissues (Storz 2018). Vertebrate Hbs also have numerous surface exposed (titratable) histidine residues that act as buffer groups (Jensen 2004; Berenbrink 2006; Northam 2022). Consequently, alterations in the proton buffering capacity of Hb (βHb)—i.e. its ability to resist changes in pH—is another potential mechanism by which small endotherms could increase O_2 delivery to the tissues (Berenbrink 2006). For example, a low Hb buffering capacity has been suggested to be beneficial for O_2 offloading in small high metabolic rate endotherms as it allows a larger pH change within the red blood cells for a given acid (CO_2) load (i.e. it increases the efficiency of the Bohr effect; Northam 2022), though the potential effect of this trait has not been quantified in hummingbirds.

As noted above, the Bohr effect stabilizes the low O_2 affinity *T*-state of Hb via the binding of H^+ to Bohr groups, ionizing them and causing them to form salt-bridges with nearby charged residues (Jensen 2004). The ability of surface exposed histidine residues to act as Bohr groups depends on their p*K*a (which affects their affinity for protons within the physiological pH range) and their proximity to charged residues (Berenbrink 2006). For example, histidine residues that have a higher p*K*a in the *T*-state compared to the *R*-state could contribute to the Bohr effect, while those whose p*K*a decreases in the *T*-state potentially counter the Bohr effect (Berenbrink 2006). The remaining solvent exposed histidine residues are solely buffering groups that bind to and remove H^+ from solution (Jensen 2004).

Little is known regarding the Bohr effect and buffering capacity of hummingbird Hb. Johansen et al. (1987) reported an average Bohr factor (φ) of -0.39 for the whole blood of three Brazilian hummingbird species, though no values appear to be available for the individual Hb components (HbA and HbD) of any hummingbird species. By

contrast, Northam (2022) revealed large, highly consistent reductions in both histidine content and predicted Hb buffering capacity (i.e. βHb ranged from 5.69 to 4.37 mol H^+ mol Hb_4^{-1} pH^{-1}) of all 20 hummingbird species examined relative to the mean value for non-passerine birds (many passerine species exhibit similar scale reductions), though measured βHb values for any hummingbird species are currently unavailable. By contrast, some research has been conducted on the role of allosteric effectors, particularly Cl^- and IPP (or inositol hexaphosphate (IHP): a chemical analog of the naturally occurring IPP) on hummingbird Hb function. The general consensus is that in the absence of the allosteric effectors (Cl^-, IHP/IPP), P_{50} values are much lower, and that HbD has a lower P_{50} than HbA under all experimental conditions (Natarajan et al. 2016; Projecto-Garcia et al. 2013). However, these studies primarily focused on the effect of altitude on the Hb-O_2 affinity of numerous (mostly South American) species at a single pH value (7.4), and did not take into account the effect of pH or temperature on P_{50}.

The pH of avian arterial blood is higher than venous blood owing to differences in CO_2 levels. While arterial and venous blood pH values of the ruby-throated hummingbird do not seem to have been measured, it is generally observed that most birds maintain an arterial pH of approximately 7.5 (Montesinos and Ardiaca 2013). Documented venous blood pH of various bird species typically fall within a range of 7.2 to 7.5 (Montesinos and Ardiaca 2013). Heightened rates of metabolism during flight leads to an increase in CO_2 production, which should contribute to a greater disparity between arterial and venous blood pH. Many small bird species, such as *A. colubris,* also undergo torpor, with some hummingbird species being able to drop their body temperature from ~40°C down to within 1°C of the ambient temperature (Lasiewski 1963). The oxygenation of Hb is exothermic (i.e. it releases heat); hence,

without compensation from other sources (e.g. exothermic allosteric effector binding) Hb-O_2 affinity is expected to decrease with increasing temperature (Weber and Campbell 2011). Therefore, variations in blood pH and temperature may both strongly influence the blood O_2 affinity of hummingbirds, but these have not been examined for *A. colubris*.

This study focused on characterizing the molecular adaptations of Hb that potentially enhance O_2 delivery in ruby-throated hummingbirds. These hummingbirds are commonly found in Central America during the winters and North America during the breading season (Weidensaul et al. 2020). Out of the 328 species of recognized hummingbirds, only 29 are long-distance migrants (Rappole and Schuchmann 2003). Among these 29 species is the ruby-throated hummingbird, which can migrate non-stop for about 20 hours across the Gulf of Mexico (Suarez 1992). Together with a high rate of O_2 consumption, these traits make their Hb-O_2 binding properties an intriguing topic of research, though no studies on the blood O_2 affinity of this species are currently available. Nonetheless, P_{50} values for both HbA and HbD of its sole congener, the black-chinned hummingbird (*Archilochus alexandri*), have been reported by Natarajan et al. (2016), but only at pH 7.4 and 37ºC. Similar to *A. colubris*, *A. alexandri* also migrate, but are predominantly medium-distance migrants (English et al. 2024). While *A. alexandri* can be found at higher elevations of up to 2500 m, both species are commonly found at low elevations and have overlapping ranges, where hybridization instances have been reported (Judd et al. 2011). Therefore, it was predicted that both species would have similar P_{50} values.

The objectives of this study were:

1. To measure the effect of temperature, pH (Bohr effect) and the allosteric effectors Cl^- and IHP on P_{50} of ruby-throated hummingbird Hb.
2. To compare the P_{50} values of the ruby-throated hummingbird with the black-chinned hummingbird (and other hummingbird species).
3. To calculate the predicted buffering capacity of ruby-throated hummingbird Hb based on Hb primary structures and HbA:HbD ratios.
4. To model the blood O_2 carrying capacity and delivery of hummingbird blood based on the calculated buffering capacity, measured Bohr effect, and estimated blood [Hb] and P_{50} values.

Methods and Materials

Hemoglobin samples. Ruby-throated hummingbird blood samples for this study were opportunistically collected in heparinized capillary tubes by collaborators at the University of Toronto (n = 3; Giulia Rossi) and Western University (n = 4; Amalie Hutchinson). Specimens were captured locally and sampled following approved animal care protocols at both institutions. Blood samples were immediately stored in sealed capillary tubes at -80°C following collection.

Hemoglobin preparation. To maximize extraction of the Hb from the limited blood sample volume available, the procedures adhered closely to the methods described by Jendroszek et al. (2018). Blood samples were extracted by cutting the ends off the sealed capillary tubes using a metal triangular file and pipetting out the contents. The extracted samples were diluted with a 4-fold volume of 10 mM HEPES buffer (pH ~ 7.4). To obtain sufficient Hb for purification/analysis, blood samples from Toronto and London were pooled separately. For cell lysis, the mixture was kept on ice for 30 min and vortexed every 10 min. The lysed cell mixture was then centrifuged at 14,000 g for 30 min at 4°C using Amicon Ultra-0.5 mL centrifugal filters (30 kDa membrane). The supernatant was collected, and 20 mg/mL of sodium dithionite was added to prevent the formation of metHb. Sodium chloride (NaCl) was then added to a final concentration of 0.2 M to displace IPP. The hemolysate solution was run through a PD-10 desalting column (Cytvia, United States) equilibrated with 20 mM HEPES buffer (pH ~ 6.8) to remove sodium dithionite, NaCl and organic phosphates from the hemolysate. In an attempt to separate the HbA and HbD isoforms, the resulting solution was then further purified using the ÄKTA Start Protein Purification System (Cytvia, United States) employing cation-exchange chromatography. The pooled blood sample from London was purified first and underwent chromatography thrice to try

and separate HbA and HbD isoforms. Briefly, the sample was first passed through an ion exchange column (HiTrap SP HP, 1 mL) equilibrated with 20 mM HEPES buffer (pH 6.8) and eluted using a linear gradient of 0–250 mM NaCl. As only a single Hb peak was detected, a second run was conducted using a linear gradient of 150-250 mM NaCl to try and separate the two Hb isoforms. As only a single Hb peak was again resolved, the sample was then eluted using a buffer of a higher pH with no NaCl (20 mM HEPES ~ pH 8.8) that was expected to elute the HbD fraction, though this step failed to elute any Hb from the column. To remove the Hb, a linear gradient of 0–250 mM NaCl was then used, resulting in a single peak (a hemolysate likely containing both HbA and HbD isoforms; hereafter referred to as Hb for convenience). As results of all four chromatography procedures were similar (i.e. only a single or no Hb peak being resolved), samples from Toronto followed the initial chromatography setting of eluting with a linear gradient of 0–250 mM NaCl. Fractions containing the purified Hb were then pooled and passed through a 10 mM HEPES buffer (pH ~ 7.4) equilibrated PD-10 column, after the addition of 20 mg/mL of sodium dithionite. The Hb samples were centrifuged at 5,000 g for 20 minutes at 4°C. The final solution was stored at -80°C.

Hemoglobin working sample preparation. The O_2 binding characteristics of the purified Toronto Hb samples were measured using the Blood Oxygen Binding System (BOBS; Loligo Systems, Denmark). The concentration of heme (mM) in the purified Hb samples was first measured spectrophotometrically using the standard extinction coefficients for human HbA (based on Zwart et al. 1984). Appropriate volumes of Hb, 1 M HEPES buffer, double-distilled water (ddH_2O), IHP (the final [IHP] was twice that of the [Hb]) and KCl (100 mM final concentration) were mixed to make working solutions. Owing to low sample volume constraints, only two allosteric effector

treatments were prepared, each comprising of 25 µL: a) stripped (without KCl or IHP), and b) 100 mM KCl + 2x IHP. Both allosteric effector treatments were made using 1M HEPES buffer of three different pH values: ~7.00, ~7.35 and ~7.75 (at 37°C). Therefore, a total of six working solutions were prepared. These three pH values were chosen to encompass the range of potential blood pH changes between the arterial and venous circulations. The pH values of the final working solutions was measured using the Thermo Scientific Orion PerpHecT Ross Combination pH Micro Electrode at both 25°C and 37°C. Owing to the low blood sample volumes, pH of the working solutions were measured in the absence of Hb.

Hemoglobin-O_2 affinity determination. The BOBS is connected to a Gas Mixing System (Loligo, Denmark) that delivers precise (and humidified) gas mixtures to the sample at a set flow rate. The machine has an airflow temperature offset function that helps minimize changes in the water content of the Hb sample, and needs to be optimized before running a curve. To obtain initial oxygenated and deoxygenated baselines, 2 µL aliquots of each of the above Hb solutions was first equilibrated at 21% O_2 (for 30 s) and then 100% nitrogen (N_2) for 10-12 min, respectively. After the initial baselines, the sample was equilibrated using 3-5 different O_2 tensions spanning 25% to 75% of pigment saturation. Equilibration steps lasted between 1-5 min each (i.e. until the sample was fully equilibrated). Hemoglobin-oxygen saturation was monitored via absorbance at 430 nm, while the wavelength of 421 nm (approximate isosbestic point) was monitored to assess potential changes to the Hb solution (formation of metHb or sample drying/wetting). In cases where abrupt or excessive absorbance drift was observed, the next trial was repeated with a slightly different airflow temperature offset (and hence humidity) value. At the conclusion of the experiment, the sample was again exposed to 21% O_2 for 30 sec and 100% N_2 for 12

min to get the second oxygenated and deoxygenated baselines, respectively. Three replicates were completed for each working solution. The P_{50} of each working solution was measured at both 25°C and 37°C, which are standardized values used for comparative purposes in the literature, resulting in a total of 12 different treatments.

The final curves were examined and classified into three groups: low, medium and high quality. Low quality curves either displayed non-linear drift (at 421 nm) or the initial/final baselines did not stabilize. Medium quality curves either exhibited moderate baseline drift or had most measurements collected either above or below 50% O_2 saturation. Finally, high quality curves had the best baselines with most of the equilibration tensions spanning 25% to 75% of pigment saturation. Only values obtained from the medium and high quality experiments were included in the results.

As P_{50} was measured at different pH values, the magnitude of the Bohr effect, which is expressed as the Bohr factor/coefficient (φ), was calculated for stripped and KCl + IHP samples at both 25 and 37°C using the formula:

$$\varphi = \Delta \log P_{50} / \Delta pH$$

The overall enthalpy change (ΔH') (kJ mol^{-1}) of the Hb oxygenation reaction was calculated for the above samples using the following equation (Weber and Campbell 2011):

$$\Delta H' = 2.303\, R \cdot \Delta \log P50 / \Delta\, (1/T)$$

Where R is the gas constant (8.314 $J \cdot K^{-1} \cdot mol^{-1}$), and T is the absolute temperature (K).

Thermal sensitivity of the Hb-O_2 binding was calculated using the formula:

$$\text{Temperature sensitivity} = \Delta \text{Log } P_{50} / \Delta T$$

Hemoglobin primary structure determination. Whole genome shotgun (WGS) sequences are available for the ruby-throated hummingbird on GenBank (https://www.ncbi.nlm.nih.gov/). Contigs containing the adult-expressed globin gene sequences were first identified using the BLAST function with publicly available coding sequences from the black-chinned hummingbird (α^A, α^D and β GenBank accession: NK250962, NK250960 and NK250955, respectively) used as the query. The contigs of interest were download in FASTA format to the Geneious (version 9.1.8) software package, and the various globin genes within each contig were annotated. The nucleotide sequences of the α^A, α^D and β globin genes were then translated into amino acid (i.e. primary structure) sequences. Alpha-globin containing contigs from both sequenced *A. colubris* specimens (GenBank accession #'s: CM041099 and JALBCY010000017) indicated that exon 2 of α^D exhibited a donor splice site mutation (GT → GC). Notably, the maximum entropy (MaxEnt) analysis, developed by Yeo and Burge (2004), of this splice site returned a low splice strength score (data not shown), further suggesting that this splice site may not be functional. Therefore, the *A. colubris* liver transcriptome (GenBank accession: SRR6148275) was downloaded to Geneious and raw SRA reads were assembled to each of the adult-expressed globin coding gene sequences. This analysis indicated that the coding (mRNA) α^D globin sequence of the ruby-throated hummingbird was not affected by the splice site mutation.

Hemoglobin structure modelling. Homology models of ruby-throated hummingbird and black-chinned hummingbird Hb were generated using SWISS-MODEL (https://swissmodel.expasy.org/), a fully automated modelling server. Protein sequences of the α^A and β globin genes were used as the target sequences. As my results indicated that the α^A orthologues and α^D orthologues of both hummingbird

species were identical to one another, modelling was based solely on the α^A globin chain. The Hb structure of *Anas platyrhynchos* (SMTL ID: 3EOK.1) was used as a template due to its relatively high similarity in primary structure with both hummingbird species (91.64 % for *A. colubris* and 91.99% for *A. alexandri*).

Estimated buffering capacity. The Henderson–Hasselbalch equation was used to predict the specific buffering capacity (βHb in mol H^+ mol Hb_4^{-1} pH^{-1}) of the ruby-throated hummingbird HbA and HbD isoforms following Northam (2022). Due to the single peak in the Hb elution profiles, quantitative estimates of the HbD and HbA isoforms from the above mentioned liver transcript assemblies were used to calculate the βHb of hummingbird blood.

Oxygen equilibrium curve modelling. Arterial and venous oxygen equilibrium curves (OECs) were modelled to estimate the O_2 saturation of hummingbird Hb at the lungs and at the tissues, hence; reflecting blood O_2 carrying capacity and delivery potential. As previously noted, several *A. colubris* specific values are currently unavailable. However, Williamson et al. (2023) reported an average [Hb] concentration of 18.84 g Hb/dL across 77 Andean hummingbird species, while Johansen et al. (1987) reported an average blood O_2 affinity of 44.00 mmHg at 37°C and pH 7.40 for three Brazilian hummingbird species. Therefore, the modelling was based on the estimated *A. colubris* Hb buffering capacity, the measured Bohr effect and previously published hummingbird [Hb] and whole blood O_2 affinity values. Due to the availability of high-quality human data, human blood values were used as a starting point to model hummingbird blood O_2 carrying capacity. The human Hb data included a buffering capacity of 10.80 mol H^+ mol Hb_4^{-1} pH^{-1} (Siggaard-Andersen 1974), a Bohr effect of -0.52 (Hlastala and Woodson 1975), and a P_{50} of 26.50 mmHg (Lahiri 1975), at 37°C and pH 7.40. An average human [Hb] of 14.00 g Hb/dL

(Fulwood et al. 1982), was also used. Similar to Northam (2022), the following re-arranged Hill equation (Willford et al. 1982) was used to generate the OECs:

$$[i]\ Y = PO_2^{nH} / (PO_2^{nH} + P_{50}^{nH})$$

Where Y is the fractional O_2 saturation and n_H is a measure of cooperativity (i.e. the Hill coefficient). Following Northam (2022), a Hill coefficient of 2.63 (Kwant et al., 1988) was used.

For comparative purposes, I modelled the effect that an acid load required to change the pH of human (venous) blood by 0.2 units had on hummingbird Hb-O_2 saturation under several different scenarios. The first calculation used human Bohr effect, [Hb] and P_{50} values, though utilized the *A. colubris* βHb value (4.88 mol H^+ mol Hb_4^{-1} pH^{-1}). This allowed me to isolate the effect that the reduced buffer capacity of ruby-throated hummingbird relative to that in humans had on O_2 delivery potential using the following equations:

[1.1] Adjusted hummingbird βHb 1= *A. colubris* βHb /Human βHb

[2] pH change = -0.2 / Adjusted hummingbird βHb 1

[3] Final pH = initial pH – pH change

[4.1] Final P_{50} 1 = 10^(human Bohr effect x pH change + log10 (human P_{50}))

To model for the lower Bohr effect in hummingbirds (-0.401) compared to humans, I followed the above steps (1.1, 2 and 3), and exchanged equation 4.1 with the following equation:

[4.2] Final P_{50} 2 = 10^(hummingbird Bohr effect x pH change + log10 (human P_{50}))

A higher [Hb] will increase blood buffering capacity due to the presence of more buffering groups per unit volume of blood (Northam 2022). Therefore, to further refine the pH change resulting from the higher hummingbird [Hb], equation 1.1 was replaced with the following calculations:

[1.2.1] Accounting for different [Hb] (A) = Hummingbird [Hb] / Human [Hb]

[1.2.2] Buffering capacity based on [Hb] (B) = *A. colubris* βHb x A

[1.2.3] Adjusted hummingbird βHb 2 = B / Human βHb

Finally, to model the combined effects of hummingbird buffering capacity, Bohr effect, [Hb] and $P_{50,}$ the same steps used to incorporate hummingbird [Hb] were followed (1.2.1, 1.2.2,1.2.3, 2 and 3); however, equation 4.2 was replaced with the following equation:

[4.3] Final P_{50} 3 = 10^(hummingbird Bohr effect x pH change + log10 (hummingbird P_{50}))

For all of the above models, blood O_2 carrying capacity and delivery were then calculated assuming a arterial blood PO_2 of 100 mmHg in the lungs, and a venous blood PCO_2 of 40 mmHg in the tissues during rest, which were derived from human values (Wagner 2015).

The maximum amount of O_2 that blood can carry (in g Hb dL^{-1}) was calculated using the following equation:

[ii] Blood O_2 max = [Hb] x 1.34 mL O_2 g Hb^{-1}

Where 1.34 mL O_2 g Hb^{-1} is the Hüfner number that indicates the maximum O_2 binding capacity per 1 g of Hb (Shimizu et al. 1986).

The amount of O_2 delivered at the tissues during rest (in mL O_2 dL^{-1}) was calculated using the following equation:

[iii] O_2 offloaded = (Y (at PO_2 = 100 mmHg) x O_2 max) – (Y (at PO_2 = 40 mmHg) x O_2 max)

Where Y and O_2 max are the previously mentioned fractional O_2 saturation [i] and maximum blood O_2 carrying capacity [ii], respectively.

Human and *A. colubris* blood O_2 carrying capacity and delivery potentials were modelled and calculated using the Tidyverse, ggplot2 and ggh4x packages on the Rstudio software (version 2023.12.0+365).

Statistical analysis: As the pH of a working solution is affected by temperature, allosteric effectors and water, P_{50} values used to calculate the Bohr factor, thermal sensitivity and oxygenation enthalpy were first standardized using linear regression analysis. To calculate the Bohr factors for the various treatments, only P_{50} values ranging between pH 7.0 and 7.5 (which exhibited a clear linear relationship) were used after standardizing them to pH 7.0 and 7.35. To calculate the oxygenation enthalpy and thermal sensitivity values, all P_{50} values were included and standardized to pH 7.0, 7.35 and 7.75. Additional statistical analyses were not conducted as some treatments only had one P_{50} value, especially the KCl + IHP working solutions.

Results

Chromatography profiles of pooled blood samples that were purified using an elution gradient of 0-250 mM NaCl exhibited a single peak (Figures 1A, 1B and 1C). The chromatography profile of the sample that was separated using a pH gradient had no distinguishable peak as no Hb came off the column (Figure 1D).

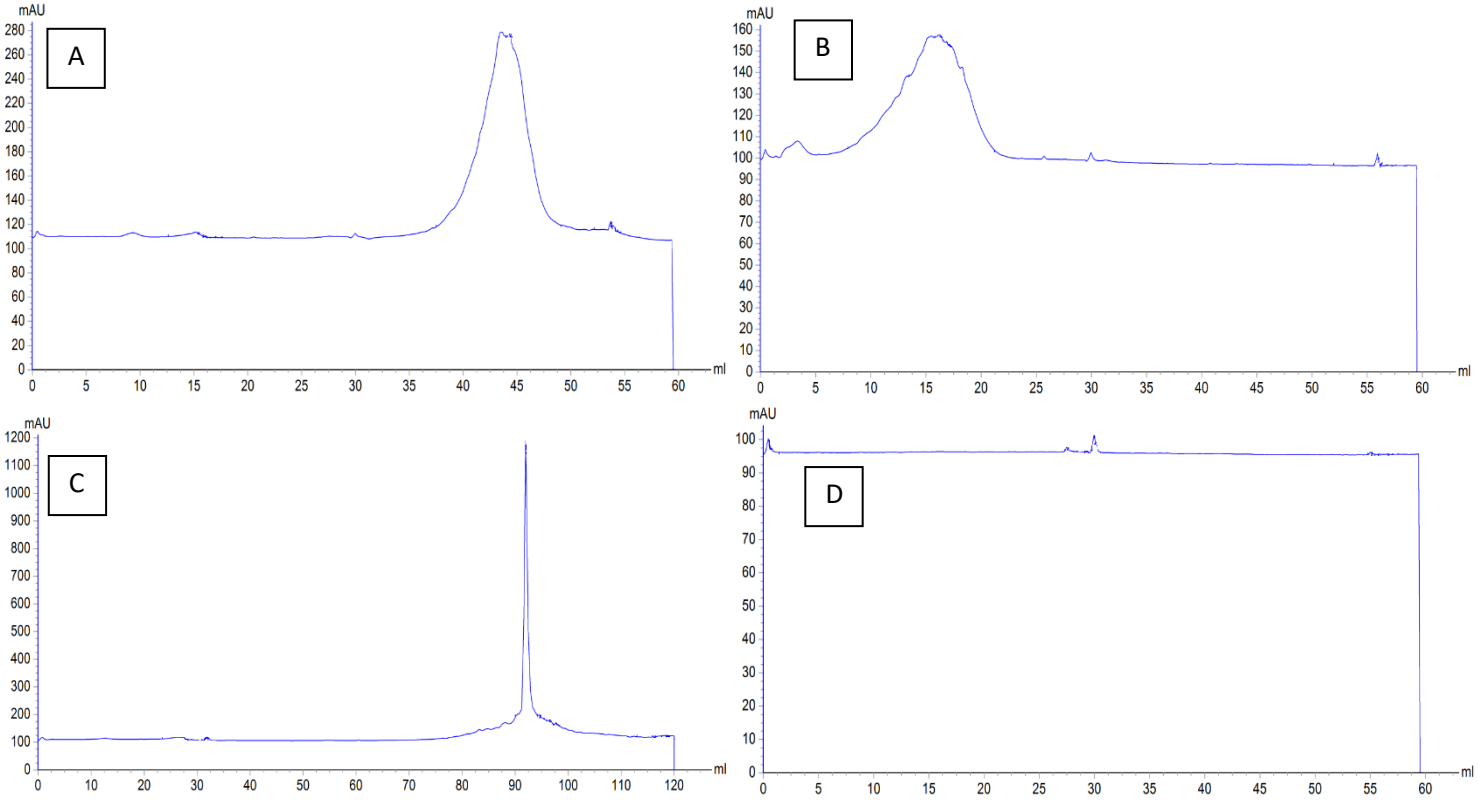

Figure 1. Elution profiles of the chromatography of the purified hummingbird blood samples. Peaks of the blue line represent hemoglobin (Hb). Figures A, B and D are elution profiles of the pooled Hb samples from London. Figure C is the elution profile of the pooled Hb sample from the Toronto. Hb samples in figures A, B and C were eluted using 250 mM NaCl, with A and C starting with 0 mM NaCl and B starting with 150 mM NaCl. The Hb sample in figure D was eluted using a pH of ~8.8, which was expected to retain the HbA isoform and elute the HbD isoform (Signore, personal communication).

The adult-expressed globin gene sequences annotated from two publicly available ruby-throated hummingbird genomes were an exact match to those assembled from the liver transcriptome of this same species. Based on the formula of Northam (2022), the predicted buffering capacity of ruby-throated HbA was estimated to be 4.67 mol H^+ mol Hb_4^{-1} pH^{-1}, while that of HbD was 5.62 mol H^+ mol Hb_4^{-1} pH^{-1}. Quantification of the Hb isoforms assembled using the liver transcriptome SRA data further indicated that the relative abundance of HbD was 21.5% while that of HbA was 78.5% (data not shown). Based on this ratio, the whole blood buffering capacity was estimated to be 4.88 mol H^+ mol Hb_4^{-1} pH^{-1}.

The translated α^A (Figure 2) sequences of *A. colubris* and *A. alexandri* were found to be identical to one another as were the α^D sequences (Figure 3). However, two amino-acid differences were observed between the β-globin chains of these species (Figure 4). Specifically, aspartic acid (D) in *A. alexandri* is substituted with glutamic acid (E) in *A. colubris* at the 22nd position, while serine (S) in *A. alexandri* is substituted with glycine (G) in *A. colubris* at the 83rd position.

α^A–globin gene sequence

Residues	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
A. colubris	V	L	S	A	A	D	K	T	N	V	K	G	V	F	A	K	I	G	G	Q	A	E	E	Y	G	A	E	T	L	A
A. alexandri	V	L	S	A	A	D	K	T	N	V	K	G	V	F	A	K	I	G	G	Q	A	E	E	Y	G	A	E	T	L	A
Residues	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
A. colubris	R	M	F	A	T	Y	P	Q	T	K	T	Y	F	P	H	F	D	L	S	P	G	S	A	Q	V	K	G	H	G	K
A. alexandri	R	M	F	A	T	Y	P	Q	T	K	T	Y	F	P	H	F	D	L	S	P	G	S	A	Q	V	K	G	H	G	K
Residues	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
A. colubris	K	V	A	A	A	L	V	E	A	V	N	N	I	D	D	I	A	G	A	L	S	K	L	S	D	L	H	A	Q	K
A. alexandri	K	V	A	A	A	L	V	E	A	V	N	N	I	D	D	I	A	G	A	L	S	K	L	S	D	L	H	A	Q	K
Residues	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
A. colubris	L	R	V	D	P	V	N	F	K	L	L	G	Q	C	F	L	V	V	V	A	I	R	N	P	A	A	L	T	P	E
A. alexandri	L	R	V	D	P	V	N	F	K	L	L	G	Q	C	F	L	V	V	V	A	I	R	N	P	A	A	L	T	P	E
Residues	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141									
A. colubris	V	H	A	S	L	D	K	F	L	C	A	V	G	T	V	L	T	A	K	Y	R									
A. alexandri	V	H	A	S	L	D	K	F	L	C	A	V	G	T	V	L	T	A	K	Y	R									

Figure 2. Comparison of ruby throated hummingbird (*A. colubris*; GenBank accession #'s: CM041099; JALBCY010000017; SRR6148275) and black-chinned hummingbird (*A. alexandri*; GenBank accession: NK250962) α^A–globin protein sequences.

α^D–globin gene sequence

Residues	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
A. colubris	M	L	T	A	E	D	K	K	L	I	T	Q	V	W	D	K	V	Q	G	C	Q	E	D	V	G	A	E	T	L	T
A. alexandri	M	L	T	A	E	D	K	K	L	I	T	Q	V	W	D	K	V	Q	G	C	Q	E	D	V	G	A	E	T	L	T
Residues	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
A. colubris	R	M	F	V	T	Y	P	Q	T	K	T	Y	F	P	H	F	D	L	S	P	G	S	D	Q	V	R	G	H	G	K
A. alexandri	R	M	F	V	T	Y	P	Q	T	K	T	Y	F	P	H	F	D	L	S	P	G	S	D	Q	V	R	G	H	G	K
Residues	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
A. colubris	K	V	V	A	A	L	G	T	A	V	K	S	L	D	N	L	S	Q	A	L	S	E	L	S	N	L	H	A	Y	N
A. alexandri	K	V	V	A	A	L	G	T	A	V	K	S	L	D	N	L	S	Q	A	L	S	E	L	S	N	L	H	A	Y	N
Residues	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
A. colubris	L	R	V	D	P	V	N	F	K	L	L	A	Q	C	L	Q	V	V	L	A	T	H	L	T	K	D	Y	T	P	E
A. alexandri	L	R	V	D	P	V	N	F	K	L	L	A	Q	C	L	Q	V	V	L	A	T	H	L	T	K	D	Y	T	P	E
Residues	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141									
A. colubris	V	H	A	A	F	D	K	F	L	S	A	V	A	A	V	L	A	E	K	Y	R									
A. alexandri	V	H	A	A	F	D	K	F	L	S	A	V	A	A	V	L	A	E	K	Y	R									

Figure 3. Comparison of ruby-throated hummingbird (*A. colubris*; GenBank accession #'s: CM041099; JALBCY010000017; SRR6148275) and black-chinned hummingbird (*A. alexandri* GenBank accession: NK250960) α^D–globin protein sequence.

β-globin gene sequence

Residues	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
A. colubris	V	H	W	T	A	E	E	K	Q	L	I	T	G	L	W	G	K	V	N	V	A	**E**	C	G	A	E	A	L	A	R
A. alexandri	V	H	W	T	A	E	E	K	Q	L	I	T	G	L	W	G	K	V	N	V	A	**D**	C	G	A	E	A	L	A	R
Residues	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
A. colubris	L	L	I	V	Y	P	W	T	Q	R	F	F	A	S	F	G	N	L	S	S	P	T	A	V	L	G	N	P	M	V
A. alexandri	L	L	I	V	Y	P	W	T	Q	R	F	F	A	S	F	G	N	L	S	S	P	T	A	V	L	G	N	P	M	V
Residues	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
A. colubris	R	A	H	G	K	K	V	L	T	S	F	G	E	A	V	K	N	L	D	S	I	K	**G**	T	F	A	Q	L	S	E
A. alexandri	R	A	H	G	K	K	V	L	T	S	F	G	E	A	V	K	N	L	D	S	I	K	**S**	T	F	A	Q	L	S	E
Residues	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
A. colubris	L	H	C	D	K	L	H	V	D	P	E	N	F	R	L	L	G	D	I	L	I	I	V	L	A	A	H	F	A	K
A. alexandri	L	H	C	D	K	L	H	V	D	P	E	N	F	R	L	L	G	D	I	L	I	I	V	L	A	A	H	F	A	K
Residues	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146
A. colubris	D	F	T	P	E	C	Q	A	V	W	Q	K	L	V	R	A	V	A	H	A	L	A	R	K	Y	H
A. alexandri	D	F	T	P	E	C	Q	A	V	W	Q	K	L	V	R	A	V	A	H	A	L	A	R	K	Y	H

Figure 4. Comparison of ruby-throated hummingbird (*A. colubris*; GenBank accession #: JALBCY010000074) and black-chinned hummingbird (*A. alexandri*; GenBank accession: NK250955) β–globin protein sequence. The amino acid differences are highlighted in red.

At both temperatures, the P_{50} values of the stripped and KCl + IHP working solutions decreased as pH increased (Figure 5). The P_{50} values of each working solution at 25°C was lower than that at 37°C. The P_{50} values of working solutions in the absence of KCl + IHP were substantially higher at 37°C than at 25°C, though the P_{50} difference between these temperatures was much smaller when KCl + IHP were present. Cooperativity values (n_{50}) ranged from 1.59 at a pH of 7.20, to 3.04 at a pH of 7.34 (Figure 5). Owing to irregularities between the *A. colubris* and *A. alexandri* P_{50} values, I re-ran several experiments (sample permitting) using the previously prepared working solutions. The new P_{50} values obtained from the KCl + IHP working solutions at 37°C (40.37 mmHg at pH 7.06 and 35.03 mmHg at pH 7.34) were notably higher than those from the original experiments (Figure 5; cf. filled purple triangles with solid and dashed lines).

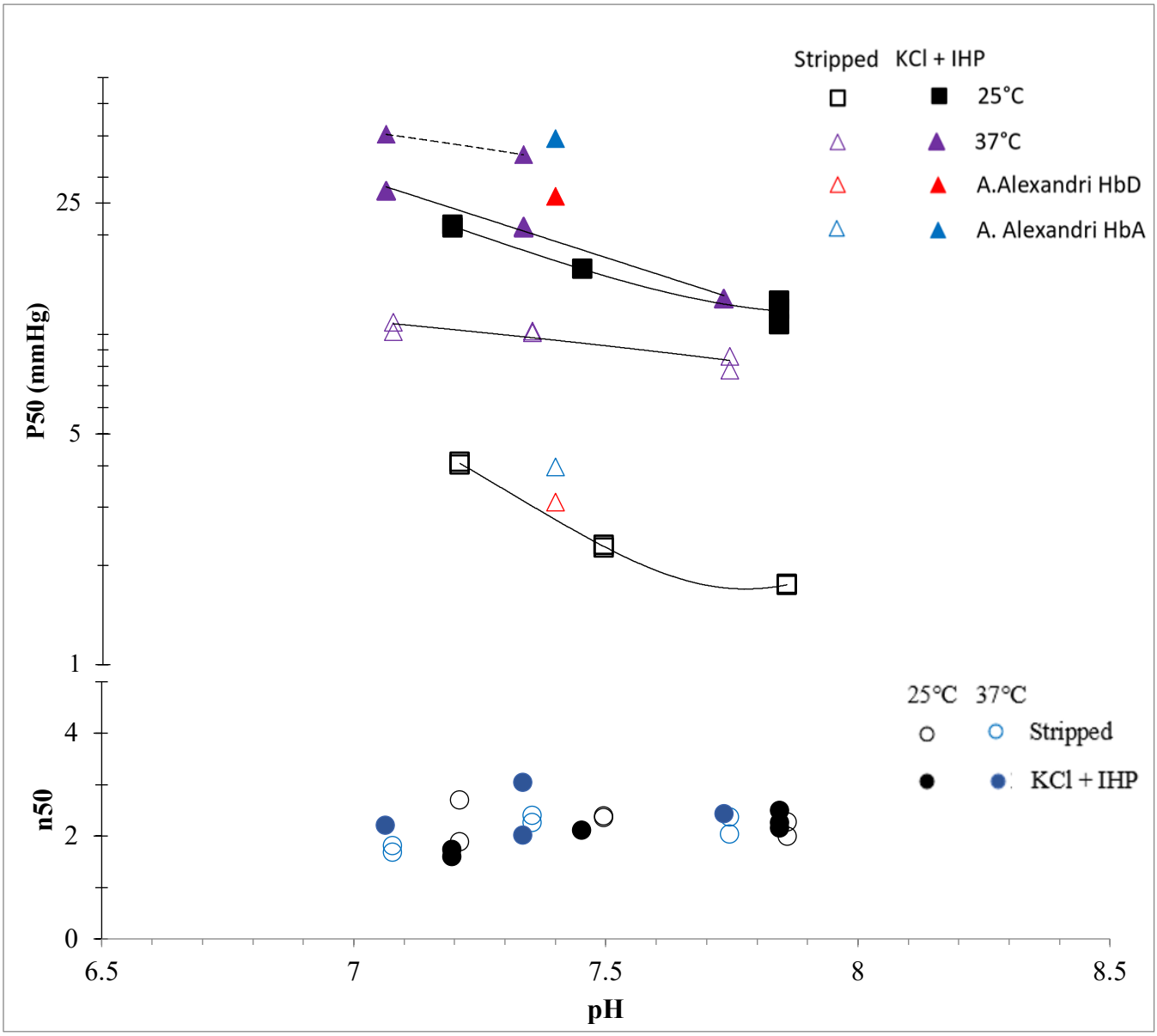

Figure 5. Effects of pH on ruby-throated hummingbird hemoglobin oxygen affinity (P_{50}) and cooperativity (n_{50}) values at 25°C and 37°C in the absence and presence of 0.1 M KCl and the two-fold excess of IHP. P_{50} values of the major (HbA) and minor (HbD) hemoglobin isoforms of *A. alexandri* at 37°C and pH 7.4 are shown for comparison (Natarajan et al. 2016). The dashed line refers to the results collected in the follow-up experiments.

The Bohr effect was higher in the presence of allosteric effectors at 37°C, while at 25°C, the Bohr effect was higher in the absence of allosteric effectors (Table 1). The Bohr effect was higher at 25°C than at 37°C, with the highest Bohr effect (-0.88) seen in the 25°C stripped Hb solution and the lowest (-0.06) seen in the 37°C stripped samples. The enthalpy of oxygenation was highly exothermic for the stripped solutions (i.e. relatively high negative values) while it was slightly endothermic in the presence of allosteric effectors. As pH increased, the stripped Hb solutions became more exothermic. Conversely, the KCl + IHP Hb solutions became more endothermic as pH increased. Similarly, stripped Hb showed a high thermal sensitivity that increased with pH, while in the presence of allosteric effectors, a reduction in thermal sensitivity at each of the measured pH values was observed (Table 1).

	Bohr factor (φ)		**Oxygenation enthalpy (ΔH') (kJ mol^{-1})**			**Thermal sensitivity (Δlog P_{50}/ΔT)**		
	37°C	**25°C**	**pH 7.0**	**pH 7.35**	**pH 7.75**	**pH 7.0**	**pH 7.35**	**pH 7.75**
Stripped	-0.055	-0.880	-39.81	-59.64	-82.32	-0.030	-0.041	-0.054
KCl + IHP	-0.401	-0.503	0.61	5.74	11.60	-0.007	-0.004	-0.001

Table 1. Bohr factor, overall enthalpy of oxygenation (ΔH'), and thermal sensitivity values for the stripped and KCl + IHP Hb solutions. Bohr effects were measured at 37°C and 25°C. The Bohr factor (φ) was calculated based on the initial experiment P_{50} values of pH ranging from 7 to 7.5. Oxygenation enthalpy and thermal sensitivity values were based on the initial experiment P_{50} values standardized to pH 7, 7.35 and 7.75.

Assuming a typical lung PO_2 of 100 mmHg, human arterial blood was shown to be ~97% saturated (18.21 mL O_2 dL^{-1}). Under standardized venous conditions (PCO_2 = 40 mmHg), modelled human venous and *A. colubris* venous blood displayed different % Hb-O_2 saturation levels (Figure 6), with the ruby-throated hummingbird venous OECs consistently being right shifted (i.e. a lower O_2 affinity) relative to human venous blood. Per single transit through the circulation, human venous blood was calculated to offload 6.74 mL O_2 dL^{-1} at the tissues during rest (Table 2). Assuming the same P_{50}, Bohr effect and [Hb] to humans, the lower estimated buffering capacity of *A. colubris* Hb offloaded ~52.5% (10.27 mL O_2 dL^{-1}) more O_2 per transit. However, accounting for the lower Bohr effect of *A. colubris* left shifted the initial *A. colubris* venous blood OEC (cf. curves 1 and 2 of Figure 6), though was still modelled to offload 30.5% (8.79 mL O_2 dL^{-1}) more O_2 than human venous blood. The further incorporation of hummingbird [Hb] into the model resulted in a further leftward shift (see curve 3 of Figure 6), though was calculated to still offload 49.9% (10.10 mL O_2 dL^{-1}) more O_2 per transit than the same volume of human venous blood. By contrast, the OEC modelled using hummingbird P_{50}, Bohr effect, buffering capacity and [Hb] (curve 4 of Figure 6) strongly shifted the hummingbird venous OEC to the right compared to human venous blood, releasing approximately 166.4% more O_2 (17.95 mL O_2 dL^{-1}) at the tissues during rest than human venous blood.

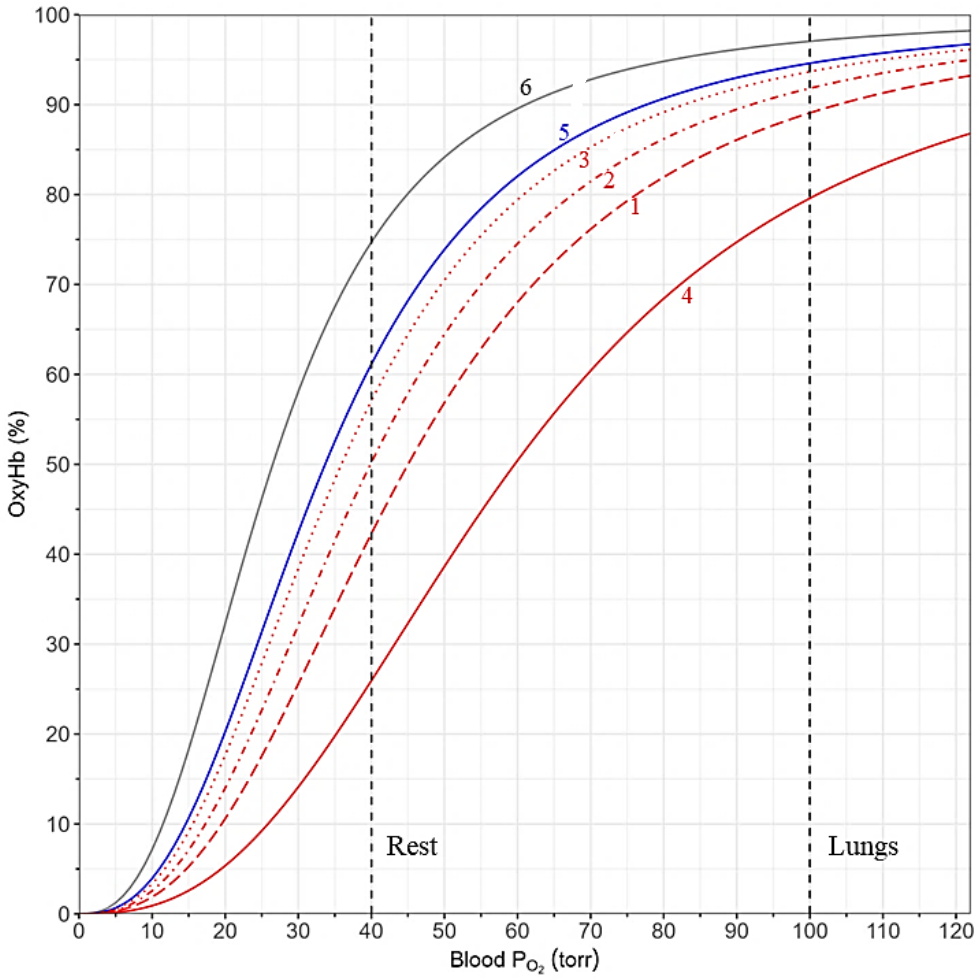

Figure 6. Modelled oxygen equilibrium curves of human arterial (black line), human venous (blue line) and ruby-throated hummingbird venous blood (red lines) at pH 7.4 and 37°C. All oxygen equilibrium curves, except for curve 4, were modelled using the human P_{50} value of 26.50 mmHg (Lahiri 1975). Oxygen equilibrium curve 1 (dashed red line) was modelled using the estimated ruby-throated hummingbird Hb buffering capacity (4.88 mol H^+ mol Hb_4^{-1} pH^{-1}). Curve 2 (dash-dot red line) additionally considered the hummingbird Bohr effect (-0.401), while curve 3 (dotted red line) further incorporated the higher [Hb] of hummingbird blood (18.84 g Hb/dL). Finally, oxygen equilibrium curve 4 (solid red line) was modelled using the hummingbird P_{50} value of Johansen et al (1987) (44.00 mmHg).

	OEC 1	OEC 2	OEC 3	OEC 4
[Hb]	14.00	14.00	18.84	18.84
*β*Hb	10.80	10.80	10.80	10.80
Bohr effect	-0.520	-0.401	-0.401	-0.401
Hemoglobin-oxygen saturation (%)				
Lungs	0.97	0.97	0.97	0.97
Human venous	0.61	0.61	0.61	0.61
Hummingbird venous	0.42	0.50	0.57	0.26
Maximum blood O_2 carrying capacity (mL O_2 dL^{-1})				
Human	18.76	18.76	18.76	18.76
Hummingbird	18.76	18.76	25.25	25.25
Arterial blood O_2 content (mL O_2 dL^{-1})				
Human	18.21	18.21	18.21	18.21
Hummingbird	18.21	18.21	24.50	24.50
Venous blood O_2 content (mL O_2 dL^{-1})				
Human	11.47	11.47	11.47	11.47
Hummingbird	7.93	9.42	14.40	6.55
Volume of O_2 offloaded (mL O_2 dL^{-1})				
Human	6.74	6.74	6.74	6.74
Hummingbird	10.27	8.79	10.10	17.95
% increase	52.49	30.45	49.86	166.37

Table 2. Ruby-throated hummingbird and human blood oxygen carrying capacity and delivery potential at pH 7.4 and 37°C. Hemoglobin-oxygen saturations were based on a typical lung PO_2 of 100 mmHg and a PCO_2 of 40 mmHg (standardized venous condition). Blood oxygen content and oxygen delivery were calculated using the Hüfner number (1.34 mL O_2 g Hb^{-1}) and oxygen equilibrium curves. Curve 1 assumed all human values expect the buffering capacity, which was adjusted to match that of the hummingbird (4.88 mol H^+ mol Hb_4^{-1} pH^{-1}). Curve 2 incorporated the measured hummingbird Bohr effect (-0.401), while curve 3 additionally accounted for the higher [Hb] of hummingbird blood (18.84 g Hb/dL). Curve 4 was modelled using all hummingbird values, including the hummingbird P_{50} value (44.00 mmHg).

The pH of the working solutions was affected by temperature, allosteric effectors and water (Table 3). The pH of all working solutions was higher at 25°C, and slightly lower in the presence of allosteric effectors.

25°C		
1 M HEPES buffer pH	**Stripped working solution pH**	**KCl + IHP working solution pH**
7.00	7.210	7.195
7.35	7.496	7.453
7.75	7.860	7.845
37°C		
1 M HEPES buffer pH	**Stripped working solution pH**	**KCl + IHP working solution pH**
7.00	7.077	7.063
7.35	7.354	7.336
7.75	7.746	7.735

Table 3. pH of the working solutions at 25°C and 37°C in the presence and absence of the allosteric effectors. Owing to the low blood sample volumes, pH of the working solutions was measured in the absence of Hb.

Discussion

As noted earlier, the ruby-throated hummingbird has one of the highest known mass-specific metabolic rates, leading to the consumption of a remarkable amount of O_2. Despite this insight, there is currently no study that characterizes the O_2 binding properties of Hb from the ruby-throated hummingbirds. In this study I was able to show how, compared to humans and other birds, the low buffering capacity of *A. colubris* Hb played a significant role in the amount of O_2 delivered to the tissues. Surprisingly, O_2 binding of their Hb was found to be thermally insensitive, which could be a potential benefit during torpor. Additionally, the chromatography elution profiles seemed to indicate the lack of the HbD isoform, which has not yet been recorded in hummingbirds.

The elution profiles over a 250 mM salt gradient (Figures 1A, 1B and 1C) displayed a single peak, while the elution profile over a pH gradient indicated no peak even at pH 8.8 (Figure 1D), which is the pH by which HbD is usually eluted. The HbD isoform tends to have an isoelectric point (i.e. the pH at which the net electrical charge of a molecule is zero) ranging from 6.8-7.3 while the isoelectric point for HbA usually ranges from 8.9-9.1 (Projecto-Garcia et al. 2013). Therefore, this data suggested that ruby-throated hummingbirds only express HbA inside their blood cells. However, the black-chinned and ruby-throated hummingbirds share identical α^A-chains and identical α^D-globin chains (Figures 2 and 3), which would not be expected if the *A. colubris* α^D-globin was inactivated and hence, no longer able to form HbD (i.e. selection against expressing α^D would be expected to increase the fixation of nucleotide substitutions in the ruby-throated hummingbird orthologue). Additionally, the black-chinned hummingbird expresses about 12.5% HbD (Natarajan et al. 2016), a percent high enough to be detectable on the elution profile if *A. colubris* similarly expressed HbD.

The observed *A. colubris* splice site mutation at exon 2 could theoretically influence the folding of the Hb molecule, which could explain the absence of HbD. However, the mutation of GT to GC is not entirely unusual and appears with a frequency of <1% in eukaryotes (Dietrich et al. 2001), including the exon 2 splice site of α^D in Anna's hummingbird and the chicken (data not shown). The relatively high transcript abundance (21.5%) of α^D mRNA in the *A. colubris* transcriptome (which was collected within the Toronto/London region) supports the lack of effect of the exon 2 GT→GC mutation, which further argues that a measurable HbD isoform should have been detected. Therefore, the single peak strongly suggests that the HbA and HbD isoforms did not separate during my chromatography trials. Therefore, I was working with a hemolysate, which is nonetheless more representative of whole blood than are the isolated Hb isoforms.

Hemoglobin-oxygen affinity

The Hb-O_2 affinity of the purified ruby-throated hummingbird Hb (hemolysate) varied depending on the presence of allosteric effectors and, to a lesser extent, temperature (Figure 5). As all n_{50} values were well above the value of 1, the Hb molecules in the various treatments exhibited cooperative O_2 binding (Soong et al. 2009). Comparable data pertinent to the results of this study are limited. However, Natarajan et al. (2016) reported P_{50} values for HbA and HbD from 18 different hummingbird species at pH 7.4 and 37°C. For the 18 species, P_{50} values for stripped Hb ranged from 2 to 4 mmHg, while those for Hb in the presence of KCl + IHP ranged from 17 to 40 mmHg (Natarajan et al. 2016). Projecto-Garcia et al. (2013) also reported P_{50} values for 10 hummingbird species at the same pH and temperature, but only for the HbA isoform; seven of these species were included in the Natarajan et al. (2016) study. For the three

species not included in Natarajan et al. (2016), P_{50} values for the stripped Hbs ranged from 2 to 3 mmHg, while those for Hb in the presence of KCl + IHP ranged from 19 to 29 mmHg (Projecto-Garcia et al. 2013). At the same temperature, and approximately same pH, the P_{50} values of the *A. colubris* KCl + IHP working solutions (21.17 mmHg and 21.05 mmHg) fell within the lower range of P_{50} values collected by Natarajan et al. (2016) and Projecto-Garcia et al. (2013). However, the P_{50} values of the stripped solutions (10.23 mmHg and 10.12 mmHg) were well above the range reported by Natarajan et al. (2016) and Projecto-Garcia et al. (2013). This aberration indicates that lingering Cl^- was likely present in my stripped working solutions (likely occurring during the final desalting column step).

The black-chinned hummingbird vs the ruby-throated hummingbird

The P_{50} values of *A. alexandri* HbA and HbD, collected by Natarajan et al. (2016) at 37ºC and pH 7.4, differed from those collected in this study. For example, *A. alexandri* stripped P_{50} values (3.97 mmHg and 3.1 mmHg, respectively), were markedly lower than *A. colubris* stripped hemolysate values (10.12 mmHg and 10.23 mmHg) at the same temperature and approximately similar pH (Figure 5). As mentioned earlier, the higher stripped P_{50} values obtained in this study were probably due to the presence of lingering Cl^-. In contrast to the stripped P_{50} values, *A. alexandri* HbA and HbD P_{50} values for the KCl + IHP Hb solutions (39.12 mmHg and 26.19 mmHg, respectively) at 37ºC and pH 7.4, were substantially higher than the *A. colubris* P_{50} values (21.17 mmHg and 21.05 mmHg) of the same temperature and approximately similar pH. Owing to these unexpectedly large differences, I conducted additional experiments on my remaining working solutions. The P_{50} values of the *A. colubris* KCl + IHP working solutions at 37°C of these follow-up experiments (Figure 5), were much closer to those

of the black-chinned hummingbird. Indeed, the value obtained at pH 7.34 (35.03 mmHg) was between those of the *A. alexandri* HbA and HbD isoforms (as was expected *a priori*). The different results between the first and second round of experiments could indicate potential problems associated with the BOBS during the initial experiments and/or the differences with the initial spectrophotometer calibration of the BOBS.

The two substitutions seen on the β-globin chain (Figure 4) likely explain the difference in P_{50} values between the black-chinned hummingbird and the (follow up) ruby-throated hummingbird experiments. For example, replacing serine (Ser) with glycine (Gly) at β83 (as is found in *A. colubris* Hb) has been shown to reduce the Hb-O_2 affinity (increase the P_{50}) of avian blood (Natarajan et al. 2016; Projecto-Garcia et al. 2013). Indeed, Projecto-Garcia et al. (2013) found that hummingbird species with the lowest Hb-O_2 affinities (highest P_{50s}) always had Gly at both positions β83 and β13, which is the case for the ruby-throated hummingbird. However, this contradicts the results of my initial KCl + IHP Hb experiments as the lower P_{50} of *A. colubris* Hb is indicative of a higher Hb-O_2 affinity. By contrast, results of my follow-up experiments are consistent with the β83 exchange, as the *A. colubris* hemolysate P_{50} values are between those of the *A. alexandri* HbA and HbD isoforms (Figure 5). In fact, assuming that my hemolysate has a 78.5:21.5 HbA to HbD ratio, the additionally collected P_{50} value at pH 7.35 (35 mmHg) was slightly higher than would be obtained if the *A. alexandri* HbD and HbA P_{50} values were combined in the same ratio (<30 mmHg). Therefore, the exchange of Ser with Gly at β83 supports the higher P_{50} values of my follow-up experiments.

The effect of the β22 aspartic acid (Asp) to glutamic acid (Glu) exchange on Hb-O_2 has not been previously studied. While Asp and Glu are both negatively charged, Glu

has a longer side chain that may influence the Hb structure, which in turn could also affect Hb-O_2 affinity and/or effector binding of *A. colubris* Hb relative to *A. alexandri*. Notably in this regard, Projecto-Garcia et al. (2013) modelled two potential IHP binding sites in the central cavity of the Hb molecule of *Adelomyia melanogenys* with one binding site being in close proximity to β22 (Figure 7A and 7B). Specifically, this study suggested that IHP preferentially binds to a site that is adjacent to β22 when β13Gly and β83Gly are present, as is found for *A. colubris*. Therefore, the longer anionic side chain of Glu in ruby-throated hummingbird Hb may alter the binding of the negatively charged IHP molecule relative to the *A. alexandri* Asp in the same position (Figure 7C and 7D). Alternatively, this replacement may impact electrostatic interactions with the adjacent alpha-chain, thereby potentially altering Hb-O_2 affinity. Future studies on *A. colubris* Hb should focus on this residue replacement as the actual effect of this exchange (if any) remains unknown.

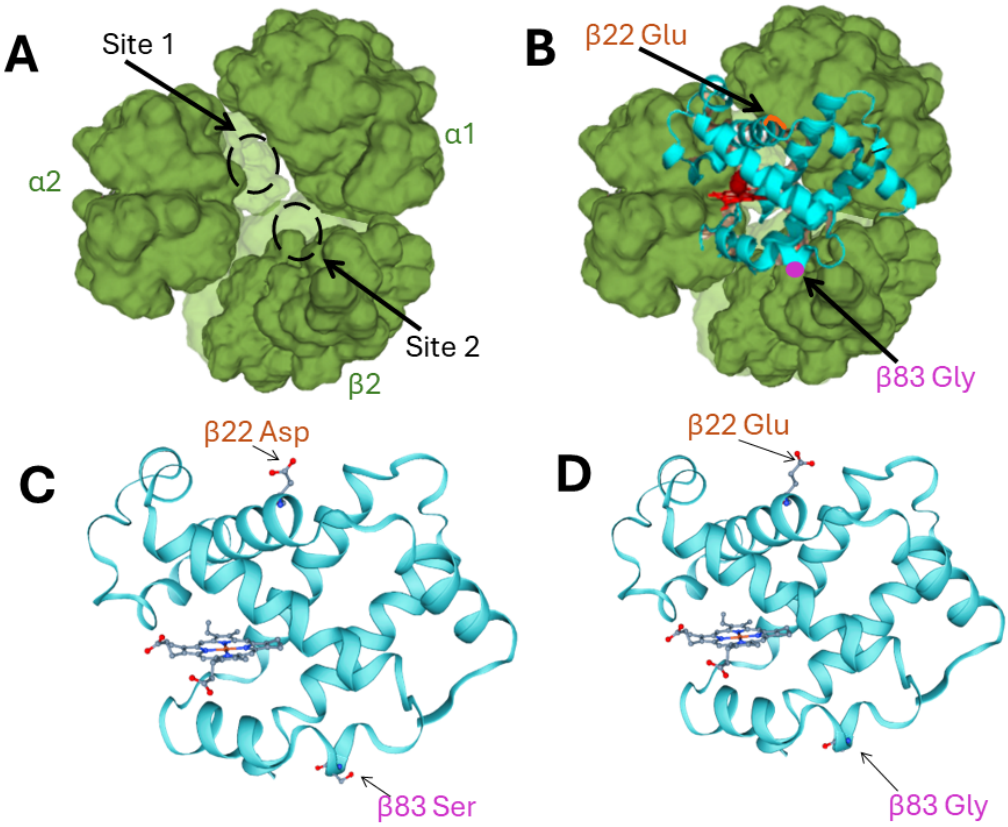

Figure 7. Structural models of ruby-throated and black-chinned hummingbird hemoglobin. The two IHP binding sites identified in hummingbird Hb by Projecto-Garcia et al. (2013) are denoted by dashed lines. Figures A and B show the positions of the β-chain residues 22 and 83 relative to two potential IHP binding sites in the central cavity: site 1 and site 2; the β1-globin chain is highlighted in cyan. Figure C is the modelled β1 subunit of the black-chinned hummingbird while figure D shows the β1 subunit of the ruby-throated hummingbird. Note the structural residue side-chain alterations arising from the β22Asp→Glu and β83Ser→Gly exchanges, and the close interaction between β22 and both the α1 chain and the proposed site 1 IHP binding site.

Overall oxygenation enthalpy and thermal sensitivity

The oxygenation of the heme groups of stripped ruby-throated hummingbird Hb was highly exothermic at all three pH values (Table 1). High oxygenation enthalpies in the absence of allosteric effectors has been reported in billfish (~ -62 kJ mol^{-1} O_2) at pH ~8, where proton binding to Hb is expected to be negligible (Weber et al. 2010). However, at the highest pH in this study (7.75), the oxygenation enthalpy of stripped Hb was even higher at -82.32 kJ mol^{-1} O_2, suggesting a highly exothermic reaction. At pH 7.5 stripped human Hb had an oxygenation enthalpy value of -59 kJ mol O_2 which is quite close to the oxygenation enthalpy of stripped ruby throated hummingbird Hb at pH 7.35 (-59.64 kJ mol O_2) (Atha and Ackers 1974).

Weber et al. (2010) observed a positive oxygenation enthalpy in marlin (between pH 7.15 to 7.55) and blue marlin (between pH 7.05 to 7.65) in the presence of ATP, the main allosteric effector of fish Hb. A slightly positive oxygenation enthalpy was also observed in *A. colubris* in the presence of the IHP and KCl allosteric effectors. The overall oxygenation enthalpy incorporates endothermic reactions resulting from the release of allosteric effectors during Hb oxygenation (Weber and Campbell 2011). Therefore, the dissociation of the allosteric effectors from the Hb molecule decreases the overall amount of heat released during oxygenation of the heme groups (i.e. the sensitivity of Hb-O_2 affinity to a change in temperature),leading to less negative oxygenation enthalpy values (Weber et al. 2014). Hemoglobin with a highly negative oxygenation enthalpy value (i.e. a high thermal sensitivity) promotes an increase in the amount of heat released during oxygenation of the heme group (Weber and Wells 1989). By contrast, a slightly positive oxygenation enthalpy, and by extension a thermally insensitive Hb, is expected to reduce the amount of heat lost at the lungs during Hb oxygenation. Campbell et al. (2012) found a reduced oxygenation enthalpy

in the Taiwanese brown-toothed shrew Hb and indicated that the reduction may aid in heat conservation. Campbell et al. (2012) suggested that given the unlikelihood of soricine shrews undergoing regional heterothermy and their relatively small size with high respiratory rates and surface area to mass ratios, a reduction in oxygenation enthalpy could function as a heat conservation mechanism. Therefore, as small-bodied birds, a similar concept of heat conservation could be applied to *A. colubris*, where the positive oxygenation enthalpy further indicates heat uptake at the lungs.

Unlike the individual P_{50} values, the thermal sensitivity values derived from the initial KCl + IHP working solution experiments are likely representative of actual *A. colubris* Hb data as they assessed the magnitude of change from 25°C to 37°C. Assuming an error in my initial experiments did occur, it likely was systematic, affecting the Hb-O_2 affinity of the same working

As noted earlier, the presence of allosteric effectors makes the Hb-O_2 affinity of *A. colubris* almost perfectly thermally insensitive (Table 1). By contrast, Johansen et al. (1987) noted a reduction in thermal sensitivity in *Melanotrochilus fuscus* and *Eupetomena macroura* Hb from -66.5 kJ mol O_2 (stripped) to -30.6 and -31.3 kJ mol O_2 (in presence of allosteric effectors) at pH 7.40, respectively. Johansen et al. (1987) attributed the cause of this reduction to the presence of cofactors, as was shown in this study. The difference in thermal sensitivity values between this study and that of Johansen et al. (1987) could be due to the presence of different IHP binding sites between these species. As shown in figure 7A, Projecto-Garcia et al. (2013) modelled two alternative IHP binding sites. Therefore, in *M. fuscus* and *E. macroura*, IHP could preferentially be binding to a site different to that of *A. colubris,* causing a difference in oxygenation enthalpy values and consequently leading to different thermal sensitivity values.

The low thermal sensitivity observed in *A. colubris* could also be beneficial during torpor. This species undergoes torpor not only when ambient temperatures are low, but also during mid-summer when their fat stores are low (<5% of body mass) (Eberts et al. 2021). Eberts et al. (2021) also found that during the late summer, *A. colubris* undergo torpor before migration, especially when their fat stores are high. While still unclear, undergoing torpor before migration is thought to accelerate fat build up (Eberts et al. 2021). Therefore, not only does torpor have a versatile role in energy conservation, it also is commonly implemented by *A. colubris.* During torpor *A. colubris* can drastically decrease their body temperature (Lasiewski 1963), by almost 20 degrees according to Eberts et al. (2021). Weber and Campbell (2011) suggested that a low thermal sensitivity may be beneficial for regionally heterothermic animals as it protects O_2 delivery to the cold extremities. A high thermal sensitivity in regionally heterothermic animals can disturb the balance between O_2 demand and release, particularly in regions that have significantly different temperatures from that of the core of their bodies (Weber and Campbell 2011). While *A. colubris* are temporal heterotherms (Shankar et al. 2022), a similar concept of protecting O_2 delivery could be applied. Being temporal heterotherms, their body temperature can fluctuate depending on ambient temperature (Lasiewski 1963). Therefore, having a low thermal sensitivity Hb phenotype can be beneficial in terms of maintaining adequate O_2 delivery during bouts of torpor. For example, cardiac output, a primary determinant of O_2 delivery to the tissues, is drastically reduced during torpor (Ambler et al. 2021). A low cardiac output coupled with a high thermally sensitivity of O_2 binding/release is thus expected to significantly impact O_2 offloading at the cool tissues. By contrast, having a thermally insensitive Hb should permit sufficient O_2 to be offloaded at the cool tissues even when cardiac output is low.

A low thermally sensitive Hb may also be beneficial when *A. colubris* arouse from torpor. During this process, *A. colubris* need to rewarm their bodies and one way they achieve this is by shivering (Ambler et al. 2021). For the mitochondria to produce the energy (ATP) required for shivering, additional O_2 is required. When the body temperature is low, a thermally insensitive Hb is better able to deliver O_2 to the muscles to initiate heat production, fulfilling the increasing demand of the mitochondria. Delivery of O_2 to the tissues is further facilitated by the increase in heart rate during rewarming (Ambler et al. 2021). Therefore, the amount of O_2 offloaded by a thermally insensitive Hb is primarily determined by cardiac output and not temperature.

Buffering capacity, Bohr effect and oxygen delivery

The following discussion will focus primarily on the Bohr effect of Hb in the presence of IHP and Cl^- ions at 37°C, as it is most relevant to *in vivo* conditions of the ruby-throated hummingbird red blood cells. The Bohr factor of Hb in the presence of allosteric effectors at 37°C was found to be -0.401 (Table 1). This value is only slightly higher than that reported for whole blood of three hummingbird species (-0.39) by Johansen et al. (1987). Moreover, the three hummingbird species studied by Johansen et al. (1987) are short-distance migrant hummingbird species in comparison to *A. colubris*. As the -0.401 Bohr factor was calculated based on the initial P_{50} values, the similarity with the value obtained by Johansen et al. (1987) further emphasizes the point that if an error in measuring Hb-O_2 affinity did occur, it was probably due to a systematic error that affected all of the Hb-O_2 affinity measurements equally. Based on available Bohr effect data in birds, Boggs and Birchard (1983) reported one of the lowest Bohr factors of -0.35, in the ring-necked pheasant (*Phasianus colchicus*), and

the highest Bohr factor (-0.61), in the greater rhea (*Rhea americana*). Within this range (~-0.35 to -0.6), a Bohr factor of -0.401 that I measured in the current study falls within the lower end of values, suggesting that *A. colubris* has a relatively low Bohr effect compared to other bird species.

On its own, a low Bohr effect may seem surprising for hummingbirds. For instance, a leftward shift of venous blood was observed when the *A. colubris* Bohr effect was reduced from -0.520 (i.e. the Bohr effect in humans; curve 1 of Figure 6) to -0.401 (i.e. the measured *A. colubris* Bohr effect; curve 2). This shift consequently decreased the amount of O_2 offloaded per single circulation from 10.27 mL O_2 dL^{-1} to 8.79 mL O_2 dL^{-1}. Despite the reduction in amount of O_2 offloaded, the net effect was an increase in the O_2 reserve (~14%) available for exercise. Therefore, a low Bohr effect may ensure that a larger O_2 reserve is available for *A. colubris* muscles during exercise.

A low Hb buffering capacity is expected to enhance O_2 offloading at the tissues by causing an exaggerated Bohr effect (Berenbrink 2006; Campbell et al. 2012). Based on the calculations of Northam (2022), the Hb buffering capacity for 19 of 20 hummingbird species ranged from ~4.7 to 4.9 mol H^+ mol Hb_4^{-1} pH^{-1} (this predominantly is due to species differences in HbA:HbD isoform ratios as the predicted HbA and HbD buffer capacities are highly conserved in this lineage). Assuming a HbA:HbD ratio of 78.5:21.5, the estimated Hb buffering capacity of *A. colubris* hemolysate (4.88 mol H^+ mol Hb_4^{-1} pH^{-1}) falls within the range of values predicted for other hummingbirds by Northam (2022). Additionally, Northam (2022) estimated the Hb buffering capacity for approximately 39 different bird species within the Strisores clade, with values ranging from ~4.7 to 9.6 mol H^+ mol Hb_4^{-1} pH^{-1}. Based on the range estimated by Northam (2022), *A. colubris* has a low buffering capacity. A low buffering capacity allows a given load of acid/CO_2 to decrease the Hb-O_2 affinity

at the tissues by a greater extent, thereby allowing for a greater offloading of O_2 (Northam 2022). Modelling *A. colubris* Hb with the same Bohr effect, P_{50} and [Hb] as humans, a decrease in buffering capacity from 10.80 mol H^+ mol Hb_4^{-1} pH^{-1} (Siggaard-Andersen 1974) to 4.88 mol H^+ mol Hb_4^{-1} pH^{-1} (estimated βHb of *A. colubris*) increased the amount of O_2 offloaded by 52.5% per trip through the circulation (Figure 6). Therefore, a low buffering capacity is shown to markedly enhance the effectiveness of the low Bohr effect of ruby-throated hummingbird Hb and offload more O_2.

The low buffering capacity of *A. colubris* may also increase blood O_2 carrying capacity by permitting a higher [Hb] (Northam, 2022). A higher [Hb] indicates an elevated amount of buffering groups per unit volume of blood, which consequently can decrease the effectiveness of the Bohr effect (Northam, 2022). Inclusion of the average hummingbird [Hb] of 18.84 g Hb/dL (Williamson et al. 2023) into the model, in conjunction with *A. colubris* Bohr effect and buffer capacity, shifted the position of hummingbird venous blood towards that of human venous blood (curve 3 of Figure 6). While this positioning indicated a similar Hb-O_2 saturation of both curves at a PO_2 of 40 mmHg, ~50% more O_2 was still offloaded in *A. colubris* than in humans owing to the lower [Hb] of ~14.00 g Hb/dL in humans (Hlastala and Woodson 1975). The advantage (in terms of O_2 delivery) of a higher [Hb] and lower buffer capacity of *A. colubris* Hb became more prominent when the average hummingbird whole-blood P_{50} of 44.00 mmHg (Johansen et al. 1987) was taken into account (curve 4 of Figure 6). At rest, *A. colubris* venous blood was modeled to release 17.95 mL O_2 dL^{-1} at the tissues per trip through the circulation, which is 166.4% more O_2 offloaded than human venous blood. As noted earlier, *A. colubris* are long distant migrants that have a very high mass-specific metabolic rate (Johansen et al. 1987; Projecto-Garcia et al. 2013); hence, per gram, *A. colubris* consume a lot of O_2 (Lasiewski 1963; Withers 1983).

Therefore, the combination of a higher P_{50}, lower buffering capacity and higher [Hb] of ruby-throated hummingbirds compared to humans offset their relatively low Bohr effects, thereby markedly increasing the amount of O_2 offloaded each trip through the circulation during rest.

Conclusions

While further experiments are needed to determine the correct O_2 affinities of ruby-throated hummingbird Hb relative to other hummingbird species, preliminary findings indicated that their affinities do fall within the range observed in available hummingbird P_{50} data, at least those for the KCl + IHP working solutions. Differences in the P_{50} values between this study and those of the black-chinned hummingbird may be due to amino acid substitutions on the β-globin chain or technical issues with the experimental setup. Nonetheless, the extremely low thermal sensitivity (slightly positive oxygenation enthalpy) of *A. colubris* Hb in the presence of allosteric effectors is suggested to minimize respiratory heat loss and ensure efficient O_2 delivery to the tissues, particularly during torpor. The similarity in the *A. colubris* Bohr factor to that of relatively short-distance migrant hummingbirds suggests that migration distance did not select for a change in the magnitude of the Bohr effect, at least not in *A. colubris*. Additionally, the low buffering capacity observed in *A. colubris*, compared to other birds, enhances their relatively low Bohr effect and presumably allows for a higher [Hb] contributing to increased O_2 carrying capacity and delivery during both rest and exercise. Based on the available whole-blood hummingbird Hb-O_2 affinity and [Hb], the addition of *A. colubris* Hb Bohr effect and buffering capacity indicates that over 2.5 times the amount of oxygen is delivered to the tissues per unit of blood compared

to humans. Therefore, these findings shed light on the remarkable physiological adaptations of the ruby-throated hummingbirds that help them fulfill their substantial oxygen requirements.

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