The *in vivo* Oxyhaemoglobin Dissociation Curve at Sea Level and High Altitude

by

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Institute of Medical Science

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Abstract

Some animals have adapted to hypoxia by increasing their haemoglobin affinity for oxygen, but *in vitro* studies have not shown any change of haemoglobin affinity for oxygen in human high altitude natives or lowlanders acutely acclimatized to high altitude. We conducted the first *in vivo* study of the oxyhaemoglobin dissociation curve by progressively reducing arterial PO_2 while maintaining normocapnia in lowlanders at sea level, lowlanders sojourning at 3600m for two weeks and native Andeans at the same altitude. We found that the *in vivo* PO_2 at which haemoglobin is half-saturated (P_{50}) is higher in lowlanders at sea level (32 mmHg) than that measured *in vitro* (27 mmHg) and that lowlanders and highlanders do significantly increase the *in vivo* affinity of their haemoglobin for oxygen with exposure to high altitude. These results indicate the value of an *in vivo* approach for studying the oxyhaemoglobin dissociation curve.

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List of Abbreviations

[2,3-DPG]	Concentration of 2,3-Diphosphoglycerate
[Hb]	Haemoglobin Concentration
2,3-DPG	2,3-Diphosphoglycerate
ABG	Arterial Blood Gases
AMREE	American Medical Research Expedition to Everest
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BE	Base Excess
BUN	Urea Nitrogen
CaO₂	Arterial Oxygen Content
Cl	Chloride
CO	Carbon Monoxide
CO₂	Carbon Dioxide
EPO	Erythropoietin
H⁺	Hydrogen Ion
Hb	Haemoglobin
HbO₂	Oxyhaemoglobin
HCO₃⁻	Bicarbonate
Hct	Hematocrit
HVD	Hypoxic Ventilatory Decline
HVR	Hypoxic Ventilatory Response
K	Potassium
Na	Sodium
NIBP	Non-Invasive Blood Pressure
NO	Nitric Oxide
O₂	Oxygen
ODC	Oxyhaemoglobin Dissociation Curve
OE II	Operation Everest II
P₅₀	Partial Pressure of Oxygen at which Haemoglobin is 50% Saturated
PaCO₂	Arterial Partial Pressure of Carbon Dioxide
PaO₂	Arterial Partial Pressure of Oxygen
PCO₂	Partial Pressure of Carbon Dioxide
PEEP	Positive End Expiratory Pressure
PetCO₂	End Tidal Partial Pressure of Carbon Dioxide
PetO₂	End Tidal Partial Pressure of Oxygen
PO₂	Partial Pressure of Oxygen
RBC	Red Blood Cell
SaO₂	Arterial Haemoglobin Saturation
SEM	Standard Error of the Mean
SO₂	Oxyhaemoglobin Saturation
SpO₂	Pulse Oximeter Haemoglobin-Oxygen Saturation

Std Dev	Standard Deviation
T	Temperature
TCO₂	Total Carbon Dioxide
VCO₂	Rate of Carbon Dioxide Elimination
VO₂	Rate of Oxygen Consumption
VO₂ max	Maximal Oxygen Consumption

1 Literature Review

1.1 Oxygen (O₂) Transport

O₂ diffuses from the air into the blood through alveolar and endothelial cells in the lung alveoli. The O₂ is then transported by the blood to the tissues, where it diffuses into the cells to be used in aerobic metabolism. The amount of O₂ available to the tissues is determined by the cardiac output, the flow of blood moving through the body, and the arterial blood O₂ content (CaO₂). CaO₂ is dependent upon haemoglobin concentration [Hb], the volume of O₂ dissolved in the plasma and arterial haemoglobin oxygen saturation (SaO₂). The SaO₂ is dependent upon the arterial partial pressure of oxygen (PaO₂) and the affinity of haemoglobin (Hb) for O₂. CaO₂ can be calculated according to Equation 1:

Equation 1

$$CaO_2 = \left(Hb \times 1.34 \times \left(\frac{SaO_2}{100} \right) \right) + (PaO_2 \times 0.0031)$$

where CaO₂ is the arterial O₂ content in volume %, Hb is in gram % and PaO₂ is in mmHg.

O₂ is transported through the body in two ways (Sladen, 1981). A relatively small amount, approximately 1.5% of the total O₂ available to the tissues of the body, is dissolved in the plasma according to Henry's law. That is, at any given temperature the volume of O₂ dissolved in a volume of plasma is directly proportional to the partial pressure of O₂ (PO₂) in equilibrium with the plasma. At a normal body temperature of 37 °C and a PaO₂ of 100 mmHg, the volume of Hb dissolved in 100 ml of blood is 0.31 cc. The remainder of the body's O₂ is transported by Hb, which is found in red blood cells (RBC). Hb makes up approximately 35% of total RBC volume, and mean corpuscular Hb concentration is approximately 32-36 g/dl.

Hb consists of four heme groups attached to a globin protein. The primary structure of Hb is its genetically determined amino acid sequence while its secondary, tertiary and quaternary structures result from both intrinsic and environmental factors. Human Hb is made of four polypeptide chains, each of which contains one heme group, the binding site for molecular oxygen. A heme group is composed of a porphyrin ring with a ferrous ion that binds one O₂

molecule (Juel & Milam, 1979). The affinity of each heme group for molecular O_2 is determined by the structure and spatial arrangement of the globin component.

Hb A, which is composed of two alpha and two beta Hb chains, constitutes 96% of the Hb in adult human red blood cells. The structures of the alpha and beta chains were discovered in 1961 (Braunitzer *et al.*; Konigsberg *et al.*, 1961). The secondary structure of Hb A contains a large proportion of alpha helices, which are linked by stretches of amino acids that are in random coil arrangements. The arrangements of these helices lead to the creation of a hydrophobic cleft where the heme moiety is inserted. The tertiary structure of the Hb is defined by the spatial positions of the helical segments, while the quaternary Hb structure is defined by the positions of the four subunit chains.

The binding of O_2 with each Hb subunit causes a conformational change in the Hb. This was first described by Muirhead *et al.* (1967) who found that the reaction of Hb with O_2 is accompanied by a change in the structure of Hb. Perutz (1970) then described the structural changes that occur in the Hb molecule upon oxygenation. He documented that the combination of an O_2 molecule with a heme group changes the position of the ferrous ion in the heme ring. This triggers a series of physicochemical events that change the position of the peptide chain, such that the salt bridge with a neighbouring chain is broken. This results in a change of the O_2 affinity of the heme group in the neighbouring subunit chain. Through this sequence of events, each combination of an O_2 molecule with a heme group facilitates the binding of the next one. This co-operativity is responsible for the sigmoidal relationship between PO_2 and the degree of saturation of Hb with O_2 .

1.2 The Oxyhaemoglobin Dissociation Curve

The oxyhaemoglobin dissociation curve (ODC) is used to depict the relationship between O_2 tension and the degree of Hb saturation with O_2 . Ward *et al.* (2000) provided a review of the early work on the ODC in the book *High Altitude Medicine and Physiology*. The early studies were published in French and German, so the information presented in this paragraph was taken from this review. In 1878 Bert created the first plot of O_2 and carbon dioxide (CO_2) dissociation curves, in which he showed the relationship between partial pressure and blood gas concentrations for CO_2 and O_2 in animals. He did not discover the sigmoidal shape of the curve because he did not use a full range of PO_2 values. In 1885 Bohr was the first to plot the ODC in

its entirety, using dilute Hb solutions. His curves were hyperbolic and were not compatible with those of Bert. In 1890 Hfner then published similar curves to those of Bohr. He attributed the hyperbolic shape of the curve to the association of Hb with O₂ according to the equation $\text{Hb} + \text{O}_2 \rightarrow \text{HbO}_2$ (oxyhaemoglobin). Barcroft and Camis confirmed these results in dilute Hb solutions (1909). Bohr finally discovered the sigmoidal shape of the curve by studying the ODC of whole blood.

The position of the ODC influences how easily O₂ is taken up at the lungs, and how easily it is offloaded at the tissues. More specifically, a parallel shift of the ODC to the right reflects a decrease in oxyhaemoglobin affinity that facilitates O₂ offloading at the tissues, while a parallel shift to the left reflects an increase in oxyhaemoglobin affinity that facilitates O₂ uptake at the lungs. The O₂ affinity of Hb is known to be influenced by a number of physiological parameters, which will be explained in detail below. Besides temperature, which affects the thermodynamics of the reaction between Hb and O₂, these parameters primarily interact with either the reduced or oxygen-bound form of Hb (HbO₂), thereby changing its affinity for O₂.

Like other biological molecules, Hb can form bonds with ligands other than O₂. These ligands bond with different groups in the globin molecule and usually have a greater affinity for one form of Hb, binding either to HbO₂ or reduced haemoglobin. The binding of these ligands to Hb alters the affinity of Hb for O₂. There are a number of ligands that preferentially bind to reduced Hb. These lower the affinity of Hb for O₂ and shift the ODC to the right. These ligands are present in red blood cells in varying concentrations under different conditions. The most important ligands that bind readily to reduced Hb and thus lower the Hb affinity for O₂ are hydrogen ions (H⁺), CO₂, inorganic anions (such as chloride) and organic phosphates like 2,3-diphosphoglycerate (2,3-DPG) and adenosine triphosphate (ATP). The ligands of Hb are interdependent, since ambient temperature and the concentration of each ligand affect the binding of other ligands to Hb (Hlastala, 1979).

1.2.1 Temperature

The first observation of the effect of temperature on the saturation of Hb with O₂ (SO₂) was attributed to Bert in 1872 (McConn, 1975) who noted that SO₂ was 90% at a PO₂ of 15 mmHg at room temperature, but only 50% at body temperature.. Barcroft and King (1909) confirmed these results and showed that increases in temperature shift the ODC to the right, decreasing the

affinity of Hb for O₂. As the combination of Hb and O₂ is exothermic, an increase in temperature favours dissociation. The converse is true as well; if temperature falls, the exothermic association of Hb and O₂ is favoured, and the ODC shifts to the left. In creating his slide rule gas calculator, Severinghaus (1966) described the changes in PO₂ with temperature (T) at constant saturation (Equation 2):

Equation 2

$$\Delta \log PO_2 = 0.024 \times \Delta T$$

1.2.2 The Bohr Effect (pH)

The relationship between hydrogen ion concentration [H⁺] and O₂ binding to Hb is twofold. Firstly, H⁺ can act as a ligand for Hb, resulting in a conformational change in Hb that makes its O₂ binding site less accessible, reducing the binding capacity of Hb for O₂. H⁺ also reduces the affinity of Hb for O₂ because reduced Hb is a weaker acid. This is because the binding of Hb with O₂ breaks salt bridges, exposing acidic groups. For these reasons, reduced Hb is favoured over HbO₂ in acidic environments, and a decrease in pH shifts the ODC to the right, favouring Hb reduction and the offloading of O₂. This lowering of affinity in acidic environments is referred to as the Bohr Effect, since it was first documented by Bohr, Hasselbalch and Krogh in 1904, though in this case they were referring to the general effects of CO₂ on the position of the ODC.

The magnitude of the Bohr Effect can be expressed for a constant oxygen saturation by the change in logPO₂ caused by a unit change in pH, as shown in Equation 3:

Equation 3

$$\Delta \log PO_2 = -0.4$$

where the pH change is induced by an acid or base other than CO₂, and where the concentration of CO₂ and the organic phosphate 2,3-DPG remains constant.

1.2.3 Carbon Dioxide

CO₂ is known to shift the ODC via two mechanisms. Firstly, most CO₂ in the blood is converted into bicarbonate (HCO₃⁻) and H⁺ ions by the enzyme carbonic anhydrase. Increases in the partial pressure of CO₂ (PCO₂) therefore increase [H⁺] and shift the ODC to the right via the Bohr Effect. In addition, some CO₂ combines with Hb, as carbamino compounds. CO₂ preferentially binds with reduced Hb, resulting in a conformational change that reduces the affinity of the Hb for O₂. This property is referred to as the Haldane Effect.

The reduction of PCO₂ in the pulmonary capillaries augments the uptake of O₂, while the increase in PCO₂ at the tissues aids in the offloading of O₂ from Hb. The influence of CO₂ on SO₂ is therefore greatest at lower SO₂, when it is most needed. When a change in PCO₂ causes a change in pH at constant PO₂, the relationship between the change in pH and the change in O₂ saturation is defined according to Equation 4:

Equation 4

$$\Delta \log PO_2 = -0.48$$

1.2.4 2,3-Diphosphoglycerate (2,3-DPG)

2,3-DPG is a by-product of glycolysis and is the most abundant organic phosphate in RBCs. Like CO₂ and H⁺, 2,3-DPG preferentially binds to, and stabilizes the reduced form of Hb, decreasing saturation for any given PO₂ (Benesch *et al.*, 1968). 2,3-DPG also affects the position of the ODC via its effect on pH; increases in 2,3-DPG lower RBC pH, and a decrease in RBC pH has been shown to shift the ODC to the right (Bellingham *et al.*, 1971). The binding of 2,3-DPG to Hb is dependent upon temperature (Benesch *et al.*, 1969), pH (Garby & De Verdier, 1971; Rorth *et al.*, 1973), PCO₂ (Bauer, 1969) and the presence of anions (Benesch *et al.*, 1969), so the extent to which it shifts the ODC (though not the direction) is variable. 2,3-DPG concentration affects both the shape and the position of the ODC (Samaja & Winslow, 1979).

1.2.5 Inorganic Salts

Since Hb is a protein made up of polar and non-polar amino acids, changes in the concentrations of inorganic ions its environment, such as chloride (Cl), may change the conformation of the protein, affecting the accessibility of the heme binding sites for O₂,

1.2.6 Carbon Monoxide

The O₂ binding site on Hb has more than 200 times the affinity for carbon monoxide (CO) as it does for O₂, so the presence of CO interferes with the ability of Hb to bind with O₂. Its presence also has the effect of shifting the ODC to the left (by increasing the affinity of free, neighbouring heme groups to O₂), and it gives the ODC a more hyperbolic shape. CO therefore severely limits the availability of O₂ to the tissues.

1.2.7 Red Blood Cells (RBCs) and Haemoglobin

Because RBC 2,3-DPG concentration [2,3-DPG] decreases with age, Hb within older RBCs tends to have a higher O₂ affinity than Hb in young RBCs (Haidas *et al.*, 1971; Edwards & Rigas, 1967). It has been reported that high concentrations of Hb are associated with decreased O₂ affinity (Bellingham *et al.*, 1971). However, there does not appear to be a systematic relationship between Hb concentration and the position of the ODC (Winslow *et al.*, 1978).

1.3 Measuring the ODC

Because the ODC is a product of the complex interplay of many different factors, its shape and position can only be determined by direct measurement. The entire ODC can be plotted by drawing one or more blood samples and analyzing them *in vitro*. Although it is useful to see the whole curve, the ODC is generally quantified by the P₅₀, which is the PO₂ at which 50% of the haemoglobin is saturated. P₅₀ may be directly measured using one of the techniques discussed below, or it can be determined from a number of points using the Hill Equation, a three parameter equation that describes the relationship between PO₂ and SO₂ (Hill, 1910). The linearized form of this equation is shown below (Equation 5).

Equation 5

$$\log \frac{SO_2}{100 - SO_2} = \log K + n \log PO_2$$

where SO_2 is percent saturation, K is a constant, n is the slope of the linearized ODC (also known as the coefficient of co-operativity), and PO_2 is the partial pressure of oxygen. First described in 1910, the Hill equation is still used to describe this relationship.

Traditionally, the “standard P_{50} ” is that which is determined under standard conditions. These include a blood serum pH of 7.4 (which translates into a RBC pH of approximately 7.1-7.2, due to excess of large anions in the cell) and a T of 37° C; the standard P_{50} may also be corrected to a PCO_2 of 40 mmHg. The “physiological P_{50} ” is that which is measured at the pH, T and PCO_2 of the blood sample. The standard P_{50} is a useful tool with which to compare the O_2 affinity of Hb of populations under different physiological conditions. At standard body temperature and pH and normal concentrations of 2,3-DPG, the standard P_{50} is 26-27 mmHg. A measured P_{50} value can be compared to reference value to determine whether an individual’s ODC is “shifted.” A lower P_{50} indicates a left-shifted ODC (with relatively increased Hb affinity for O_2) that favours O_2 uptake from the environment, while a higher P_{50} indicates a right-shifted curve (with relatively decreased Hb affinity for O_2) that favours O_2 offloading at the tissues. The ODC and some of the factors that influence its position are shown below, in Figure 1.

Figure 1: The ODC

1.3.1 *In vitro* methods for measuring the shape and position of the ODC

1.3.1.1 *In Vitro* Equilibration

Astrup et al. (1965) described a method for measuring the ODC *in vitro*. A small sample of blood is placed in a tonometer and exposed to a series of gases of varying PO₂ tensions at constant PCO₂, with the balance composed of nitrogen. Once the sample comes into equilibrium with each gas, the pH and saturation are measured. By using a wide range of gas compositions, the entire curve can be plotted. A few of these points can be used with the Hill equation to allow the calculation of the P₅₀.

1.3.1.2 Mixing Technique

An alternate method of designating Hb-O₂ affinity was described by Edwards and Martin (1966). In this method fully oxygenated and deoxygenated samples of blood are mixed in various proportions in order to produce samples of varying saturations. P₅₀ is measured by mixing equal volumes of desaturated and saturated blood.

1.3.1.3 Dissociation Curve Analyzer

Duvelleroy *et al.* (1970) described a method that plots the entire ODC from a 10 ml sample of blood by measuring the O₂ uptake of completely deoxygenated blood. Blood is first completely desaturated by exposure to 95% nitrogen per 5% CO₂. It is then exposed to a gas mixture of 95% O₂ and 5% CO₂ while being stirred. Two oxygen electrodes take continuous readings of PO₂ within both the gas and blood until the two phases are equilibrated.

1.3.1.4 Canizaro's Nomogram

This nomogram (Canizaro *et al.*, 1974), which has been validated against the mixing technique mentioned above, estimates P₅₀ based on the PO₂ and SO₂ values of a single venous blood sample. Because it relies on interpolation based on two measurements, the caveat of this method is that the two measurements must be extremely accurate, or the resulting P₅₀ may be very inaccurate. Unfortunately, saturation monitors and gas analyzers vary in their accuracies at different saturation levels, so the estimated P₅₀ may also be quite inaccurate.

1.3.1.5 Severinghaus Equation and Slide Rule

P₅₀ can be found using the Severinghaus equation (Equation 6).

Equation 6

$$P_{50} = 26.6 \times \frac{PO_2}{PO_{2sat}}$$

where the PO_{2sat} is the PO₂ on the standard dissociation curve that corresponds with the measured saturation. Alternatively, the Severinghaus (1966) slide rule can be used. The slide rule offers a simple method in which P₅₀ is estimated by aligning PO₂ on one scale with

saturation on another. The P_{50} can then be read off as the PO_2 on the first scale that corresponds with the 50% saturation mark on the second scale.

1.3.2 Comparing P_{50} s

P_{50} values under standard conditions are calculated to facilitate comparisons of experimentally determined values. Blood gases are usually measured at 37.5°C so temperature need not be adjusted. On the other hand, pH may deviate from standard values (serum pH of 7.4 and RBC pH of 7.2), especially under conditions of illness or environmental stress. Therefore, pH of the serum and RBCs should be measured. If they deviate from the standard values, correction for pH is made using the Bohr equations. The magnitude of the Bohr Effect has been previously reported as 0.4 when the pH change is due to H^+ (Equation 3) and 0.48 when it is due to a change in PCO_2 (Equation 4). The final P_{50} will also depend on the effect of other ligands in the RBC, such as 2,3-DPG and inorganic salts. Correcting only for pH may not result in a correct P_{50} .

1.3.3 Predicting Saturation

The accurate measurement of SO_2 requires photometric equipment, which is expensive and can be difficult to use. A number of models have therefore been created to predict saturation from more readily available arterial blood measures, such as PCO_2 , PO_2 and pH. The Kelman model (Kelman, 1966) uses PCO_2 , pH, and temperature to calculate a “virtual PO_2 ” (the PO_2 that would lead to the same saturation if measured under standard conditions of T, PCO_2 and pH), which is then used to predict SO_2 . This model was shown to be both reliable and valid at sea level (Breuer *et al.*, 1989). Because it assumes normal acid-base status and 2,3-DPG levels, this model may be inaccurate at altitude.

A number of newer algorithms that incorporate additional variables have been recently introduced. Dash and Bassingthwaighe (2004) included temperature and 2,3-DPG. The latter addition makes this equation especially suitable for measurements taken at altitude, where changes in [2,3-DPG] becomes a significant influence on SO_2 . Results using their model correlate well with published values. Chiari *et al.* (1997) presented a comprehensive model taking into account ventilatory, circulatory and chemical parameters. This model is a useful simulation tool, but its practical use in predicting saturation is limited because of its complexity.

1.4 Physiological Effects of High Altitude

Atmospheric pressure decreases with increasing altitude. This is associated with a decrease in PO_2 , despite a constant fractional percent of O_2 . This drop in atmospheric PO_2 will lead to a decrease in PaO_2 and thereby, without compensation, the amount of O_2 available to the tissues. This is at least partially prevented by changes in ventilation and the presence of factors that influence the way that O_2 binds to Hb.

1.4.1 Hypoxic Ventilatory Response

The first response upon acute ascent is an increase in ventilation, which is known as the hypoxic ventilatory response (HVR). It is usually caused by both a decrease in the ventilatory recruitment threshold (as seen by a leftward shift of the isoxic relationship between PCO_2 and ventilation as compared to normoxia), as well as an increase in sensitivity to PCO_2 (as seen by an increase in the slope of the relationship between PCO_2 and ventilation) (Mohan & Duffin, 1997; Duffin & Mahamed, 2003). HVR is followed by a ventilatory decline (HVD), which occurs with continued exposure to hypoxia.

The hyperventilation of HVR leads to a fall in PCO_2 as the ratio of ventilation to CO_2 production increases. Similarly, alveolar PO_2 increases as the turn-over of air in the lungs increases while O_2 consumption remains constant. The fall in PCO_2 increases the affinity of Hb for O_2 via the Haldane effect and the development of a respiratory alkalosis. HVR therefore increases Hb- O_2 saturation via increasing alveolar PO_2 and the Hb affinity for O_2 .

exceeds 17 g/dl, reducing tissue perfusion and offsetting the benefits with respect to tissue O₂ delivery. As mentioned above, young Hb cells contain more 2,3-DPG than older cells, so accelerated erythropoiesis results in RBCs with increases in [2,3-DPG], which reduces Hb-O₂ affinity. The benefit of this, if any, to overall tissue O₂ delivery with hypoxia will be discussed in greater detail in the following sections.

1.4.3 2,3-DPG

2,3-DPG is a metabolic intermediate produced in the glycolytic pathway of RBCs. It is produced during the anaerobic Embden Meyerhof pathway by a side reaction called the Rapoport Leubering shuttle, which converts 1,3-diphosphoglycerate to 3-phosphoglycerate without the creation of ATP. This side reaction occurs in 20% of the glycolytic flux during RBC metabolism (Juel & Milam, 1979). 2,3-DPG levels vary with the rate of glycolysis or with changes in the activity of the enzymes diphosphoglyceromutase and diphosphoglycerate phosphatase.

The rate of glycolysis can be affected by the internal milieu, such as pH, PO₂ and the concentration of inorganic phosphates. Acidosis decreases 2,3-DPG by down regulating phosphofructokinase and by altering the equilibrium between 1,3-DPG and 2,3-DPG. Inorganic phosphates stimulate glycolysis (and therefore 2,3-DPG production) independently of pH. In renal disease, 2,3-DPG increases in proportion to the increase in serum inorganic phosphate levels. However, inorganic phosphates also have a small dampening effect on diphosphoglyceromutase, an enzyme involved in 2,3-DPG production from 1,3-DPG, though this effect must be small because the overall effect of inorganic phosphates is to increase [2,3-DPG].

Under hypoxic conditions there is an increase in reduced Hb levels. This increases the binding of 2,3-DPG to Hb and reduces the intracellular [2,3-DPG]. This results in less feedback inhibition of the enzyme diphosphoglyceromutase and subsequently leads to an increase in the conversion of 1,3-DPG to 2,3-DPG. Hypoxia also increases [2,3-DPG] through its induction of respiratory alkalosis, the major mechanism of increasing [2,3-DPG] in response to ascent to high altitude.

1.5 Population Studies at High Altitude

Andeans have been at high altitude for approximately 11,000 years and Tibetans for almost 25,000 years (Aldenderfer, 2003). These high altitude populations differ from each other, as

well as from other high altitude populations and acutely acclimatized lowlanders, in a number of ways. The information presented below was reviewed by Beall (2007c).

While acutely acclimatized lowlanders show a decreased maximal O_2 consumption and an increased basal metabolic rate (showing a limited capacity to increase O_2 consumption), Andeans and Tibetans have a normal range of O_2 consumption, with expected basal metabolic rates and expected O_2 consumption capacity based on their age, sex and weight, which are comparable to those of lowlanders at sea level.

Lowlanders born at sea level exhibit increased ventilation at high altitude. This increase occurs immediately after initial exposure to high altitude and decreases over time, but resting ventilation tends to remain increased above sea level values while the lowlanders remain at altitude.

Tibetans born and raised at high altitude also retain an increase in ventilation over sea level values. Andeans, as well as Europeans and Chinese who were raised at high altitude, display a relative hypoventilation as compared to the other groups at high altitude, including Tibetans and lowlanders born at sea level.

With acute exposure to high altitude, lowlanders show an increased HVR as compared to sea level. Over a period of days their HVR returns to normal levels and after weeks or months it falls below normal levels. This is in contrast with Tibetan natives, who show an HVR that is similar to that of lowlanders at sea level, and Andeans, who show an HVR that is lower than that of lowlanders at sea level. The indefinite sustained HVR in Tibetans likely plays a role in their adaptation to life at high altitude.

Although Tibetans have relatively high ventilation and HVR responses that allow them to respond more to fluctuations in blood O_2 levels, they have lower CaO_2 than Andeans natives at high altitude, lowlanders at sea level, and Ethiopians at high altitude. Andean high altitude natives have increased CaO_2 over the other three groups. This can be partially explained by their relatively high EPO and Hb levels. Blood O_2 content in Andeans is also increased because of their relatively high O_2 saturation. Andean and Tibetan natives at high altitude both have resting SO_2 values that are lower than the normal sea level values. However, ambient Andean SO_2 values have been found to be more than one standard deviation higher than those of Tibetans at high altitude. Surprisingly, Ethiopians at high altitude have SO_2 values that are within the normal sea level range.

Another mechanism that is involved in acclimatization and adaptation to high altitude is that of changes in blood flow. Along with SO_2 , tissue blood flow determines tissue O_2 delivery. Lowlanders at high altitude often exhibit pulmonary hypertension, which results from hypoxic pulmonary vasoconstriction, a protective reflex that shunts blood away from areas of the lung with poor ventilation or oxygenation. At high altitude, the entire lung becomes hypoxic and widespread pulmonary arteriole constriction can lead to pulmonary hypertension. Tibetans do not usually exhibit pulmonary hypertension (they have normal or slightly elevated pulmonary artery pressure or hypoxic pulmonary vasoconstriction (they have normal pulmonary blood flow). This is probably due to increased levels of nitric oxide (NO) as compared to Andeans at high altitude, lowlanders at high altitude and lowlanders at sea level. Consistent with this is that nitric oxide synthesis tends to be down regulated in lowlanders at high altitude, and Andeans are known to exhibit a high incidence of pulmonary hypertension. Tibetans also increase blood flow through increased capillary density in muscles as compared to Andean high altitude natives. They therefore have greater diffusion capacity that can compensate for their decreased CaO_2 .

Muscle mitochondria levels also differ across populations at altitude and sea level. Lowlanders tend to lose leg muscle mitochondria during the first three weeks of exposure to high altitude. Similarly, leg muscle mitochondrial volume is lower in Andeans and Tibetans at high altitude than in lowlanders at sea level. Decreased mitochondria volume is even found in Tibetans who are born and raised closer to sea level, indicating that, in the Tibetan population, the decrease in mitochondria is not dependent upon exposure to high altitude. The functional benefit of a decrease in mitochondrial number at high altitude is not clear, since basal O_2 metabolism is not lower. In Tibetans, this decrease in mitochondria somehow supports normal metabolic rates, perhaps indicating greater metabolic efficiency.

Effect size is equal to the mean difference between two samples, divided by their pooled variance. An effect size of 0.8 or greater is usually considered as large, and indicates that there is no overlap in almost 50% of the observations in the two samples that are being compared. Using this method, certain effect sizes between Tibetans and Andeans are large. These include the higher ventilation of Tibetans, the lower haematological traits of the Tibetans especially for Hb, the higher NO and capillary density in Tibetans, lower Tibetan arterial O_2 levels, lower Tibetan SO_2 , and lower Tibetan pulmonary artery pressure. It therefore seems that the two high altitude populations have evolved different sets of adaptations to life at high altitude.

1.6 The ODC at Altitude

It has been known since the turn of the 20th century that altitude hypoxia causes physiological stress. Many investigations have since been conducted in order to uncover the mechanisms with which the body is able to deal with this stress. A great focus of this research has been on the ODC. As mentioned above, there are a number of known physiological changes that occur with exposure to hypoxia. Many of these have the ability to influence the shape and position of the ODC. It is for this reason that the net effects of these changes on the ODC have been looked at in native highlanders and lowlanders sojourning at high altitude.

These studies have been carried out at various altitudes, in different populations with different acclimatization profiles. They involve either the calculation or *in vitro* measurement of P_{50} . Because of different measurement methods and conditions, the results have not been consistent. A few key studies will be presented along with their results.

1.6.1 The ODC in Lowlanders at High Altitude

The position of the ODC in acclimatized lowlanders sojourning at high altitude was first measured by Barcroft (1911). He was aware that hypoxia is associated with hyperventilation and a fall in PCO_2 that is associated with a leftward shift of the ODC. For that reason he expected to find that the ODCs of the lowlanders were left-shifted at altitude as compared to sea level. Instead, he found that the physiological curves were identical at sea level and altitudes of 2130 and 3000 m, and that the standard curves (at a PCO_2 of 40 mmHg) were right shifted. He posited that his data provided evidence for the presence of some acid that was compensating for the decrease in carbonic acid, and that the increase in this acid corresponded to increases in altitude so that it matched the change in carbonic acid and the physiological ODC would not be shifted.

In a subsequent study Barcroft *et al.* (1915) found that, above 3048 m, the ODC at the prevailing PCO_2 is slightly right-shifted. This was assumed to reflect an excess of lactic acid, which is present at high altitude due to the lack of oxygen and which counteracts the alkalosis caused by hyperventilation. He suggested that at lower altitudes there is the presence of some other acid, due to an acid-base adjustment performed by the kidneys. In yet another study Barcroft *et al.* (1923) reported a left-shifted ODC at altitude as measured at the prevailing PCO_2 , which in this particular study was between 25-30 mmHg. This leftward shift was attributed to alkalemia and

was argued to be beneficial, as it would enhance O₂ loading at the lung. These results were not reconciled with those previously reported.

Keys *et al.* (1936) studied the position of the ODC in acclimatizing lowlanders at six different altitudes, from sea level to 6140 m. The ODCs of the subjects at altitude standardized for pH were right shifted (P₅₀ increase of 3.5 mmHg). At physiological pH, the ODC's were left-shifted up to an altitude of approximately 4267 m, above which they became increasingly right-shifted. This decrease in affinity at the higher altitudes was considered advantageous, as it would facilitate O₂ unloading at the tissues.

Two groups of researchers simultaneously discovered the effects of 2,3-DPG on the ODC (Chanutin & Curnish, 1967; Benesch & Benesch, 1967). They discovered that this organic phosphate, a by-product of RBC metabolism, is an allosteric modulator of Hb that binds to its reduced form and decreases its affinity for O₂ (Benesch *et al.*, 1968). Soon thereafter, Lenfant *et al.* (1968) reported that the increase in the standard P₅₀ in lowlanders during acclimatization to high altitude is accompanied by an increase in [2,3-DPG]. Both P₅₀ and [2,3-DPG] normalized upon return to sea level. With this discovery, all previous speculation regarding the direction and cause of shifts of the ODC at altitude were rendered disputable, as they did not measure [2,3-DPG]. In previous studies the blood samples were not always analyzed in a timely fashion. Therefore, the 2,3-DPG may have been metabolized before measurement, as [2,3-DPG] rapidly decreases under *in vitro* conditions.

Once it was established that [2,3-DPG] increases upon ascent to high altitude, Lenfant *et al.* (1971) conducted an experiment to determine the relationship between pH change, [2,3-DPG] and shifts of the ODC at high altitude. Two subjects were made acidotic with the carbonic anhydrase inhibitor acetazolamide before ascending to high altitude along with four control subjects, to whom no drugs were administered. The experimental group did not exhibit an increase in pH, an increase in [2,3-DPG] or a shift of the ODC. Meanwhile, the control group developed a respiratory alkalosis and increased their P₅₀ and [2,3-DPG]. Half of the changes in both P₅₀ and [2,3-DPG] occurred within 15 hours of altitude exposure. They interpreted the similar time course as an indication the two changes were probably related. That the acidotic experimental group did not exhibit an increase in [2,3-DPG] or P₅₀ provided further confirmation that the increase in [2,3-DPG] is a response to the respiratory alkalosis. A later study (Milles *et*

al., 1987) found that those receiving acetazolamide did have an increase of pH and [2,3-DPG] above baseline levels, though to less of an extent than those who did not. They also had greater PO₂ levels than the controls.

According to the results of Lenfant *et al.* (1971), the increase in 2,3-DPG upon ascent to high altitude provides some metabolic compensation for the respiratory alkalosis, though this 'compensatory' mechanism does not seem to be complete at extremely high altitudes. Blood pH does not return to the normal sea level value of 7.4, even after a few weeks at altitude (Lahiri & Milledge, 1967). This issue was studied by Winslow *et al.* (1984) during the 1981 American Medical Research Expedition to Everest (AMREE). While [2,3-DPG] is assumed to be affected by pH, above 6300 m they found large changes in pH but only small changes in [2,3-DPG]. They also found that up to 6300 m there is no physiological shift of the ODC, since 2,3-DPG counteracts the effect of respiratory alkalosis on the ODC. Above 6300 m the physiological ODC was shifted progressively to the left. This was primarily attributed to the respiratory alkalosis that overwhelmed the effect of increases in [2,3-DPG]. Meanwhile, standard P₅₀ was slightly increased when measured at pH 7.4. Torrance *et al.* (1970a) showed that 2,3-DPG increases only up to 4500 m). Incomplete compensation was thought to be beneficial because it protects against reductions in SaO₂ at severe hypoxia. It should be noted that the data from above 6300 m was made in only one subject, and it was derived from blood samples collected at 6300 m, which were adjusted based on alveolar gas samples collected on the summit.

Mairbaurl *et al.* (1990) looked at the physiological P₅₀s of lowlanders during progressive ascent to an altitude of 4559 m. This was the first study to directly measure P₅₀ under physiological acid-base conditions, where the normal Bohr and Hill coefficients may not be applicable. They found an initial decrease in physiological P₅₀ with initial altitude exposure, followed by a return to the sea level value within a few days despite continued exposure to altitude, reflecting a balance between respiratory alkalosis and the effects of 2,3-DPG. Meanwhile, standard P₅₀ increased upon altitude exposure and continued to increase with continued exposure. This is a reflection of the effect of the increase in [2,3-DPG] on the ODC. These data also confirmed that early acclimatization is associated with young RBCs that are high in 2,3-DPG, which is associated with decreased Hb-O₂ affinity. However, it was not clear whether the increased hematocrit itself or age of the RBCs or a combination of both was responsible for the decreased affinity and increase in [2,3-DPG].

The topic was re-examined during Operation Everest II (OE II), a simulated ascent of Mount Everest (Wagner *et al.*, 2007). In this study there was a progressive increase in standard P_{50} as barometric pressure fell from 760 to 282 mmHg. This increase was attributed to a progressive increase in [2,3-DPG], assumed to be induced by progressive hypoxia, because standard P_{50} accounts for PCO_2 and pH. Meanwhile, physiological P_{50} was steady at the normal value (between 26-27 mmHg) despite decreases in atmospheric pressure. These data agree with those of AMREE up to an altitude of 6300 m, but do not agree with the AMREE data from above 6300 m. This is because the PCO_2 values at extreme altitude were higher, and pH lower, in the case of OE II. There are a number of possible reasons for this apparent contradiction. Firstly, the AMREE data beyond this altitude was based on only one subject, who may not have had a typical response. Alternatively, it could be due to differences in how the summit measurements were made, or due to the different ascent profiles of the participants of AMREE and OE II, since the OE II subjects “ascended” more quickly than those who participated in AMREE. Lastly, it could be because the AMREE subject had a greater reduction in PCO_2 and a greater increase in pH than those in OE II, though these differences should only have translated to a P_{50} difference of 0.5 mmHg.

Based on these studies, it seems that the increase in [2,3-DPG] with exposure to high altitude is associated with an increased standard P_{50} , while the physiological P_{50} remains at the normal sea level value and may become progressively lower at extreme altitudes.

1.6.2 The ODC in Highlanders

Previous studies have indicated that the extent of altitude exposure affects the degree of acclimatization to high altitude. Those who are native to high altitude, or those who have dwelled there for many years, may exhibit different responses to the altitude than do lowlanders who are acutely acclimatized. Additionally, those whose ancestors have been at altitude for thousands of years may have evolved adaptations that affect the affinity of Hb for O_2 . This is the case in genetically adapted animals, such as llamas and yaks (Beall, 2007b). Other instances of such genetic adaptations and their associated benefits will be discussed below.

The ODC was first measured in highlanders at altitude by Barcroft *et al.* (1923), who reported a left-shifted curve in Peruvian highlanders as compared to that of lowlanders at sea level. This was attributed to alkalemia of acclimatization, since a similar shift was observed in the

lowlanders studied at the same altitude. Keys *et al.* (1936) conducted a similar study in Peruvian highlanders living at 5340 m. They found that the standardized ODCs of the highlanders (measured at a cellular pH of 7.1) were right shifted with respect to lowlanders at sea level, but left shifted with respect to lowlanders at high altitude, the latter of which was attributed to a greater degree of acclimatization due to a longer period of exposure to high altitude. Because, however, the lowlanders at altitude exhibited a respiratory alkalosis (whereas the altitude residents had pH values similar to those of lowlanders at sea level), their ODCs at physiological pH were similar to those of the altitude residents.

A later study by Aste-Salazar and Hurtado (1944) looked at standard and physiological ODCs in Peruvian highlanders as well as in lowlanders at sea level and lowlanders at 4540 m. They found that the high altitude residents had right-shifted curves as compared to lowlanders at sea level, both at standard serum pH of 7.4 and at physiological pH. This was explained as being beneficial for offloading O₂ at the tissues.

Morpurgo *et al.* (1970) conducted a study on Peruvian high altitude natives and found that their ODCs were right shifted with respect to lowlanders at sea level and with respect to Europeans native to similar altitudes (though the ODCs of the Europeans were also right shifted with respect to the normal sea level values). The Peruvian high altitude natives were also found to have a greater Bohr Effect. This was not found to be due to a genetic Hb modification, so it was attributed to the presence of some unknown factor that lowers Hb-O₂ affinity. This higher Bohr Effect was seen as an evolutionary adaptive mechanism for increasing O₂ delivery to the tissues, since it was present in those who grew up at altitude whose families had been at altitude for many years, but not in those who grew up at altitude but whose ancestors were from lowland regions. The right shift may also have been due to the increase of [2,3-DPG] (Benesch *et al.*, 1968).

Morpurgo *et al.* (1976) later conducted a study of Sherpas native to an altitude of 3800-3900 m. Unlike Andean highlanders and Europeans residing at altitude, these Sherpas did not have increased haematological values (RBC number, [Hb] or [2,3-DPG]) as compared to lowlanders at sea level. Their ODCs were also shifted strongly to the left as compared to Caucasians living at sea level, and the fact that the standard ODCs of their haemolysed blood were similar to their standard whole blood ODCs gives evidence that the Hb-O₂ affinity of Sherpas at high altitude is

not greatly affected by the RBC environment. Meanwhile, Sherpas permanently living at low altitudes (Kathmandu) exhibited a lower Hb-O₂ affinity than the Caucasian lowlander controls and they were found to be somewhat anaemic, as defined by lower Hb levels (however, their tissues were still well oxygenated because of the lower Hb-O₂ affinity). The lower affinity was attributed in part to an increased 2,3-DPG/Hb ratio. Meanwhile, Sherpas living at altitude had [2,3-DPG] similar to Caucasians living at sea level and much lower than Caucasians acclimatized to high altitude. They posited that Sherpas, unlike Andean natives, have genetically adapted to life at high altitude due to prolonged exposure over many more generations to the stress of hypoxic hypoxia, which would have increased the possibility of natural selection and evolution. However, some of the observations they made may have been physiological, and not genetic, in nature. While their findings were interesting, it must be noted that their blood samples were analyzed 5-6 days after being collected, so their results may not be valid. This is because *in vitro* P₅₀ and 2,3-DPG are known to decrease over time. (Aberman *et al.*, 1976)

Samaja *et al.* (1979) were dissatisfied with the conflicting results from previous studies. They identified possible sources of variation in the data—that previous studies may have overlooked certain factors that are known to influence Hb-O₂ affinity (for example, studies performed before the identification of the role of 2,3-DPG may have missed the influence of this important molecule). Another possible source is that blood collection, storing, transfer, and analysis techniques may have differed between studies. They therefore conducted their own studies on Sherpas living at high altitude (3850 m), high altitude Sherpas who had been living at 1450 m (tested at sea level), Caucasians temporarily dwelling at 3500 m and sea level controls. All blood samples were analyzed within 6 hours of being collected. Their results indicated no difference in the standard P₅₀s (at serum pH 7.4 and PCO₂ of 40 mmHg) of the different groups, despite the fact that the two groups at altitude had increased ratios of 2,3-DPG to Hb concentrations. Electrophoresis did not reveal the presence of any abnormal haemoglobin. This indicates that high altitude Sherpas do not have a genetic or acquired facilitated O₂ transport mechanism.

Winslow *et al.* (1981) used a technique to plot the entire ODC rapidly and accurately under both standard and physiological conditions. This method was first applied to a population at sea-level, where it was found that there is great variation in P₅₀ (Winslow *et al.*, 1978). The study was then performed in a population of Peruvians who were native to 4540 m and in sea level

controls. Results of this study indicated that the high altitude natives had elevated [2,3-DPG] and subsequently had standard P_{50} s that were higher than the sea level controls, but due to a compensated respiratory alkalosis the physiological P_{50} s of the two groups did not differ. As in their previous study, they found great variation in the P_{50} s of the sea level controls and the high altitude dwellers. The range of P_{50} s was greater for the altitude population than it was for the sea level population.

In summary, as was the case with lowlanders at high altitude, there is no consensus about the position of the ODC in native highlanders. However, since the discovery of the influence of 2,3-DPG on the Hb affinity for O_2 , studies performed at physiological pH and PCO_2 have not found a shift of the ODC. This has been attributed to a balance between alkalosis and an increase in [2,3-DPG]. Meanwhile, studies performed under standard conditions of pH and PCO_2 tended to find a rightward shifted ODC, resulting from increases in [2,3-DPG].

1.6.3 What type of shift is beneficial at altitude?

Some animals native to hypoxic environments have adapted by developing increased Hb affinity for O_2 . High altitude ostriches and huallata have higher O_2 affinities than bird species that are not native to high altitude. Similarly, the P_{50} of bar-headed geese, which fly over the Himalayas during migration, is more than 10 mmHg lower than that of closely-related birds from moderate altitudes (Black & Tenney, 1980). The degree of the change in affinity seems to be related to the degree of hypoxia, as determined from the case of deer mice (Snyder *et al.*, 1982; Snyder, 1985). Those that live at higher altitudes have higher affinities than their lower altitude relatives, and when these mice are relocated to lower altitudes the changes persist into the next generation of animals. This genetic basis can also be seen in llamas, which exhibit a high Hb affinity for O_2 for generations after being raised in zoos at sea level (Banchero *et al.*, 1971; Banchero & Grover, 1972).

Another interesting case is that of the tadpole. Tadpoles often live in stagnant water that is low in O_2 and high in CO_2 . Tadpole Hb has a higher affinity for O_2 than that of adult frogs and a smaller Bohr Effect, so the high CO_2 in the stagnant water in which tadpoles usually live does not lead to further offloading of O_2 .

The globin chain of human foetal Hb has a different amino acid sequence than the adult form. In dilute solutions the P_{50} of the foetal Hb is approximately the same as that of adult Hb, but in whole blood solutions the foetal P_{50} is only 19 mmHg. In the foetal environment, the PO_2 is less than 30 mmHg, equivalent to that faced by a climber on the summit of Everest. Interestingly, both the foetus and climber deal with this by increasing the affinity of their Hb for O_2 . In some mammalian foetuses (e.g. dog, horse, pig) the increase in affinity is due to a decrease in organic phosphate concentrations, while in others [2,3-DPG] is similar to that in the adult, but the Hb in foetal RBCs is less sensitive to it.

Given that these animals that live in low O_2 environments have genetic adaptations that shift their ODCs to the left, it seems that this leftward shift confers an advantage at altitude. In one of their studies, Barcroft *et al.* (1923) found a slightly increased affinity of Hb for O_2 . They argued that this was beneficial, as it would enhance O_2 loading from the atmosphere at the lungs. In contrast, Aste-Salazar and Hurtado (1944) reported a slightly decreased Hb affinity for O_2 in high altitude natives. They argued that a decreased affinity is beneficial because it enhances O_2 offloading at the tissues, where it is needed. This argument was also used by Lenfant and Sullivan (1971). They noted that the increased [2,3-DPG] leads to a decrease in Hb affinity for O_2 . They explained that this is beneficial, as it allows for peripheral O_2 unloading. They posited that this decreased affinity plays an important role in the acclimatization of lowlanders to altitude, and in the genetic adaptations of those native to altitude.

Eaton *et al.* (1974) wished to test the hypothesis that decreased Hb affinity for O_2 is advantageous at altitude. They manipulated Hb- O_2 affinity of rats, via carbamylation of the Hb, to create high and low affinity groups. They then exposed both groups of rats to hypobaria to simulate altitude conditions. They found that those with higher Hb- O_2 affinity had better survival outcomes than those with decreased affinity.

During a theoretical study, Turek *et al.* (1978) showed that high Hb affinities for O_2 are beneficial at altitude, especially during exercise. They then found that cyanate-treated rats maintained better oxygen transfer to tissues during severe hypoxia than did normal animals.

Hebbel *et al.* (1978) studied a family in which two out of the four children have mutant Andrew-Minneapolis Hb, with a P_{50} of 17.1 mmHg. The increased O_2 affinity of the mutant Hb seemed to confer a sort of pre-adaptation to altitude; at a moderate altitude of 3100 m, the mutant Hb

siblings exhibited lower increases in resting heart rates than their normal siblings and minimal increases in plasma and urinary EPO levels, while they did not exhibit thrombocytopenia or decrements in O₂ consumption. They had similar 2,3-DPG increases to their normal siblings.

A theoretical study by Bencowitz *et al.* (1982) examined the effects of changes in Hb-O₂ affinity on exercise tolerance at altitude to reconcile the conflicting evidence from previous studies. They determined that the effects of shifts in the ODC depend on whether or not diffusion limitation is present. When complete blood-gas equilibration is allowed to occur via diffusion, a rightward shift that favours O₂ offloading at the tissues is beneficial. However, when diffusion limitation is present, such as during exercise at altitude, a leftward shift is beneficial, since it allows for greater O₂ uptake. They demonstrated that a leftward shift can be beneficial, especially at higher altitudes and especially during exercise.

Wagner (1997) performed a theoretical modelling study to determine the optimal P₅₀ for O₂ transport during maximal exercise at varying altitudes (sea level, intermediate altitude and extreme altitude equivalent to the summit of Everest). He found that maximal oxygen consumption (VO₂ max) is insensitive to P₅₀ at all altitudes (from sea level to extreme altitude), such that neither a right nor left shift of the ODC will enhance or compromise VO₂ max during maximal exercise. However, as altitude increases, VO₂ max technically peaks at decreasing P₅₀s, as the difference between arterial and venous PO₂ decreases.

Animals that have adapted to hypoxic conditions generally have Hb with a higher affinity for O₂ than other animals. It therefore seems that increased affinity is adaptive. Indeed, a number of human and animal studies have found that an increased Hb-O₂ affinity can be beneficial under hypoxic conditions. However, no such shift has been found before in the ODC of human blood measured under physiological acid-base conditions.

2 Statement of the Problem

2.1 Drawbacks of Measuring the ODC *in vitro*

While *in vitro* methods of measuring the ODC, when used properly, yield consistent and reliable results, they may not accurately represent what is actually happening physiologically, even if the measurements are taken under naturally occurring conditions. The actual position of the curve depends on the metabolic and respiratory status at the time of measurement. The human body is dynamic and the changes that take place in one system are highly dependent on others, but the physiological integration of the body systems cannot be easily captured in a test tube. To understand how the body interacts with the environment, one must take a more systemic approach that reflects the conditions present in a breathing human that is continuously responding to changes in the internal and external environment.

The limited potential of *in vitro* measurement becomes even more important under non-standard conditions. This is because *in vitro* measurement imposes specific constraints on the blood, which may not be realistic, without accounting for other factors that may be present. For example, it has already been mentioned that ions may interact with Hb and affect its affinity for O₂. Many diseases are associated with ion imbalances, which will affect the shape and position of the ODC. *In vitro* analysis of blood samples taken under disease conditions may not consider the physiological interactions of such ions with the Hb-O₂ relationship and may therefore ignore factors that play important roles in SO₂. Further, environmental factors, such as altitude hypoxia, may influence the ODC in ways other than those that are reflected by *in vitro* methods. *In vitro* methods of measurement may not be sensitive to the acute and chronic changes that occur with altitude hypoxia, and predictive methods often completely ignore the effects of 2,3-DPG, increased at altitude, on the shape and position of the ODC. Illnesses such as high altitude sickness, high altitude pulmonary oedema and high altitude cerebral oedema, which occur with acute exposure to hypobaric hypoxia, may be related to shifts in the ODC. Characteristic *in vivo* data of the shifts of the ODC are necessary for the understanding of the development of such illnesses.

In addition to not providing a full picture of how changes in PO₂ are reflected in SO₂, *in vitro* methods of measuring the ODC require highly specialized equipment, which is expensive to buy and can be difficult to operate. Meanwhile, predictive methods, which are based upon *in vitro*

data, are subject to large errors because they rely on the accuracy of one or two samples to be used with extrapolation. Therefore, an *in vivo* method for studying the ODC would more faithfully reflect the natural changes of O₂ affinity of Hb with progressive hypoxia.

2.2 Difficulty of Measuring the ODC *in vivo*

PCO₂, PO₂, and ventilation are usually coupled in a breathing human (as depicted in Figure 3). For example, hypoxia causes an increase in ventilation, which is subsequently associated with increased PO₂ as well as with decreased PCO₂.

Figure 3: Coupling of PO₂, Ventilation & PCO₂

This coupling is what makes *in vivo* measurement of the ODC difficult; since PCO₂ is known to shift the ODC via two mechanisms (through its effect on pH and through its direct effect on Hb), a single ODC cannot be graphed unless PCO₂ is kept constant at varying PO₂ levels. Because PCO₂ and PO₂ are linked under normal *in vivo* conditions, such measurement cannot easily be done.

Without *in vivo* measurements of the ODC, it is not possible to gain a clear understanding of the shape and position of the ODC, either under normal conditions or those of physical or environmental stress. As well, because the different *in vitro* methods use different mechanisms for constraining the variables that affect the position and shape of the ODC, it is difficult to draw true conclusions from their data and to compare results from different studies. An *in vivo* method of measurement is therefore needed.

2.3 Using Prospective End-Tidal Targeting to Measure the ODC *in vivo*

The problem of independent control of PCO_2 and PO_2 has been approached by researchers for almost 50 years. Early methods (Richardson *et al.*, 1966; Weil *et al.*, 1970) controlled inspired gas concentrations on a breath by breath basis in order to control end tidal values. Because these methods did not control for the effects of breathing frequency and tidal volume, they did not yield reliable end-tidal values for PO_2 (PetO_2) and PCO_2 (PetCO_2). More sophisticated methods of dynamic end-tidal forcing (Robbins *et al.*, 1982) use new and improved algorithms to control PetO_2 , PetCO_2 and ventilation independently of one another. However, these methods use high inspiratory flows and complex, expensive systems, so they are not suitable for use in clinical settings.

Our laboratory has developed a relatively portable, simple method and apparatus that can be used to prospectively target and control PetCO_2 and PetO_2 independently of each other and independently of the ventilatory response to O_2 (Slessarev *et al.*, 2007). Figure 4 depicts these variables dissociated.

Figure 4: Uncoupling of PO_2 , ventilation & PCO_2

Prospective end-tidal targeting makes use of a gas-blending device, called the RespirAct™, in combination with a modified sequential gas delivery system. This system can be used to induce

step changes in PetCO_2 and PetO_2 , which take place in just a few breaths, independent of breathing rate or frequency. These changes can be sustained for the duration of a breathing sequence, within ± 2 mmHg. It has recently been shown that PetCO_2 achieved while using this system is equal to arterial PCO_2 (PaCO_2) in healthy subjects (Ito *et al.*, 2008). A full description of this method for prospective end-tidal targeting was described by Slessarev *et al.* (2007). A schematic of the system is pictured in the representation below, in Figure 5.

Figure 5: RespirAct™ Device and Breathing Circuit

This method works by sequentially supplying the subject with a limited amount of fresh gas (from gas reservoir 5) followed by rebreathed gas (from gas reservoir 6), which is neutral with respect to gas exchange because it was previously exhaled and therefore equilibrated with the gas

in the subject's lungs. Since this rebreathed gas does not contribute to gas exchange, only the fresh gas flow contributes to alveolar ventilation.

The sequential gas delivery circuit contains 3 one-way valves, which are used to control the flow of gas through the circuit. Expiration opens valve 3, a low-pressure valve, so the exhaled gas can enter the rebreathed gas reservoir (6) and the atmosphere. This valve closes upon inspiration, in which the negative inspiratory pressure initially opens valve 2, a low-pressure valve that controls the passage of gas from the fresh gas reservoir (5). Once the fresh gas reservoir collapses, further negative pressure opens valve 4, a cross-over valve, so the balance of the subject's breath is composed of previously rebreathed gas. Upon expiration the cycle repeats.

This system relies on the fact that alveolar ventilation is preset, so it can only be used to control end-tidal values when subjects consistently empty the fresh gas reservoir on every breath. Because any further inhalation does not affect gas exchange, subjects can breathe at any level beyond this minimum. It is for this reason that PetCO_2 and PetO_2 can be kept constant with this system, even with extreme hyperventilation.

The fresh gas flow is prospectively controlled by a computer in combination with the RespirAct™ gas blender. An algorithm in the computer takes into account the subjects CO_2 production (VCO_2), O_2 consumption (VO_2), resting PCO_2 and resting PCO_2 (all which can be measured using the system) to maintain the subject's PetCO_2 and PetO_2 at predetermined values. This is done through fluctuations in fresh gas flow and through fluctuations in the amounts of each of the three input gases, which contain various combinations of CO_2 , O_2 and nitrogen.

3 Research Aims and Hypotheses

The ODC has not been previously studied *in vivo* because of the difficulty of controlling PO_2 independently of PCO_2 and independently of ventilation. We are the first laboratory to perform *in vivo* ODC mapping studies, using prospective end-tidal targeting. We hope that our method will eventually be helpful in understanding the role of the ODC in the development and effects of illness, both at sea level and high altitude.

3.1 Aim One: Method Demonstration

The first aim of this study is to demonstrate the ability to measure the ODC *in vivo*, using prospective end-tidal targeting. This will require the maintenance of normocapnia during progressive hypoxia. We will carry out this protocol in lowlanders at sea level as well as in lowlanders and highlanders at high altitude. We will also demonstrate the ability to shift the ODC *in vivo* by inducing progressive hypoxia while maintaining hypocapnia and hypercapnia in a sample of lowlanders at sea level. Based on the results of previous studies using prospective end-tidal targeting, we hypothesize that this method will allow the dissociation of $P_{et}O_2$ and $P_{et}CO_2$, such that $P_{et}CO_2$ may be kept constant during stepwise changes in $P_{et}O_2$ leading to progressive hypoxia. We also hypothesize that PCO_2 can be maintained at hypocapnic and hypercapnic levels during a similar protocol, which will be carried out in lowlanders at sea level.

3.2 Aim Two: Characterization of *in vivo* Curves and Comparison to *in vitro* Predictions

The second aim of this study is to characterize physiological (at physiological PCO_2) *in vivo* ODCs in lowlanders at sea level, lowlanders at high altitude, and native Andean highlanders at high altitude. We will also characterize the shifted *in vivo* curves (under conditions of hypocapnia and hypercapnia) in a group of lowlanders at sea level. Characterization will be done by calculating P_{50} , n and K values for all subjects in each condition, using the Hill equation. It will also be done by comparing the P_{50} s, n values and K values using the measured SO_2 to those achieved by the Severinghaus and Kelman methods. We hypothesize that our data will fit the Hill equation and that we will be able to characterize our curves using P_{50} , n and K . We expect that our measured SO_2 data will match the predicted SO_2 data at sea level, though less so at lower saturations. This is because metabolic compensation for hypoxia will become

influential in the lower ranges. These differences will be reflected in differences in P_{50} , n and K . At high altitude, we expect measured SO_2 to be lower than predicted SO_2 . This is because [2,3-DPG] increases at high altitude and decreases the Hb affinity for O_2 , but is not taken into account by these models. As a result, we expect that the P_{50} s of the subjects at high altitude using measured SO_2 will be higher (right-shifted) than those achieved using the predicted SO_2 values.

3.3 Aim Three: Comparison of the *in vivo* ODC Across Experimental Groups

The third aim of this study is to calculate and compare P_{50} , n values and K values in lowlanders at sea level, lowlanders at high altitude and highlanders at high altitude. *In vitro* studies have found no shift of the physiological ODC at high altitude. This has been attributed to a balance between respiratory alkalosis and an increase in [2,3-DPG]. However, animals that are native to hypoxic environments have adapted by increasing their Hb affinity for O_2 . We therefore expect to find a leftward shift of the *in vivo* ODC in both groups of subjects at high altitude as compared to the position of the *in vivo* ODC in lowlanders at sea level.

4 Methods

This study consists of four experiments that were conducted in different populations. The four groups underwent similar protocols, with differences only in the details. The four experiments were:

- Experiment 1: Lowlanders at sea level (at normocapnia)
- Experiment 2: Lowlanders at sea level (at hypocapnia, normocapnia and hypercapnia)
- Experiment 3: Lowlanders at 3600 m for two weeks (at normocapnia)
- Experiment 4: Native Andeans at 3600 m (at normocapnia)

4.1 Subjects

4.1.1 Experiments 1 & 2

Subjects in Experiment 1 were 13 healthy sea level residents (5 females), between the ages of 21 and 35. Subjects in Experiment 2 were 19 healthy sea level residents (8 females). Subjects were recruited by the means of ads placed on the campus of Duke University in Duke Hospital North and South Clinics. They contacted a study coordinator, who booked a screening session in which subjects were briefed about the requirements and risks of the study and tested for eligibility. The study took place on the campus of Duke University.

Exclusion criteria included allergies to lidocaine or heparin, haemoglobinopathy or gastroesophageal reflux, history of or anatomy suggestive of difficult airways, $Hb < 12$, platelets $< 150,000$, Hepatitis A, Hepatitis B, Hepatitis C, HIV, pregnancy, cardiac or pulmonary disease, coagulation abnormalities, tobacco, drugs or alcohol abuse, hypertension, abnormal electrocardiogram results, abnormal pulmonary function test results, abnormal diffusion capacity of the lung for carbon monoxide, abnormal arterial blood gases or any other conditions that would potentially increase subject risk.

Subjects who completed the study were reimbursed monetarily.

Anthropometric data for each subject in Experiments 1 and 2 are shown below, in Table 1 and Table 2.

Table 1: Anthropometric Data for Subjects in Experiment 1

Subject	Age	Sex	Ethnicity	Weight (Kg)	Height (cm)
21	25	F	Caucasian	59.3	173
22	25	M	Caucasian	93.7	180
23	21	M	African American	80.5	178
24	29	M	Caucasian	85.3	180
25	23	M	Caucasian	72.5	172
26	24	F	Caucasian	57.2	168
27	21	F	Caucasian	58.4	155
28	21	F	Caucasian	56.5	162
29	34	M	African American	95.3	177
31	21	M	Caucasian	90.7	183
32	22	M	Caucasian	77.1	175
33	22	F	African American	58.9	157
34	22	M	Caucasian	79.4	172

Table 2: Anthropometric Data for Subjects in Experiment 2

Subject	Age	Sex	Ethnicity	Weight (Kg)	Height (cm)
1	26	M	Caucasian	67.4	183
3	21	F	Caucasian	56.6	163
5	24	F	African American	67.9	170
6	31	M	Caucasian	98.1	188
7	24	F	Caucasian	61.5	165
8	32	M	Caucasian	99.5	180
9	28	M	Hispanic	58.8	165
10	26	F	Asian - Pacific Islander	72.4	163
11	27	M	Caucasian	83.3	183
12	22	M	Caucasian	77.8	191
13	35	F	Caucasian	63.8	170
14	23	F	Asian - Pacific Islander	74.7	178
15	29	M	Caucasian	81.4	180
16	25	M	Caucasian	75.1	182
17	21	M	Caucasian	90.4	182
19	21	M	Caucasian	72.3	177
20	34	M	African American	94.3	177
21	24	F	Caucasian	56.5	167
22	25	F	Caucasian	58.8	165

4.1.2 Experiment 3

Subjects were 6 healthy sea-level residents (2 females), who were taken to La Paz, Bolivia (3600 m) and tested at high altitude after two weeks of exposure to hypoxia. Due to the difficulty in bringing subjects to altitude, all of them also acted as co-investigators in the study. As such, they were not blind regarding the purposes and hypotheses of the experiment. Participation was strictly voluntary and the subjects were not compensated for their participation. However, they were reimbursed for any out of pocket expenses. All subjects were healthy, with no history of cardiopulmonary abnormalities or disease. None of the subjects exhibited acute altitude sickness.

Anthropometric data for each subject in Experiment 3 are shown below in Table 3.

Table 3: Anthropometric Data for Subjects in Experiment 3

Subject	Age	Sex	Ethnicity	Weight (Kg)	Height (cm)
1	26	F	Caucasian	61	172
2	32	M	Caucasian	73	170
3	22	F	Caucasian	44	150
4	30	M	Caucasian	84	188
5	60	M	Caucasian	80	180
6	26	M	Caucasian	90	184

4.1.3 Experiment 4

Subjects in this study were 8 healthy residents of high altitude (2 females) native to La Paz, Bolivia or the surrounding highlands of El Alto. They were recruited and screened by our collaborators in Bolivia, who run a clinic in La Paz that specializes in treatment of patients with altitude-related illness. Specific exclusion criteria included history of cardiopulmonary abnormalities, fingertip clubbing, and hematocrit >50. Subjects were not included if they exhibited any health risks or abnormalities that may have put them at risk or influenced the outcome of the study. Subjects received monetary remuneration for participation in the study of an amount that was appropriate by Bolivian standards.

Anthropometric data for each subject in Experiment 4 are shown below in Table 4.

Table 4: Anthropometric Data for Subjects in Experiment 4

Subject	Age	Sex	Ethnicity	Weight (Kg)	Height (cm)
1	46	M	South Amerindian	70	160
2	41	M	South Amerindian	62	169
3	33	M	South Amerindian	70	160
4	30	M	South Amerindian	60	145
5	26	F	South Amerindian	56	150
6	50	F	South Amerindian	missing	missing
8	26	M	South Amerindian	60	169
9	34	F	South Amerindian	58	150

4.2 Informed Consent

Consent was collected as per the requirements of the research ethics boards at Duke University, at the University Health Network in Toronto and in Bolivia. All participants in all experiments

were briefed about the goals and procedures of the study and gave written informed consent before study participation. For those who spoke only Spanish, the consent form was verbally translated and then signed. Subjects were advised that there were no inherent benefits of participation in this study, and that the only benefit would be contribution to the existing knowledge-base.

4.3 Risks

All study participants were assessed prior to testing, in order to confirm that they did not exhibit any of the exclusion criteria, which would have increased study risks.

4.3.1 Hypoxia

The oxyhaemoglobin saturation range between 60 and 100% is well tolerated and safe in most otherwise healthy people. At altitude of about 4500-6000 m, SO_2 typically ranges from 75 to 65%. In those that climb higher (Mt. Everest is at 8000 m) SO_2 is even lower. SO_2 in the range of 100-70% is frequently used in experimental protocols. At O_2 concentrations resulting in SO_2 approaching 65% with fixed PCO_2 , some subjects may experience shortness of breath and even some distress. In that case, the experiment was terminated and subject given 100% O_2 . This terminated the distress within 2-3 breaths. Additionally, SO_2 was continuously monitored with a pulse oximeter (yielding SpO_2). If SpO_2 fell below 70%, the study was immediately terminated and the subject received 100% O_2 . Non invasive blood pressure and heart rate were also monitored throughout the study, and the study was terminated if the protocol was deemed too stressful. Decreased consciousness or alertness was immediately treated with 100% O_2 .

4.3.2 Arterial Blood Sampling

Puncture of a radial artery to sample arterial blood is performed ubiquitously throughout the world and is considered a safe procedure. The discomfort of the puncture depends partly on the skill of the person drawing the blood and the size of the needle. In this study the arterial puncture was performed by anaesthesiologists who have performed thousands of such punctures over about 30 years. The puncture was performed using a smaller needle (25 G) than is commonly used clinically (20 G) so as to lessen the discomfort.

4.4 Measurement Devices

4.4.1 Arterial Blood Gases (ABG)

In Experiments 1 and 2, haematological parameters were analyzed using the Instrumentation Laboratory GEM Premier 4000, using cartridges with the part number 00027445008.

In Experiments 3 and 4, haematological parameters were analyzed using the i-STAT (Abbott Point of Care, Illinois, USA), a handheld, portable system. EC3+ cartridges were used to get baseline sodium (Na), potassium (K), hematocrit (Hct) and Hb (calculated) in all subjects in Experiment 4 and two subjects in Experiment 3. For all subjects in Experiment 4 and two subjects in Experiment 3 the CG4+ cartridges were used to analyze blood samples. This cartridge measures pH, PCO₂ and PO₂ and calculates total CO₂ (TCO₂), base excess (BE), HCO₃⁻ and SO₂. For the other 4 subjects in Experiment 3, EC8+ cartridges were used. This cartridge measures Na, K, Cl, urea nitrogen (BUN), glucose, Hct, pH, PCO₂, HCO₃⁻, BE (calculated), anion gap and Hb (calculated). PO₂ was measured separately by Radiometer PhMK2 (Radiometer Co., Copenhagen, Denmark). Values with this device were equivalent to those measured by the i-STAT.

4.4.2 Measured SO₂

In Experiments 1 and 2, SaO₂ was measured by the co-oximeter in the Instrumentation Laboratory GEM Premier 4000. SpO₂ was also measured by means of a pulse oximeter, which analyzes the pattern of absorption/transmission of infra-red light through a finger or ear lobe to determine non-invasive peripheral SO₂. The device used in Experiments 1 and 2 was the Conmed PRO₂ Monitor (Conmed, Utica, NY, USA), from which the data were continuously recorded. Only co-oximeter measurements were used for data analysis in these experiments because the co-oximeter provided direct invasive measurement of SaO₂ in each blood sample and was therefore the most accurate measurement available. We did not have access to a co-oximeter in Bolivia, where Experiments 3 and 4 were carried out. As such, a finger pulse oximeter, the Onyx II 9550 (Nonin Medical, Inc., Plymouth MN), was used to measure SpO₂ in lieu of SaO₂ in these experiments. This device is stated as accurate within $\pm 2\%$ (this is the root mean square difference between the SpO₂ values and reference SaO₂ values from a co-oximeter) in the saturation range between 70-100%.

4.4.3 Blood Pressure

In Experiments 1 and 2 invasive blood pressure was measured continuously by a Propaq CS (Welch Allyn, Skaneateles Falls, NY, USA). In Experiments 3 and 4 non-invasive blood pressure (NIBP) was measured using an arm cuff, which inflated every minute. NIBP data was recorded manually.

4.4.4 Heart Rate (HR)

In all experiments, HR was measured non-invasively using pulse oximeters. In Experiments 1 and 2 the Conmed PRO₂ Monitor (Conmed, Utica, NY, USA) was used and continuously recorded. In Experiments 3 and 4 the Onyx II was used. This information was recorded by hand at the time of blood sampling in Experiments 3 and 4.

4.4.5 End Tidal PCO₂ (PetCO₂) and End Tidal PO₂ (PetO₂)

PetCO₂ and PetO₂ were continuously measured from a sampling port in the mask of the subject's sequential gas delivery system, using sensors in the RespirAct™. The CO₂ sensor within the RespirAct™ (Ir3107, Servomex Group Ltd, Sugar Land, TX, USA) is accurate to within $\pm 0.1\%$ of the CO₂ within the range of 0-10% CO₂. The O₂ sensor (UFO 130, Teledyne Analytical Instruments, City of Industry, CA, USA) is accurate within 1%. Both sensors were calibrated, according to specifications, at least once a day, or between each subject. A computer program (Labview, National Instruments, Austin, TX, USA) was used to display these PetCO₂ and PetO₂ on a screen throughout testing. Breath-by-breath values were calculated by the program and displayed as well. The raw data and breath-by-breath values were continuously recorded.

4.5 Experimental Protocol

4.5.1 Subject Setup (common to all subjects)

A catheter and arterial line was inserted into the subject's radial artery, and a blood sample was taken and analyzed to get resting, baseline data. The pulse oximeter was placed on the index or middle finger of the hand with the arterial line. The modified sequential gas delivery apparatus was then affixed to the subject's face using Tagederm opsite (3M, London, ON, Canada), which prevented air from leaking in or out of the mask. Before any tests were started, the subject was allowed to sit for 5-10 minutes in order to acclimatize to wearing the mask. During this time, the

subject either breathed room air (by opening the port in the mask) or medical air (the equivalent of dry room air, from compressed gas cylinders) with a flow greater than the subject's alveolar ventilation, according to the subject's preference. The subject was told to relax and breathe at a level of ventilation that was most comfortable.

4.5.2 Equipment Setup (common to all subjects)

The subject's age, height and weight were entered into the computer program. A computer algorithm was used to automatically compute $\dot{V}CO_2$ and $\dot{V}O_2$ from these parameters. Once the subject began breathing into the sequential gas delivery system, $PetO_2$ and $PetCO_2$ were measured over ten breaths using the RespirAct™ software. These values were saved to the RespirAct™ program.

4.5.3 Experiment 1

The subject was told to get as comfortable as possible while semi-reclining on a bed. Once the subject's breathing became steady, a test sequence was run with the RespirAct™. This test sequence had two purposes: to determine the subject's tolerance for hypoxia and to determine whether the estimated $\dot{V}O_2$ and $\dot{V}CO_2$ were correct. This sequence involved maintaining $PetCO_2$ at 40 mmHg while targeting a series of $PetO_2$ steps, starting at 100 mmHg, leading to a minimum of 35 mmHg. Each step lasted approximately 1 minute in length. If the subject's end tidal values were not stable at the targeted values, the resting parameters ($\dot{V}CO_2$, $\dot{V}O_2$, resting $PetCO_2$ and resting $PetO_2$) were adjusted accordingly. The subject was told to indicate as soon as the test became too uncomfortable. If the $PetO_2$ at that point was above 35 mmHg, this value was then used as the minimum $PetO_2$ during testing. Once the proper resting values and the minimum $PetO_2$ were determined, the experiment commenced.

The protocol was initiated with baseline measurements followed by the study protocol. The delivered gas mixture was adjusted by the RespirAct™ to maintain a $PetCO_2$ of 40 ± 3 mmHg, while progressively decreasing $PetO_2$ from 100 mmHg to a predetermined minimum value (either 35 mmHg or the lowest value above 35 mmHg that would be tolerated by the subject). Once the minimum value was achieved, $PetO_2$ was progressively brought back to 100 mmHg in a manner that mirrored the steps taken to bring the subject from $PetO_2$ of 100 mmHg to that of the minimum value. This was done in 9 steps (e.g. 100, 60, 45, 40, 35, 40, 45, 60, 100 mmHg),

for which the target values depended on what the minimum PetO_2 would be. In those who had low tolerance to hypoxia, the minimum PetO_2 was increased (e.g. to 42 mmHg) but the number of steps was kept constant, such that the PetO_2 increments were smaller. The protocol was followed by the administration of 100% oxygen. The subject was then allowed to rest while breathing room air, to allow PetCO_2 , PetO_2 and SpO_2 to return to baseline levels.

Each step lasted 4 minutes. During the last 30 seconds of each PetO_2 step one arterial sample was taken. All blood samples were analyzed immediately by co-oximetry. PetO_2 , PetCO_2 and SpO_2 were continuously measured and automatically recorded.

Upon completion of the study all catheters were removed and sites inspected for haematoma. Pressure dressing was applied to the radial arterial catheter site.

4.5.4 Experiment 2

The subject was told to get as comfortable as possible while semi-reclining on a bed. Once the subject's breathing became steady, a test sequence was run with the RespirAct™. This test sequence had two purposes: to determine the subject's tolerance for hypoxia and to determine whether the estimated VO_2 and VCO_2 were correct. This sequence involved maintaining PetCO_2 at 40 mmHg while targeting a series of PetO_2 steps, starting at 100 mmHg, leading to a minimum of 35 mmHg. Each step lasted approximately 1 minute in length. If the subject's end tidal values were not stable at the targeted values, the resting parameters were adjusted accordingly. The subject was told to indicate as soon as the test became too uncomfortable. If the PetO_2 at that point was above 35, this value was then used as the minimum PetO_2 during testing. Once the proper resting values and the minimum PetO_2 were determined, the experiment commenced.

The protocol started with baseline measurements followed by two study stages. For the first study stage the delivered gas mixture was adjusted by the RespirAct™ to maintain a PetCO_2 of 40 ± 3 mmHg, while reducing SpO_2 in increments of approximately 5% from baseline of 95 – 100% (e.g. 95%, 90%, 85%, 80%, 75% and 70%). This was done using a series of PetO_2 targets (e.g. 100, 80, 60, 48, 42, 38, 35 mmHg). In those who had low tolerance to hypoxia, the minimum PetO_2 was increased (e.g. to 42 mmHg) but the number of steps was kept constant, such that the SpO_2 increments were smaller. After the last hypoxic step an ascent to room air was carried out to return the SpO_2 to 100%. This was followed by the administration of 100%

oxygen. The subject was then allowed to rest while breathing room air (by removing the plug from the mask) for 10 minutes before starting the second stage of the study, to allow PetCO₂, PetO₂ and SpO₂ to return to baseline levels.

For the second stage of the study, the delivered gas mixture was adjusted to provide descending SpO₂ steps, this time in 10% increments, using a series of PetO₂ targets (eg. 100, 60, 42, 35 mmHg). At each step there were two sub-steps to provide a PetCO₂ of 35 and 45 mmHg. The 70% SpO₂ step was followed by a return to baseline SpO₂ and then administration of 100% oxygen.

Each step lasted 3 minutes. During the last 30 seconds of each step of progressive hypoxia, two arterial samples were taken. All blood samples were analyzed immediately by co-oximetry. PetO₂, PetCO₂ and SpO₂ were continuously measured and automatically recorded.

Upon completion of the study all catheters were removed and sites inspected for haematoma. Pressure dressing was applied to the radial arterial catheter site.

4.5.5 Experiments 3 and 4

The subject was seated comfortably in an armchair. Once the subject's breathing became steady, the experimental sequence was started. This sequence entailed the maintenance of PCO₂ at the subject's resting level (as determined from the arterial sample) during progressive hypoxia. There were 6 PO₂ targets, 100, 70, 60, 50, 40, 35 mmHg. If PCO₂ or PO₂ drifted up or down during the sequence or if the targets were not hit, the test was aborted and the subject's parameters (VCO₂, VO₂, resting PetO₂ and resting PetCO₂) were adjusted in order to correct for the problem. The sequence was then restarted and completed. The test ended when saturation fell below 70% or when the subject felt too uncomfortable to continue. Each PetO₂ step was 2 minutes in length, and a blood sample was drawn at the end of each step. PetCO₂ and PetO₂ were continuously measured and recorded during testing. SpO₂, HR and BP were manually recorded when blood samples were drawn, at the end of each PetO₂ step.

4.6 Data Analysis

4.6.1 Comparing Subjects across Experimental Group

Control haematological values (pH, PCO_2 , PO_2 , BE, HCO_3^- , Hct and Hb) were collected for each subject. One-way ANOVA tests with an alpha level of 0.05 were used to compare these parameters across experimental groups in order to characterize the differences between the lowlanders at sea level (all were put into one group, since they came from the same population), lowlanders at high altitude and native Andeans at high altitude. Tukey pair-wise tests were used for post-hoc analysis.

4.6.2 Method Demonstration

4.6.2.1 Stability and Control of PCO_2 and pH

PCO_2 and pH are known to affect the position of the ODC. PCO_2 is one variable that influences pH, but pH can still change independently of changes in PCO_2 . Our method of measuring the ODC has means of controlling PCO_2 . However, the efficacy of the device in maintaining PCO_2 at the desired level is dependent on the diligence and ability of the operator. If PCO_2 is not maintained at a single level throughout a test, the resulting saturations may not fall on a single ODC. Similarly, if PCO_2 is not maintained at similar levels between subjects, it will be difficult to define the effect of PCO_2 on the *in vivo* ODC. pH cannot be controlled by the RespirAct™ independently of PCO_2 . pH variability also influences the ability to graph a single ODC, so it was measured throughout the experiment. In order to determine the efficacy of our method for measuring the ODC, PCO_2 and pH variability were tracked throughout each subject's data. Average and standard deviation of both variables for all subjects have been reported. We also reported the PetO_2 and PetCO_2 stability during the last 60 s of each PO_2 step during testing at high altitude, as well as the PetCO_2 stability throughout testing for these same subjects. Such data is not available for the lowlanders at sea level, but similar variability is expected.

4.6.2.2 Minimum PO_2

Because the ODC has a sigmoidal shape, the steep part of the curve is that which is most important. In order to characterize the curve, it is therefore imperative to achieve a number of PaO_2 values that fall on the steep part of the ODC. If PaO_2 is not reduced enough in the

protocol, there may not be sufficient information to characterize the curve. It is for this reason that the minimum PaO₂ values achieved have been reported for each subject in each condition.

4.6.2.3 Number of PO₂ Steps/Number of Blood Samples

Our *in vivo* ODCs were constructed by linearizing a number of PaO₂/SO₂ data pairs for each subject and finding the parameters of the line that best fits the data using the Hill Equation (Equation 12). The process by which this was done will be explained, in detail, below. The more data pairs that are available, the better the line of best fit will represent the data. We have therefore presented the number of PO₂ steps or blood samples for each subject.

4.6.3 Comparison of Measured SO₂ with Calculated SO₂

One of the aims of this study is to compare our *in vivo* method to previously used *in vitro* methods, by comparing our measured saturations to those predicted by some of the models that were created from *in vitro* data. There were two models chosen for the sake of this comparison, that of Severinghaus and that of Kelman. These models will be shown, in detail, below. SO₂ was calculated using each of the two models, and results were compared graphically. Scatter plots were used to compare Measured SO₂ and Severinghaus SO₂ for each of the four experimental groups, and a second set of scatter plots were used to compare Measured SO₂ and Kelman SO₂ for each of the four groups. Bland and Altman difference plots were also used to separately compare all Measured SO₂ values to all Severinghaus values and all Measured SO₂ values to all Kelman values. The Bland and Altman plots show the bias and 95% confidence intervals for each set of data.

4.6.3.1 Severinghaus SO₂

The Severinghaus (1979) model was chosen because it is widely used to predict saturation from blood gas measurements. It is a modification of the Hill equation that has been reported as being accurate within 0.55%. The Severinghaus equation (Equation 7) calculates SO₂ from PO₂ under standard conditions (pH of 7.4 and T of 37°).

Equation 7

$$SO_2 = 100 \div \left[1 + \left(\frac{23400}{(PO_2)^3 + (150 \times PO_2)} \right) \right] \%$$

where PO_2 is the partial pressure of O_2 in mmHg.

However, most measurements are not made under standard conditions. Severinghaus therefore provided an alternate form of the equation that takes into account the Bohr Effect, factoring in the difference between the *in vivo* pH and standard pH. His adjustment for the Bohr Effect takes into account the changes in the ODC due to both pH and PCO_2 . When the Bohr Effect is present, the above equation is used, but PO_2 is substituted with Equation 8:

Equation 8

$$PO_2 \times e^{\left[\left(\left(\frac{PO_2}{26.7} \right)^{0.184} + 0.003 \times BE - 2.2 \right) \times (7.4 - pH) \right]}$$

If base excess cannot be directly measured, it can be derived using an equation from Siggaard Andersen (Equation 9).

Equation 9

$$BE = [1 - (0.014 \times tHb)] \times [HCO_3^- - 24.8 + (1.43 \times tHb + 7.7) \times (pH - 7.4)]$$

where THb is the total concentration of Hb (g/dL) and HCO_3^- (mmol/L) is the concentration of bicarbonate.

These equations were also used by our co-oximeter to calculate SO_2 in Experiments 1 and 2.

4.6.3.2 Kelman SO_2

The other model that was used with our data is that of Kelman (1966). This model preceded that of Severinghaus. It is robust because it uses PO_2 , PCO_2 , T and pH to calculate a “virtual PO_2 ,” which it then uses to predict saturation. It is a popular model because it allows for easy calculation of SO_2 and is thus very convenient. This is a mathematical model in which coefficients were found by using the least squares method on a data set containing 38 pairs of PO_2/SO_2 data. The model equation is shown in Equation 10.

Equation 10

$$SO_2 = \frac{100 \times (a_1x + a_2x^2 + a_3x^3 + x^4)}{(a_4 + a_5x + a_6x^2 + a_7x^3 + x^4)}$$

The coefficients have the following values:

$$a_1 = -8.5322289 \times 10^3$$

$$a_2 = 2.1214010 \times 10^3$$

$$a_3 = -6.7073989 \times 10^1$$

$$a_4 = 9.3596087 \times 10^5$$

$$a_5 = -3.1346258 \times 10^4$$

$$a_6 = 2.3961674 \times 10^3$$

$$a_7 = -6.7104406 \times 10^1$$

Lastly, the x in the Kelman model represents the “virtual PO₂”, which accounts for changes in saturation due to deviation from standard conditions (Equation 11).

Equation 11

$$PO_2vir = PO_2 \times 10^{\{0.024(37-T)+0.40(pH-7.40)+0.06(\log 40-\log PCO_2)\}}$$

This model works under the assumption that, besides for pH at high saturations, these variables that are part of the “virtual PO₂” change the scale, but not the shape of the PO₂ axis. The factors used here were originally suggested by Severinghaus in formulating his nomogram (1966).

4.6.4 Hill Analysis and ODC Comparisons

The ODC is usually quantified by a single value, the P₅₀. While some *in vitro* systems can measure the PO₂ at which Hb is 50% saturated, this cannot be done safely *in vivo*. We therefore calculated P₅₀ by using the linearized form of the Hill equation (Equation 12), which was mathematically used to fit the data.

Equation 12

$$\log \frac{SO_2}{100 - SO_2} = \log K + n \log PO_2$$

The linearized Hill equation was used in the following manner:

In each condition $\log(PaO_2)$ was graphed on the x axis against $\log[SO_2/(100-SO_2)]$ for all cases where PaO_2 was less than 90 mmHg. Data where PaO_2 was greater than 90 mmHg were excluded because there tends to be SO_2 variability in this range, and this variability has the potential to greatly influence P_{50} even though it falls on the flattest part of the ODC. A line of best fit was drawn through the data in order to determine the values for K and n, the Hill coefficient of sigmoidicity, which represents the co-operativity of Hb. Genetics and the environment will influence the n value and strength of co-operativity of the binding of Hb and O_2 . These values were then used with the Hill equation to determine the P_{50} . For each data set R^2 was calculated in order to determine how well the line of best fit matched the linearized data. The n and K values were then used in the non-linearized form of the equation and R^2 was calculated to see how well the resulting curve fit the non-linearized data. This linearization process was not used on data sets with fewer than 3 PaO_2/SO_2 pairs, because one or two sets of points may not be characteristic of the entire curve.

Not only was the Hill equation used to determine P_{50} using measured SO_2 values, it was also used with the Kelman and Severinghaus SO_2 values to determine the P_{50} s that would be predicted by these models. The above method yielded a P_{50} , n and K value for each subject in each condition using each type of SO_2 (measured, Severinghaus, Kelman).

We used bar graphs to show the average and standard errors of P_{50} and n values for each experimental group at normocapnia, using each SO_2 type. K values were also graphed, but medians, 25% and 75% were shown instead of averages because the K value data were not normally distributed.

We attempted to apply a mathematical exponential fit to the data, but this idea was discarded because, while an exponential often fit our data well, this good fit was only in the measured part of the curve, as an exponential does not reflect the sigmoidal shape of the ODC.

4.6.4.1 Comparison within Groups, across SO₂ Type

A one-way repeated measures ANOVA with an alpha level of .05 was used to compare the three types of each value within each subject of each condition. Thus, for each experiment, 3 such tests were done; one on P₅₀, one on n, and one on K. These were performed in order to further assess how our data compares with predictions based on *in vitro* data. If differences were found between measurement types, the appropriate post-hoc test was used to determine which SO₂ types differed significantly from one another. If the normality test failed, a Kruskal-Wallis test was done on ranks. Frequency distributions were also used to show how the three SO₂ types influenced P₅₀ and n values for each experimental group. The two groups of lowlanders at sea level were combined for the graphical representation.

4.6.4.2 Comparison across Groups, within SO₂ Type

P₅₀, n, and K were compared across groups, using measured, Severinghaus, and Kelman SO₂ types. This was done in order to address the third aim of this thesis, which was comparison of lowlanders at sea level, lowlanders at high altitude, and highlanders at high altitude. These were compared using 1 way ANOVAs, with an alpha level of .05. If normality tests failed, a Kruskal-Wallis test was done on ranks. If differences were found between groups, the appropriate post-hoc test was used to determine which groups significantly differed from one another.

4.6.4.3 Composite ODC Comparison across Groups using Measured SO₂

Graphs were also used to compare the experimental groups, using Measured SO₂. Average ODCs for each group were drawn by using the average Hill parameters. Outliers were excluded so the parameters would be normally distributed. Frequency distributions for P₅₀ and n values were also created.

4.6.4.4 Influence of PCO₂ on Hill Parameters

In Experiment 2 we explored the influence of PCO₂ on the *in vivo* ODC by having three PCO₂ conditions: hypocapnia, normocapnia and hypercapnia. We assessed the influence of PCO₂ on the ODC by performing a two-way repeated measures ANOVA at an alpha level of 0.05 on each of the three Hill parameters (P₅₀, n and K), using SO₂ type and PCO₂ condition as factors. The appropriate post-hoc tests were used for pair-wise comparison.

4.6.4.5 Descending and Ascending PO₂ Comparison

The protocol for Experiment 1 entailed progressive hypoxia followed by a progressive return to normoxia in lowlanders at sea level. We assessed whether the order of the PO₂ steps influenced the position of the ODC by splitting the data into descending and ascending PO₂ segments. Hill parameters were calculated separately for each segment, and a two-tailed paired t-test was used to compare them at an alpha level of 0.05.

5 Results

5.1 Control Haematological Values

Control haematological parameters for each subject are shown in Appendix A. In order to characterize our samples and determine how their haematological environments differed from one another, these were compared across groups using a one way ANOVA at an alpha level of 0.05. Because the two groups of lowlanders at sea level (Experiments 1 and 2) were from the same population and did not differ from one another, they were combined into one group for the sake of comparison. For all variables a Tukey pair-wise test was performed in order to determine which experimental group averages differed significantly from one another. Averages and standard deviations for each parameter for each experimental group are shown below, in Table 5. An asterisk (*) denotes that the group differs significantly from the lowlanders at sea level. A double dagger (‡) in the column for Andeans at Altitude denotes that the two high altitude groups (Experiments 3 and 4) differed significantly from one another. Detailed ANOVA results can also be found in Appendix A.

Table 5: Control Haematological Values (Mean $\pm$ Std Dev) for Subjects in All Experiments

	Lowlanders at Sea Level Experiments 1 & 2	Lowlanders at Altitude Experiment 3	Andeans at Altitude Experiment 4
pH	7.40 $\pm$ 0.03	7.44 $\pm$ 0.02*	7.44 $\pm$ 0.02*
PCO₂ (mmHg)	41.0 $\pm$ 2.7	29.1 $\pm$ 2.9*	28.9 $\pm$ 3.4*
PO₂ (mmHg)	104.6 $\pm$ 8.3	70.2 $\pm$ 15.3*	57.5 $\pm$ 4.3*‡
BE	0.7 $\pm$ 1.9	-4.3 $\pm$ 1.9*	-4.8 $\pm$ 2.1*
HCO₃⁻ (mmol/L)	25.6 $\pm$ 1.9	19.8 $\pm$ 1.7*	19.5 $\pm$ 2.1*
Hct (%)	41.0 $\pm$ 3.8	43.8 $\pm$ 3.7	46.9 $\pm$ 3.0*
Hb (g/dL)	14.3 $\pm$ 1.5	14.9 $\pm$ 1.3	16.0 $\pm$ 1.0*

The above data provide evidence that the high altitude groups differed haematologically from the sea level groups. Such significant differences under baseline conditions may lead to different ODC positions across groups if the differences are not compensated for.

5.2 Method Demonstration

The current method for measuring the *in vivo* ODC involves progressive hypoxia during isocapnia. This was done by maintaining PetCO₂ while inducing square-wave changes in PetO₂.

Figure 6 shows breath by breath PetCO₂ and PetO₂ data for a typical subject during ODC testing. It demonstrates the precision with which PetCO₂ and PetO₂ can be independently controlled using this method.

For all subjects in Experiments 3 and 4, PetO₂ remained within ± 1.5 mmHg standard deviation (Std Dev) during at least the last 60 s of each step, while PetCO₂ remained within ± 0.5 mmHg. Throughout testing, PetCO₂ remained within ± 1.0 mmHg for all subjects. Similar data are not available for those in Experiments 1 and 2, but the methodology and technology was the same so the extent of control is expected to be similar.

Figure 6: Raw Data Example: Breath by Breath End Tidal Data for a Typical Subject

In vivo study of the ODC requires the maintenance of PCO₂ and pH during progressive hypoxia, despite changes in ventilation. This is important because arterial samples taken at different times will not lie on the same ODC if PCO₂ and pH are allowed to vary. While PCO₂ influences pH, pH can also be influenced by metabolism, ion shifts and other factors. PCO₂ was specifically controlled in this study, while pH was not. For each subject in each group, averages and standard deviations were calculated for both PaCO₂ and pH. The standard deviations reflect how well the parameters were controlled. The means $\pm$ standard deviations for each group, based on the individual means and standard deviations are presented in Table 6, below. Individual data are provided in Appendix B.

Table 6: Mean and Std Dev Data for PaCO₂ and pH during *in vivo* ODC Testing

Experiment	Avg PaCO ₂ (mmHg)	Std Dev PaCO ₂ (mmHg)	Avg pH	Std Dev pH
1	42.2 ± 2.0	1.5 ± 0.6	7.39 ± 0.03	0.01 ± 0.01
2 Hypocap	38.1 ± 1.5	1.7 ± 1.0	7.40 ± 0.03	0.02 ± 0.01
2 Normocap	42.4 ± 1.4	1.6 ± 0.6	7.37 ± 0.04	0.01 ± 0.01
2 Hypercap	46.8 ± 1.6	1.7 ± 0.9	7.33 ± 0.03	0.01 ± 0.01
3	27.4 ± 2.7	0.6 ± 0.2	7.47 ± 0.03	0.01 ± 0.00
4	26.9 ± 3.4	1.1 ± 0.3	7.43 ± 0.02	0.02 ± 0.00

Mean PaCO₂ and pH varied by experimental condition in a manner that was consistent with the experimental protocol. In Experiments 1, 2 normocapnia, 3 and 4 the PaCO₂ and pH were maintained roughly at control levels. PaCO₂ was lower in the hypocapnia condition of Experiment 2 than in the normocapnia condition, so the pH in the hypocapnia condition was consequently increased. PaCO₂ was higher in the hypercapnia condition of Experiment 2 than in the normocapnia condition, so the pH was consequently decreased.

Standard deviation for PaCO₂ was greatest in the hypocapnia and hypercapnia conditions of Experiment 2. This is probably due to the fact that PCO₂ alternated with each PO₂ step for the two conditions, while the other conditions and experiments had only a single PCO₂ target. Standard deviation for pH was greatest in the hypocapnia condition of Experiment 2 as well as in Experiment 4. It is not known whether the standard deviations of PaCO₂ and pH for these conditions were physiologically significant, but it is possible that they impacted the accuracy of ODC measurement in the studies.

Because the ODC is best constrained by data on its steep portion, it is important to achieve some low PO₂ levels *in vivo* in order to characterize the *in vivo* curve. The greater the number of samples, the better the characterization can be. Table 7 shows the minimum PaO₂ and number of samples taken for each experiment. Data for each subject are presented in Appendix B.

Table 7: Average and Std Dev Data for Minimum PaO₂ and Number of Samples during *in vivo* ODC Testing

Experiment	Minimum PaO ₂ (mmHg)	Number of Samples
1	42.85 ± 5.49	8.38 ± 1.66
2 Hypocapnia	45.76 ± 6.31	6.29 ± 1.49
2 Normocapnia	39.00 ± 3.21	25.21 ± 1.27
2 Hypercapnia	50.83 ± 12.39	6.17 ± 1.98
3	34.83 ± 2.14	5.33 ± 0.52
4	35.88 ± 3.94	5.75 ± 0.46

5.3 Comparison of Measured SO₂ Values with those of Severinghaus and Kelman Predictions

In order to determine how our *in vivo* SO₂ data compared to predictions based on previous *in vitro* studies, we then compared our *in vivo* SO₂ results to those calculated using the Severinghaus (1966) and Kelman (1966) methods.

Graphs depicting the correlation between the Measured SO₂ values and the Severinghaus SO₂ values are shown below for each experiment (Figure 7). The diagonal lines represent the lines of identity. In Experiments 1 through 3 the line of identity generally fits the data, except at the topmost SO₂ points where the Measured SO₂ values tend to be slightly higher than the Severinghaus SO₂ values. The data are heteroskedastic, with increased variability at low SO₂ values.

Figure 7: Graphs Showing the Correlation between Measured and Severinghaus SO_2 Values

Graphs depicting the correlation between the Measured SO_2 values and the Kelman SO_2 values are shown below for each experiment (Figure 8). The data comparing Measured SO_2 and that predicted by Kelman generally follow what was seen above, in the comparison of the Measured and Severinghaus SO_2 data. In Experiments 1 through 3 the line of identity generally fits the data, except at the topmost SO_2 points where the Measured SO_2 values tend to be slightly higher than the Severinghaus SO_2 values. In Experiment 4 the data clearly depart from the line of

identity, especially towards lower SO_2 values, where measured SO_2 tends to be higher than the Severinghaus SO_2 .

Figure 8: Graphs Showing the Correlation between Measured and Kelman SO_2 Values

Shown below (Figure 9 and Figure 10) are the Bland & Altman difference plots for the data shown above. These show the bias and 95% confidence intervals for each data set. These plots offer an alternate depiction of the above data.

Figure 9: Difference Plots for Measured and Severinghaus SO_2

Figure 10: Difference Plots for Measured and Kelman SO_2

5.3.1 Lowlanders at Sea Level

There was no difference between measured SO_2 and those predicted by the Kelman and Severinghaus models. The data were heteroskedastic, showing greater variability at low SO_2 values.

5.3.2 Lowlanders at High Altitude

We expected the measured SO_2 values to be lower than the Severinghaus and Kelman values in the sample of Lowlanders at high altitude. This is because [2,3-DPG], which has been shown *in vitro* to decrease the affinity of Hb for O_2 , is increased at high altitude and is not taken into account by these models for predicting SO_2 . Contrary to our expectations, there was no difference between the measured SO_2 and predicted SO_2 in the sample of lowlanders at high altitude. The data are heteroskedastic, with greater variability at low SO_2 values.

5.3.3 Andeans at High Altitude

In Andeans at high altitude predicted SO_2 values are consistently lower than the measured values. At high SO_2 values the predictions are very close to the measured values, with a few of the measured values even falling below the predicted values (as opposed to the other two groups, in which high SO_2 values were associated with a more consistent positive measured SO_2 bias). As SO_2 decreased, the difference between measured and predicted SO_2 became progressively greater. Some measured values are more than 10 units above the predicted values at low saturations. Our data for this sample strongly suggest a true difference between measured SO_2 and predicted SO_2 (both Severinghaus and Kelman) in our sample of Andeans at high altitude.

5.4 Hill Parameters (P_{50} , n , K)

For each experimental group, the three Hill parameters (P_{50} , n and K) were determined in three different ways, using measured SO_2 , Severinghaus SO_2 and Kelman SO_2 . This was done linearizing our data using the Hill Equation (Equation 12). We then drew a line of best fit in order to determine the P_{50} for the curve, in addition to the n and K values that characterize it. A detailed explanation of this process is explained above in the methods. This analysis was not conducted on data sets that included fewer than 3 pairs of PaO_2/SO_2 . Hill parameters for each individual in each experimental condition are presented in Appendix C.

5.4.1 Comparison of Hill Parameters (P_{50} , n , K) Across SO_2 Types (Measured, Severinghaus & Kelman) within each Experiment

In order to further assess how my data compare to those from *in vitro* studies, we compared the Hill parameters for our *in vivo* ODCs to those predicted using the Kelman and Severinghaus SO_2 values. This was done on each variable within each experimental group, using one-way repeated measures ANOVA, with an alpha level of 0.05. However, in the case of Experiment 2, a two way repeated measures ANOVA was done, in order to take into account the effects of both PCO_2 and SO_2 type on the Hill Parameters. Some of the K value data was not normally distributed, so the ANOVA was carried out on ranks instead of on means. Tukey-post hoc tests were used for pair-wise comparisons.

ANOVA results for all experiments comparing all parameters are presented in Appendix C. The data for P_{50} and n value (Hill Coefficient) will be summarized below.

The graphs below (Figure 11, Figure 12, and Figure 13) show the Hill Parameters for all normocapnic conditions, using all three SO_2 types. Significant differences within experimental group across each SO_2 type are indicated.

Figure 11: Comparing P_{50} Values (mean $\pm$ SEM) within Experimental Group across SO_2 Types

Figure 12: Comparing n Values (mean $\pm$ SEM) within Experimental Groups across SO₂ Types

Figure 13: Comparing K Values within Experimental Groups across SO₂ Types

(This graph shows the medians and 25% and 75% as the error bars)

5.4.1.1 P₅₀ Comparison

In Experiments 1, 2 and 3, Measured P₅₀ values were higher than those of Severinghaus and Kelman predictions, indicating that the curves were right shifted with respect to the predicted curves. In Experiment 1 the difference was significant only between Measured and Severinghaus (p=0.003). In Experiment 2 the difference was significant between Measured and Severinghaus (p<0.001) as well as between Measured and Kelman (p<0.001). In Experiment 3, neither predicted P₅₀ was significantly lower than the measured P₅₀, despite the presence of 2,3-DPG which would be expected to shift the Measured curve rightward from its predicted position. In the case of Experiment 4, Measured P₅₀ was significantly lower than that of Severinghaus

($p=0.016$) and that of Kelman ($p=0.014$), indicating a significant left shift of the curve with respect to its expected position.

It is unexpected that the curves in Experiments 1 and 2 were right shifted with respect to those predicted by the Severinghaus and Kelman models while that of Experiment 3 was not. This is because the models do not take into account the increase in [2,3-DPG] that occurs at high altitude (as in Experiment 3) and should further shift the ODC to the right. The fact that such a shift was not seen in this data may be a reflection of the low power (the data set had only 6 subjects), or it may reflect the presence of some *in vivo* factor that compensates for the increase in [2,3-DPG] at high altitude and shifts the ODC in the opposite direction of where it is expected to be. The fact that the *in vivo* curve from Experiment 4 was left shifted as compared to those predicted by the models further supports this possibility.

Figure 14 shows the frequency distributions for P_{50} according to SO_2 type, for each group of subjects at normocapnia. The lowlanders at sea level were combined into a single group.

Figure 14: P_{50} Frequency Distributions across SO_2 Type for each Subject Group

5.4.1.2 Hill Coefficient (n Value) Comparison

As was the case with P_{50} , the Hill Coefficients using Measured SO_2 values in Experiments 1, 2 and 3 were higher than those predicted using Severinghaus and Kelman SO_2 values. Measured n was significantly higher in Experiment 1 than both Severinghaus and Kelman n (both at $p < 0.001$). It was also significantly higher in Experiment 2 than both Severinghaus ($p < 0.001$) and Kelman n ($p = 0.025$). While the overall effect of SO_2 type was significant in Experiment 3 ($p = 0.043$), none of the pair-wise comparisons were significant at the 0.05 level. However, the test had low power and the differences approached significance in the cases of Measured n and Severinghaus n ($p = 0.056$) and Measured n and Kelman n ($p = 0.079$). The Measured Hill Coefficient in Experiment 4 was lower than both predicted values, but the difference was not significant.

The Measured and Kelman Hill Coefficients in Experiments 1, 2 and 3 were all higher than have been previously reported in the literature (2.8-3), while the Severinghaus values tended to fall in this range.

Figure 15 shows the frequency distributions for n value according to SO_2 type, for each group of subjects at normocapnia. The lowlanders at sea level were combined into a single group.

Figure 15: n Value Frequency Distributions across SO₂ Type for each Subject Group

5.4.2 Comparison of Hill Parameters (P_{50} , n , K) Across Normocapnia Groups within each SO_2 Type

The comparisons of the results for each parameter within each group were presented above. This section deals only with comparisons across groups, within each SO_2 method. One-way ANOVA tests were used to compare P_{50} and Hill Coefficients across groups, with an alpha level of 0.05 for each SO_2 type. Tukey pair-wise tests were used for post-hoc analysis. We did not expect to find any differences between those in Experimental Group 1 and Experimental Group 2, since both groups comprised of lowlanders at sea-level. However, the method of testing varied slightly between these two groups, so differences reflecting the method are possible. The results are summarized below for P_{50} and n values. Detailed ANOVA results are provided in Appendix C.

The graphs below (Figure 16, Figure 17 and Figure 18) show the Hill Parameters for all normocapnic conditions, using all three SO_2 types. Significant differences across experimental groups within each SO_2 type are indicated.

Figure 16: Comparing P_{50} Values (mean $\pm$ SEM) across Experimental Groups within SO_2 Type

Figure 17: Comparing n Values (mean $\pm$ SEM) across Experimental Groups within SO₂ Type

Figure 18: Comparing K Values across Experimental Groups within SO₂ Type

(This graph shows the medians and 25% and 75% as the error bars)

5.4.2.1 P₅₀ Comparison

P₅₀ values were compared across Experiments. For all SO₂ types, P₅₀ values in Experiments 1 and 2 were significantly higher than those in Experiments 3 and 4, indicating that those at high altitude had significantly left shifted curves with respect to those at sea level. In the cases of Measured and Kelman, P₅₀ in Experiment 4 was almost significantly lower than that in Experiment 3 (p=0.054 and p=0.67). With more subjects, these differences would probably have been significant. This seems to indicate that chronically acclimatized Andeans have a left-

shifted curve with respect to that of acutely acclimatized lowlanders who have been at high altitude for two weeks.

5.4.2.2 Hill Coefficient (n Value) Comparison

Hill Coefficients in Experiments 1 and 2 were higher than those in experiments 3 and 4, using all SO_2 types. For Measured SO_2 the Hill Coefficients in Experiments 1 and 2 differed significantly from those in Experiment 4. For Severinghaus SO_2 those in Experiments 1 and 2 differed significantly from those in Experiment 3. For Kelman SO_2 , those in Experiment 1 differed significantly from those in Experiments 3 and 4. Those in Experiment 4 also had higher n values than those in Experiment 3 when using Kelman SO_2 .

The Hill Coefficient data is less consistent than the P_{50} data, and seems to depend on SO_2 type.

5.4.3 Composite ODC Comparison across Groups using Measured SO_2

The average ODCs for each normocapnic experiment are show below, in Figure 19. Measured SO_2 was used in order to generate n and K values for each subject in each condition. The average n and K value for each condition were used to create a curve. A few subjects were excluded from this analysis because they were outliers (had data more than 3 interquartile ranges from the mean). Figure 19

Figure 19: Average ODCs for Each Experimental Group at Normocapnia using Measured SO_2

Figure 20 shows the frequency distribution for P_{50} , using measured SO_2 , comparing across experimental groups. Figure 21 shows the frequency distribution for n value, using measured SO_2 , comparing across experimental groups. Lowlanders at sea level were combined into a single group for these graphs.

Figure 20: P_{50} Frequency Distribution using Measured SO_2 across Experimental Groups

Figure 21: n Value Frequency Distribution using Measured SO_2 across Experimental Groups

5.4.4 Influence of PCO₂ on the Hill Parameters

The influence of PCO₂ on the Hill parameters was investigated in Experiment 2, in which subjects were tested under three PCO₂ conditions. A two-way repeated measures ANOVA was done on the data from this group in order to assess the influence of both SO₂ type and PCO₂ level on the Hill Parameters. The detailed ANOVA results are presented in Appendix C. This analysis showed that there was a main effect of PCO₂ on P₅₀. P₅₀ in the hypercapnia condition was significantly higher (right-shifted) from those in the hypocapnia ($p < 0.001$) and normocapnia conditions ($p < 0.001$). The difference between P₅₀ in the normocapnia and hypocapnia conditions was not significant, even though the PCO₂ difference was greater between these two conditions than between normocapnia and hypercapnia. PCO₂ did not significantly influence the Hill Coefficient (n value).

5.4.5 Descending and Ascending PO₂ Comparison

The protocol for Experiment 1 included progressive hypoxia followed by a reversal of PO₂ steps to return to a PO₂ of 100 mmHg. It was hypothesized that each subject's descent profile might differ from his or her ascent profile due to carryover effects of hypoxia. To test this hypothesis each subject's data was split into descending and ascending data. The lowest PO₂ value appeared in both data sets. P₅₀ analysis was then separately conducted on each data set, and a paired t-test was used to compare results for the 3 P₅₀ parameters. Two subjects were excluded from this analysis. One subject had only one data set while the other subject's data did not fit the Hill equation. Analysis for the other subjects did not reveal any significant differences between descending and ascending P₅₀ ($t(10)=0.797, p=0.444$), n ($t(10)=0.0715, p=0.944$) and K ($t(10)=0.445, p=0.666$). It was therefore concluded that the *in vivo* ODC does not depend on the order of PO₂ steps.

6 Discussion

6.1 Main Findings

Our knowledge about the O₂ affinity of Hb comes from *in vitro* studies in which the temperature, PCO₂ and pH of blood are maintained while PO₂, the independent variable, is changed over a wide range of partial pressures and the corresponding SO₂ values are recorded. This method allows the characterization of the PO₂-SO₂ relationship of blood for an animal species or human ethnic-genetic pool, which may be subdivided by age (including foetal versus post natal differentiation), sex and altitude exposure history. The curve showing the Hb affinity for O₂ over a range of PO₂ values is not linear; it is sigmoidal as a result of increased cooperative binding of Hb subunits at low PO₂ values and reduced cooperative binding as Hb becomes saturated with O₂. By varying the PO₂ while holding constant the temperature, PCO₂ and pH, the intrinsic O₂ affinity of Hb, usually presented as an oxyhaemoglobin dissociation curve, can then be designated by a single PO₂-SO₂ point. By convention this point has been the P₅₀, the PO₂ at which 50% saturation occurs. Temperature, pH, PCO₂, [2,3-DPG] and other factors have the ability to offset the position of the ODC by increasing or decreasing the O₂ affinity of Hb throughout the PO₂ range. P₅₀ measured under standard conditions can then be used to quantitate the change in affinity (i.e., offset) or to identify any intrinsic differences of the O₂ affinity of Hb in different study populations.

The great advantage of mapping out the ODC by applying progressive reductions of PO₂ to blood in a test tube is that it allows the maintenance of standard conditions without uncontrolled exposure to the products of body processes that are triggered by hypoxia, which may independently affect the P₅₀. By the same token, the great limitation of these *in vitro* methods is that they cannot be used to measure the O₂ affinity of Hb in response to progressive hypoxia *in vivo*. The results of studies showing increased P₅₀ in animals adapted to chronic hypoxia and high altitude led us to propose that despite *in vitro* evidence to the contrary, similar changes in the O₂ affinity of Hb take place in humans. We proposed to study the question *in vivo* in anticipation that this approach would support our hypothesis; a negative study would support the previous *in vitro* results.

This is the first study to characterize the ODC *in vivo*. In this study we exposed volunteers to progressive isocapnic hypoxia while measuring PaO₂, PaCO₂ and pH and monitoring the

corresponding SO_2 as measured directly, by co-oximetry, or non-invasively, by pulse oximetry. We found that the P_{50} of lowlanders at sea level is greater than those previously reported using *in vitro* blood analysis. Whereas the same *in vitro* methods have led us to believe that the P_{50} in native Andeans living at 3600 m is near that of lowlanders living near sea level, our *in vivo* measurements indicate a marked relative left shift of the Andean ODCs. This shift was even present, to a significant, but lesser extent, in acclimatized lowlanders who spent two weeks at the same altitude.

When two of the most widely accepted models of *in vitro* ODC data, those of Severinghaus and Kelman, were applied to sets of our *in vivo* data, the models poorly predicted the *in vivo* ODC. Even at sea level, where we expected the Severinghaus and Kelman models to be accurate, they both over-predicted the P_{50} values. We expected the models, which assume sea level [2,3-DPG], to underestimate P_{50} at high altitude (Benesch & Benesch, 1967). Meanwhile, the P_{50} values predicted by the models were considerably greater than those measured *in vivo* for the Andeans at high altitude, while the P_{50} values measured *in vivo* were similar to those predicted by the models for the lowlanders acclimatized for two weeks to an altitude of 3600 m. Overall, these findings suggest that during progressive hypoxia *in vivo* there is *at least* one other mechanism that controls the O_2 affinity of Hb but is not taken into account in the models of *in vitro* ODC prediction.

I will now discuss my *in vivo* findings and then return to further discussions regarding *in vitro* studies.

6.2 The *in vivo* Approach to Measuring the ODC

This *in vivo* study provides evidence that, like the animals mentioned above, humans respond to hypoxia by increasing their Hb affinity for O_2 . This new finding is not supported by previous studies that were done using *in vitro* methods, probably because it reflects *in vivo* conditions that cannot be attained during progressive hypoxia in a blood sample. Using an *in vivo* approach to study the ODC is advantageous because it highlights the changes that happen in the body during progressive hypoxia. However, it is very difficult to measure the ODC under *in vivo* conditions because *in vivo* hypoxia leads to hyperventilation, which leads to a fall in PCO_2 and an increase in pH. Because both PCO_2 and pH influence the position of the ODC, it is impossible to measure a single ODC if these factors are allowed to change. PCO_2 and pH can be held stable in

the blood during progressive hypoxia *in vitro*, but doing so excludes any effects induced by hypoxia on the other tissues of the body, singly or in concert. *In vivo* manipulation of PO_2 and SO_2 would provide a more natural method of examining the ODC. However, it is very difficult to carry out because of the tight link between PO_2 , ventilation, PCO_2 and pH. There are few practical methods of dissociating these variables in the laboratory, and there are no methods that are suitable for studies at altitude. It is for this reason that the ODC has not been previously studied *in vivo*.

We used an automated method using previously described principles to control $PetCO_2$ and $PetO_2$ independently of one another and of ventilation (Slessarev *et al.*, 2007). The method was used to hold $PetCO_2$ steady at a predetermined level while inducing step changes in $PetO_2$ that led to progressive hypoxia. Both $PetO_2$ and $PetCO_2$ were held stable independent of ventilation. Below (Figure 22) is an example of such a study in a subject at sea level. Because PCO_2 is one of the factors that influence *in vivo* pH, by controlling $PetCO_2$ we are able to hold pH roughly constant. However, pH may still vary due to changes in metabolism and other factors that may occur during *in vivo* hypoxia. Some considerations and caveats of measuring the ODC *in vivo*, with respect to PCO_2 , pH and PO_2 , will be discussed below.

Figure 22: Sample ODC Data in a Subject at Sea Level

6.2.1 PCO₂

In order to successfully target a specific PetCO₂ and prevent PetCO₂ drift, it is important to ensure that the subject's resting parameters, resting PetCO₂, PetO₂, VCO₂ and VO₂, have been properly measured (Slessarev *et al.*, 2007). However, these parameters may change after they are measured, as speaking, subject movement or exertion can influence VCO₂, VO₂, resting PetO₂ and resting PetCO₂. For all subjects at altitude, PetCO₂ remained within ± 0.5 mmHg (Std Dev) during at least the last 60 s of each step. Throughout testing, PetCO₂ remained within ± 1.0 mmHg for all subjects. Similar data were not calculated for the lowlanders at sea level in Experiments 1 and 2, but the methodology and technology was the same so the extent of control is expected to be similar. PetCO₂ with this method was shown to be equal to PaCO₂ (Ito *et al.*, 2008), but we did not depend on this and measured PaCO₂ as well. The difference between PetCO₂ and PaCO₂ for lowlanders at altitude was 2.09 ± 0.89 mmHg and for Andeans at altitude it was 3.47 ± 1.70 mmHg.

6.2.2 pH

pH directly influences the position of the ODC via the Bohr effect. One factor that affects pH is PCO₂. pH can also be influenced by hypoxia, via lactic acid production, and by other factors such as strong ion difference and organic phosphate levels. As such, even if PCO₂ is controlled perfectly, pH may still change throughout the study, especially with progressive hypoxia, affecting the position of the ODC. In this study, pH was kept constant and no progressive changes in pH were seen in subjects. For each subject, pH varied less than ± 0.02 (Std Dev) within a trial. These pH changes were minor and too small to account for the observed left shift of ODC.

6.2.3 PO₂

Unlike *in vitro* methods, *in vivo* measurement of the ODC is limited by the safety of the subject and the ability of the subject to withstand progressive hypoxia. Because the steep portion of the ODC is what defines it, it is important to achieve SO₂ values that fall on this part of the curve. A limitation of *in vivo* measurement of the ODC is the tolerance of subjects for hypoxia. Another caveat of this method is that physical discomfort of *in vivo* hypoxia may influence the study results.

Unlike the case of PetCO_2 being equal to PaCO_2 and independent of shunt fraction, when using the RespirAct™ (Ito *et al.*, 2008), targeting PaO_2 is more problematic because the size the shunt fraction cannot be anticipated. Nevertheless, we found that at PetO_2 less than 80 mmHg, the average difference between PetO_2 and PaO_2 was 6.89 ± 2.9 mmHg for the lowlanders at high altitude and 3.69 ± 3.07 mmHg for the Andeans. For all subjects at high altitude, PetO_2 remained within ± 1.5 mmHg (Std Dev) during at least the last 60 s of each step. Similar data were not calculated for the lowlanders at sea level in Experiments 1 and 2, but the methodology and technology was the same so the extent of control is expected to be similar. As the PaO_2 was taken from invasive values, the PetO_2 did not affect the characterization of the *in vivo* ODCs.

6.2.4 Blood Gas and SO_2 Measurements

PetCO_2 and PetO_2 were continuously measured by gas sensors in the RespirAct™ during *in vivo* ODC testing. The CO_2 sensor within the RespirAct™ (Ir3107, Servomex Group Ltd, Sugar Land, TX, USA) is accurate to within $\pm 0.1\%$ of the CO_2 within the range of 0-10% CO_2 . The O_2 sensor (UFO 130, Teledyne Analytical Instruments, City of Industry, CA, USA) is accurate to within 1%. Both sensors were calibrated prior to each experiment.

Each PetO_2 step in our experiments lasted at least 2 minutes (in Experiment 1 they lasted 4 minutes and in Experiment 2 they lasted 3 minutes), and blood samples were drawn at the very end of each step. In the experiments at sea level the blood samples were analyzed using the Instrumentation Laboratory GEM Premier 4000, and in the experiments at high altitude the blood samples were analyzed using the the i-STAT (Abbott Point of Care, Illinois, USA), a handheld, portable system. These are commercially available devices that conform to required specifications. In the case of the lowlanders at sea level SO_2 was directly measured in the blood sample using the Gem 4000 device. We did not have access to a co-oximeter at high altitude. We instead used a finger pulse oximeter, the Onyx II 9550 (Nonin Medical, Inc., Plymouth MN). This device is rated as accurate $\pm 2\%$ in the saturation range between 70-100%. SpO_2 was recorded manually from this device at the time of each blood sampling.

6.2.5 P_{50} Calculation

The Hill equation (Hill, 1910) was used in this study to describe the *in vivo* ODCs and compare them across conditions and across methods of measurement. This equation describes the

relationship between a ligand and its enzyme or receptor. The Hill coefficient, n , defines whether there is cooperative binding, negative cooperative binding, or non-cooperative binding between the two molecules. Traditionally, the Hill co-efficient for the association of Hb and O_2 has been taken to be some value between 2.8 and 3, indicating co-operative binding between the two molecules. This is consistent with the observation that the ODC has a sigmoidal shape. The actual value of the Hill coefficient will depend upon the conditions under which the blood was taken and analyzed. This robust equation is still widely used to describe the Hb- O_2 relationship and calculate the P_{50} .

This is the first time that the Hill equation has been used to describe the ODC using multiple data points obtained *in vivo*. As such, the question remains as to how well it fits such data, especially given that the data in this study were only taken from the top-most portion of the ODC. While this is the physiological range of the curve, using only data from this range may decrease the accuracy with which this equation represents the *in vivo* ODC.

When we first fit our data to the Hill equation, it became apparent that data points at high PO_2 values greatly reduced the fit of the equation. Fit was assessed by calculating the R^2 value describing the relationship between the measured data and the line of best fit. When using the data points at high PO_2 values the R^2 was often below 0.90. When these data points were removed, the R^2 value was, in most cases, greater than 0.95. Because the highest data points fall on the flat part of the curve and are therefore less representative of the curve than the points that fall on the steeper portion, these data points were removed for the P_{50} analysis. This was specifically done because, if they were included, the curve-fitting program would accommodate these points to the exclusion of the points in the middle range.

6.3 The *in vivo* ODC at Sea Level and High Altitude

In this study we used the Hill equation to identify the P_{50} from the data generated *in vivo*. We found that the P_{50} values of both the lowlanders at high altitude (26.2 ± 2.59 mmHg) and the Andeans at high altitude (23.2 ± 2.66 mmHg) were significantly left-shifted from those of the lowlanders at sea level (30.5 ± 1.85 mmHg). There was also a strong trend for the P_{50} of the Andeans at high altitude to be lower (left-shifted) than those of the lowlanders at high altitude ($p=0.054$). It is possible that a larger sample would provide the power to confirm such a difference.

The difference in the position of the ODCs of Andeans and lowlanders at high altitude may have been due to the duration of acclimatization (two weeks vs. a lifetime, including a pre-natal period). On the other hand, as the mechanism that increases O₂ affinity of the Hb in the course of progressive hypoxia is unknown, it is possible that there are inducible responses to hypoxia in lowlanders and genetic or epigenetic factors acquired by the highlanders over the potentially 11,000 years of residency at altitude. This conjecture is still appropriate even though at the moment there is no proof of such adaptation in this population. On the other hand, such a mechanism may not have been previously discernable because there has not been a means of detecting the left shift of the ODC using *in vitro* methods.

6.3.1 Previous Studies in Lowlanders and Native Highlanders at High Altitude

Previous *in vitro* studies performed on the blood of both highlanders and lowlanders at high altitude have not always found a shift of the ODC and, when a shift was found, its direction was inconsistent. Since the discovery of 2,3-DPG, most *in vitro* studies have not found a shift in the ODC in blood of either lowlanders or high altitude natives at high altitude. The lack of a shift of the ODC at high altitude has been attributed to a balance between the leftward shift caused by respiratory alkalosis resulting from the hypoxic ventilatory response, and the rightward shift caused by hypoxia-induced increases in [2,3-DPG].

During AMREE, Winslow *et al.* (1984) measured P₅₀ *in vitro* from blood taken at sea level and on Mount Everest, at 6300 m, 8050 m and the summit, at 8848 m. There was no shift in the physiological ODC of lowlanders up to an altitude of 6300 m. However, above this altitude, the ODC was found to be shifted progressively leftward (in two subjects at 8050 m and only one subject at 8848 m). They attributed the leftward shift above 6300 m to the fact that above 6300 m there is a marked uncompensated respiratory alkalosis that dominates over the effect of increased [2,3-DPG]. The leftward shift at extreme altitude was considered to be an adaptive mechanism that preserves arterial Hb by enhancing lung O₂ uptake in the presence of severe reductions in atmospheric PO₂.

During OE II (Wagner *et al.*, 2007), which took place in 1985, five subjects were decompressed in a hypobaric chamber to the equivalent altitude of the summit of Mount Everest over a period of 42 days. The ODCs measured in the blood of those who participated in OE II, defined by

their P_{50} values, were not shifted from their sea level control values at any altitude, even beyond 6300 m. These P_{50} results, published in 2007, differed from those gathered above 6300 m during AMREE. The authors gave a few possible reasons for the discrepancy between the data from AMREE and OE II. Firstly, AMREE only had data from one subject at the summit of Everest and two subjects at 8050 m, while OE II had multiple samples from each of the 5 subjects above 6300 m. With such a small sample size and only one data sample per subject, the data from above 6300 m for the subjects in AMREE may not have been representative. Furthermore, the data from above 6300 m in AMREE were from venous samples, so arterial pH was calculated and not directly measured. Lastly, the ascent profiles of the two groups were different, with those in OE II ascending more quickly than those who reached the summit in AMREE. The difference in ascent profiles may account for the fact that those in AMREE had a greater degree of respiratory alkalosis, which would have led to a greater shift in their physiological P_{50} s.

In 1990, Mairbaurl *et al.*, (1990) published the first study of the physiological ODC in acclimatizing lowlanders under natural acid-base conditions. They tested 11 mountaineers at sea level and after 1 and 5 days at an altitude of 4559 m, and measured the *in vitro* ODC at physiological pH, PCO_2 and [2,3-DPG] without assuming any relationship between these variables and Hb- O_2 affinity. On the first night after arrival at 4559 m the physiological P_{50} s were significantly lower than their sea level values, but by the fifth day of altitude exposure the P_{50} s returned to baseline levels. The initial decrease in P_{50} upon exposure to high altitude was attributed to a respiratory alkalosis in the beginning stages of metabolic compensation. Although the compensation for the alkalosis remained incomplete with continued exposure, the P_{50} returned to the sea level value under the influence of increased [2,3-DPG]. Another interesting finding from this study is that the Bohr Effect is increased in sojourners at high altitude. This means that both O_2 uptake at the lungs and O_2 offloading at the tissues are enhanced, because PCO_2 is low at the lungs and high at the tissues.

Few studies have examined the ODC in native highlanders under their physiological acid-base conditions. In one such study, Winslow *et al.* (1981) measured the complete ODCs of 46 Peruvian highlanders at 4540 m and 25 controls at sea level. When the ODC measurements for the Peruvians were corrected to physiological pH (7.439 ± 0.065), there was no difference between the P_{50} values of the Peruvians at altitude and the sea level controls. Great variability was found in both groups, but the range of P_{50} values was greater at high altitude. As with the

instances above of acclimatized lowlanders, the similarity between the P_{50} values of the high altitude natives and sea level controls was attributed to an *in vivo* balance of the influences of alkalosis and increased [2,3-DPG] on the affinity of Hb for O_2 . A later study by Winslow *et al.* (1989) showed similar results in a group of high altitude Sherpas living at 3700 m, whose physiological ODCs were not shifted as compared to controls.

6.3.2 Adaptation to Hypoxia by Animals

In contrast to what previous *in vitro* studies have concluded about the ODC in humans, there is consistent evidence of left shift of ODC in animals adapted to hypoxia. It has been postulated that a leftward shift in hypoxic animals can be due to a) an intrinsically high Hb- O_2 affinity, b) an decrease in organic phosphate concentration, or c) a decrease in sensitivity to organic phosphates (Petschow *et al.*, 1977).

The bar headed goose flies over the Himalayas and has remarkably adapted to high altitude hypoxia (Petschow *et al.*, 1977). This bird is native to high altitude and has an Hb- O_2 affinity (standard P_{50} =29.7 mmHg) that is much greater than its relatives that are native to sea level (e.g. Canada goose standard P_{50} =42 mmHg). In the case of the bar headed goose, the difference in Hb- O_2 affinity is not due to intrinsic characteristics of the Hb. Rather, it is due to reduced interaction between Hb and organic phosphates, such as 2,3-DPG.

High altitude camelids, such as llamas and alpacas, have a number of adaptations to hypoxia. These animals have a relatively high number of RBCs but low hematocrit because their RBCs are small. Their RBCs are elliptical in shape, and live only around 60 days. Despite similar amounts of 2,3-DPG, the ODCs of camelids are left-shifted with respect to those of lowlanders at sea level (P_{50} of llamas is approximately 21 mmHg), and this shift persists even in llamas that are born and raised at sea level (Banchero *et al.*, 1971; Reynafarje *et al.*, 1975). This left shift is at least partly due to a persistence into adulthood of foetal Hb (55% foetal Hb), which has higher O_2 affinity than adult Hb. It has also been shown that llamas have lower venous PO_2 and SO_2 than humans, indicating a greater capacity for O_2 extraction (Banchero *et al.*, 1971). Thus, despite an increase in affinity, camelids do not seem to have difficulty extracting O_2 at the tissues.

Tadpoles tend to live in stagnant water that is high in CO_2 and low in O_2 . However, they still manage to have a significantly higher Hb- O_2 affinity than adult bull frogs (Pinder & Burggren, 1983). The difference in Hb- O_2 affinity that occurs with metamorphosis results from a change from larval Hb to adult Hb. It cannot be due to changes in organic phosphate, as organic phosphate levels are actually lower in adult frogs than in tadpoles. The increased affinity of tadpole Hb for O_2 may be due to a latent or reversed Bohr Effect, which has also been referred to as an “acid” Bohr Effect (Watt *et al.*, 1980). In addition, binding of organic phosphates to tadpole Hb does not seem to greatly influence the affinity of Hb for O_2 (Pinder & Burggren, 1983).

6.4 Explanation for the *in vivo* Left Shift of the ODC at High Altitude

The purpose of this study was to determine the *in vivo* position of the ODC at sea level and high altitude. It was not designed to address the causes of the observed left shift of the ODC at high altitude, which was greater in chronically acclimatized Andeans than in acutely acclimatized lowlanders after two weeks of exposure to an altitude of 3600 m. However, I can discuss possible causes for the left shift based on information from the published literature.

A number of factors are known to increase the affinity of Hb for O_2 in humans: increased pH, decreased temperature, decreased PCO_2 and decreased [2,3-DPG]. These and perhaps other factors are controlled and regulated during exposure to high altitude and influence the shift of the ODC in both lowlanders and native Andeans. In native Andeans, genetic and epigenetic mechanisms may also be at work.

6.4.1 Temperature

The combination of Hb with O_2 is an exothermic reaction, so when temperature is high the reverse reaction, which is endothermic, predominates. Temperature was not measured in the current study. However, it was assumed to be normal throughout. Hence, it is not expected that temperature played a role in the results of this study.

6.4.2 PCO_2 and pH

CO_2 binds to Hb and reduces its affinity for O_2 via the Haldane Effect. Additionally, it lowers pH and decreases the affinity of Hb for O_2 via the Bohr Effect. Oxyhaemoglobin is more acidic

than reduced haemoglobin, so decreases in pH decrease the affinity of Hb for O₂. H⁺ can also act as a ligand that binds to reduced Hb, thereby decreasing its affinity for O₂. pH can influence the position of the ODC via these mechanisms, independently of changes in PCO₂. These factors act as offsets that affect the ODC throughout the range of PO₂.

Exposure to high altitude is associated with the hypoxic ventilatory response. The increase in ventilation influences O₂ transport in two ways. It increases PaO₂ by increasing gas exchange with the atmosphere and it leads to a decrease in PCO₂ and an associated respiratory alkalosis, both of which facilitate O₂ loading from the atmosphere at the lungs by increasing Hb affinity for O₂. While the alkalosis caused by hyperventilation is compensated for metabolically by renal excretion of the base HCO₃⁻, this compensation is usually incomplete.

Compared to lowlanders at sea level (PCO₂ 41.0 ± 2.7 mmHg) PCO₂ was reduced in lowlanders after two weeks at high altitude (29.1 ± 2.9 mmHg) and in native Andeans (28.9 ± 3.4 mmHg). Neither group at high altitude had complete metabolic compensation for respiratory alkalosis. Compared to the pH values of the lowlanders at sea level (7.40 ± 0.03), lowlanders after two weeks at high altitude and native Andeans had higher pH values (7.44 ± 0.02 and 7.44 ± 0.02 respectively), which would tend to shift the ODC to the left. It is therefore likely that these factors contributed to the left shifts of the ODC that were seen in both groups of subjects at high altitude in this study. However, there were clearly additional factors involved, as the small degree of alkalosis was insufficient to account for the extent of the shift.

6.4.3 2,3-DPG

The relationship between [2,3-DPG] and Hb affinity for O₂ was first discovered by Benesch and Benesch (1967) and Chanutin and Curnish (1967), who demonstrated the allosteric modulation of the Hb-O₂ relationship by 2,3-DPG. Lenfant *et al.* (1968) then found that exposure to high altitude is associated with both an increase in *in vitro* P₅₀ and a parallel increase in [2,3-DPG]. While this study provided evidence of a correlation between exposure to high altitude, P₅₀ and [2,3-DPG], causality was not explored.

Evidence of a causal relationship between [2,3-DPG] and ODC position at high altitude came from a later study by Lenfant *et al.* (1971), in which subjects made acidotic by acetazolamide before exposure to high altitude did not show the rightward shift in ODC position that was seen

in the group members who were not given the drug. It was also shown that the respiratory alkalosis in response to hypoxia at high altitude increases the activity of phosphofructokinase (Minakami & Yoshikawa, 1966), a rate limiting enzyme of glycolysis, increasing 2,3-DPG production. Hypoxia also increases the concentration of reduced Hb (Juel & Milam, 1979), which has a higher affinity for 2,3-DPG than does HbO₂. The reduced Hb binds to the available 2,3-DPG in the RBC, decreasing the concentration of free 2,3-DPG in the cell. Free 2,3-DPG inhibits one of the enzymes in its synthetic pathway, diphosphoglyceromutase (Rose, 1968), so when the concentration of free 2,3-DPG in the RBC decreases, the production of 2,3-DPG is increased.

The high altitude subjects in this study were tested at 3600 m, where atmospheric pressure is roughly 495 mmHg and atmospheric PO₂ is roughly 100 mmHg. Despite increases in ventilation at high altitude and the associated beneficial effect on PaO₂, the subjects were nevertheless hypoxic as compared to sea level. In the group of lowlanders the average PaO₂ was 70.2 ± 15.3 mmHg, and in the group of Andeans the average PaO₂ was only 57.5 ± 4.3 mmHg. These values were significantly lower than 104.6 ± 8.3 mmHg, the average PaO₂ in the subjects at sea level, so high altitude hypoxia was likely.

2,3-DPG was not measured in our studies, but previous data suggest that its concentration was increased in both the lowlanders and highlanders at high altitude (Lenfant *et al.*, 1968; Torrance *et al.*, 1970b). It is possible that the lowlanders at high altitude had a higher [2,3-DPG] than the Andean natives, as this trend has been shown in other samples of lowlanders and highlanders in the Andes (Torrance *et al.*, 1970b). This might explain a higher Hb-O₂ affinity in the Andeans as compared to the lowlanders at high altitude.

6.4.4 Genetic Factors

Genetic factors play a role in increasing O₂ affinity in a variety of animals. Animals native to high altitude (such as llamas and yaks), but not animals that have recently been introduced to high altitude (such as sheep and cows), exhibit high haemoglobin affinity (Beall, 2007a) that is retained, to a large extent, when they are taken lower altitudes. Increased affinity has been achieved by changes in haemoglobin structure, as in deer mice in the Rocky Mountains (Storz *et al.*, 2007) and high altitude chickens that were introduced to the Andes only 500 years ago (Velarde *et al.*, 1991). Alternatively, it has also been achieved by a decrease in [2,3-DPG], as in

Tibetan pika (Ge *et al.*, 1998), or by decreased influence of 2,3-DPG, as in bar-headed geese (Petschow *et al.*, 1977).

Given the prevalence of genetic changes in some organisms native to hypoxic conditions, it seems possible that part of the *in vivo* left shift of the ODCs of our Andean subjects at high altitude may have been caused by genetic or epigenetic factors. The presence of genetic adaptation could explain the greater left shift in Andean natives than in acutely acclimatized lowlanders, although the difference between the positions of the ODCs in the two groups may simply be a function of more complete acclimatization in the Andean group due a greater extent of altitude exposure.

Genetic changes have been shown to occur in less than 500 years in Andean chickens (Velarde *et al.*, 1991), so it therefore seems possible that natural selection could have occurred since people arrived in the Andes 11,000 years ago. However, if genetic adaptations have occurred in human high altitude natives, they do not seem to be manifested in Hb or [2,3-DPG] changes. Tibetans and Andeans are reported to have normal Hb A (Beall *et al.*, 1998), and high altitude natives have been shown to have increased [2,3-DPG], which is associated with a rightward shift of the ODC (Torrance *et al.*, 1970b).

An autosomal dominant major gene (an inferred genetic locus whose alleles have a large quantitative effect) for increased Hb-O₂ affinity has been found in the Tibetan population (Beall *et al.*, 2004), which has been at altitude for approximately 25,000 years. Tibetans with the autosomal dominant allele have higher resting SO₂ values than those at the same altitude who are homozygous for the low saturation allele. Similarly, those who are homozygous or heterozygous for the high SO₂ allele have greater reproductive fitness, having more surviving children, primarily due to lower infant mortality. When the Darwinian fitness of those with the high saturation genotype was assigned as 1.0, the relative Darwinian fitness of those with the low saturation genotype was only 0.44. For the sake of comparison, those who are homozygous for Hb A have a Darwinian fitness coefficient of 0.66 in the case of Malaria. This indicates that the low saturation allele may be decreasing rapidly in the Tibetan population.

No such gene has been found in the Andean population, though Andean natives have greater SO₂ values than expected under conditions of ambient hypoxia (this should not be confused with a left-shifted ODC, which has not been shown *in vitro*) (Beall, 2007c). The average SO₂ in native

Andeans at 4000 m has been reported as 92%, which is greater than the average SO_2 of 88% in Tibetans at a similar altitude. Quantitative genetic techniques, which calculate the heritability for a trait by collecting phenotypic data from large samples of genetic relatives, have not shown that there is significant heritability for the trait of high resting SO_2 in Andean natives (Beall *et al.*, 1999). While the lack of heritability indicates that natural selection is not currently acting on the trait for Hb- O_2 affinity, it does not indicate whether or not the trait has been acted upon in the past. As seen in the case of Tibetans, the low saturation allele is decreasing rapidly. If this is the case, it is possible that natural selection has already occurred in the Andean population, such that the current population is now relatively homozygous for the trait.

One pathway that may be responsible for adaptation and acclimatization to high altitude is that of the hypoxia-induced transcription factor, HIF1 (Semenza, 2000). The oxygen dependent subunit of HIF1 is HIF1 α . HIF1 α is constantly being synthesized and degraded when PO_2 is normal. Hypoxia stabilizes HIF1 α and it is allowed to combine with HIF1 β to form HIF1. Hypoxia therefore leads to the increase of HIF1. This gene is often called the “master regulator” of homeostasis of oxygen because it induces more than 70 genes that respond to hypoxia. It is for this reason that HIF1 α and HIF1 have been implicated as important in acclimatization and adaptation to high altitude.

While it is possible that changes in the gene for HIF1 are responsible for physiological differences across high altitude populations (for example, there are differences in the HIF1 nucleotide sequence of Tibetans and Japanese controls, though not in the sequences of Andeans vs. controls), it is more likely that such differences are due to downstream, less global changes in the HIF1 pathway. This may include changes in the many genes induced by this factor or in the biochemical pathways that they influence. HIF1 is known to influence the production of erythropoietin (stimulates RBC production), vascular endothelial growth factor (stimulates blood vessel growth and permeability), endothelin (a vasoconstrictor produced in the blood vessels), and many other factors (Beall, 2007b). HIF1 also increases the rate of glycolysis and subsequently the production of 2,3-DPG. If HIF1 does influence the affinity of Hb for O_2 during *in vivo* hypoxia, its influence would not be discovered through *in vitro* methods. This is because the role of HIF1 is confined to genetic material, and such material is not contained in RBCs.

6.4.5 Summary

Altitude hypoxia is known to be associated with a decrease in PCO_2 , an increase in pH, and an increase in [2,3-DPG]. While the increase in pH and decrease in PCO_2 are known to shift the ODC to the left, increases in [2,3-DPG] are known to shift the ODC to the right. Previous studies that were performed *in vitro* have reported that these effects cancel each other out under physiological conditions at high altitude (Winslow *et al.*, 1981; Winslow *et al.*, 1984; Wagner *et al.*, 2007). However, both of our groups of subjects at high altitude exhibited marked leftward shifts in their *in vivo* ODCs. Furthermore, the leftward shift of the Andean natives was greater than that of the acutely acclimatized lowlanders. The *in vivo* leftward shift may indicate that 2,3-DPG does not balance out the influence of the respiratory alkalosis. Alternatively, it is likely that there are previously undiscovered factors that influence the position of the ODC during *in vivo* progressive hypoxia. Hb is an unstable molecule that is made up of charged amino acids. Its conformation is highly dependent on its environment, so any changes in the RBC environment, such as during metabolism, have the potential to influence the way Hb binds to O_2 . Additionally, the ODCs of the Andeans may have been influenced by genetic or epigenetic factors that become important during progressive hypoxia *in vivo*. However, it is impossible to know at this time whether the differences between Andean natives and acclimatized lowlanders were due to differences in the extent of acclimatization or the influence of adaptation in the native subjects.

6.4.6 Benefits of a Leftward Shift

Because a leftward shift of the ODC enhances O_2 uptake at the lungs, it increases the O_2 carrying capacity of the blood at high altitude. The benefit of increased Hb affinity for O_2 was assessed during an investigation of two children with mutant Andrew-Minneapolis Hb, which has a higher than normal affinity for O_2 , and their two siblings with normal Hb (Hebbel *et al.*, 1978). When taken to high altitude the children with mutant Hb had better exercise tolerance, as indicated by lower increments in resting heart rate. They had no decrease in maximal O_2 consumption, no increase in plasma or urinary EPO and no thrombocytopenia.

Some animal studies have confirmed such benefits of increased Hb- O_2 affinity. In one study, Eaton *et al.* (1974) investigated how increased Hb- O_2 affinity influences rat survival after exposure to extreme and sudden hypoxia. The ODCs of the experimental rats were shifted leftward by their ingestion of sodium cyanate. The P_{50} of these rats became 21 mmHg, much

lower than the P_{50} of the control rats (37.3 mmHg). These and control rats were then exposed to an atmospheric pressure of 223 mmHg (equivalent to 9180 m) over a 12 minute period and remained there for 78 minutes. The experimental rats had significantly lower increases in HR than the controls. They also had better survival, as all experimental rats survived the experimental protocol while only 2/10 of the control rats survived. Based on these studies and the fact that many high altitude animals have developed genetic adaptations that shift their ODCs to the left, this shift seems beneficial.

One potential problem created by a leftward shift of the ODC is reduced O_2 release at the tissues, since the Hb has a higher affinity for O_2 . It seems that a number of animals with left-shifted curves have mechanisms that manage to shift the curve leftward while enhancing offloading at the tissues. Evidence of this is that some animals with left-shifted curves, such as llamas, have especially low venous PO_2 and SO_2 (Banchero *et al.*, 1971). This indicates that they are able to extract the O_2 well, but does not explain how it is done. One possible way that has been shown in both animal and human studies is through an increased Bohr effect (Mairbaur *et al.*, 1990), which may be linked to increases in [2,3-DPG] (Benesch *et al.*, 1969; Samaja & Winslow, 1979). An increased Bohr Effect facilitates binding of O_2 at the lungs where PCO_2 is low and facilitates O_2 offloading at the tissues where PCO_2 is high. Thus, while a leftward shift of the ODC causes a greater Hb- O_2 affinity, O_2 need not be impaired. Rather, a leftward shift may be advantageous for both O_2 loading and offloading under hypoxic conditions.

6.5 *In vitro* Methods of ODC Measurement and Prediction

In vitro methods of ODC measurement are stable and repeatable. They involve the measurement of SO_2 during manipulation of PO_2 in a blood sample under controlled and constant conditions of pH and PCO_2 , which can be set to standard or physiological levels. While some methods, such as *in vitro* equilibration, are used to measure the ODC over its entire range, others, like the Canizaro Nomogram, rely on previous results to predict the position of the ODC under current conditions. Predictive methods of determining the position of the ODC are less reliable than direct measurement because they necessarily make assumptions about relationships between the variables that are known to contribute to the position of the ODC. Our *in vivo* data measured the net effect of these variables plus any other unmeasured influences on the ODC.

Measurement of Hb saturation in arterial blood can be difficult and expensive. A number of methods have therefore been created from *in vitro* data to allow the prediction of SO_2 for the entire range of the ODC. These predictions may be physiological or mathematical in nature, and predict SO_2 based on specific parameters, such as temperature and acid base status. Two such models are those of Severinghaus (1979) and Kelman (1966). The Severinghaus model is a modification of the Hill equation (1910), which describes the sigmoidal shape of the ODC for a given set of points that lie on a single ODC. This widely used model predicts SO_2 from PO_2 , base excess, pH and temperature. It is reported as accurate within 0.55% saturation. The Kelman model is robust because it uses PO_2 , PCO_2 , T and pH to calculate a “virtual PO_2 ,” which it then uses to predict saturation. It is a popular model because it allows for easy calculation of SO_2 and is thus very convenient. This is a mathematical model in which coefficients were found by using the least squares method on a data set containing 38 pairs of PO_2/SO_2 data. This model works under the assumption that, besides for pH at high saturations, these variables that are part of the “virtual PO_2 ” change the scale, but not the shape of the PO_2 axis. The factors used here were originally suggested by Severinghaus in formulating his nomogram (1966).

The Severinghaus and Kelman models were both created from *in vitro* data and are considered highly accurate (Breuer *et al.*, 1989). We therefore used these models to compare our *in vivo* data to what would have been expected if we measured our ODCs *in vitro*. We expected our *in vivo* data to differ from the model predictions because progressive hypoxia in a blood sample does not induce metabolic and other changes that are present during *in vivo* hypoxia. Similarly, these models do not take into account the increase in [2,3-DPG] that occurs with exposure to high altitude.

6.5.1 Comparison of *in vivo* data with *in vitro* Predictions

We graphically compared our *in vivo* SO_2 values to those predicted using the Kelman and Severinghaus models. We found that the predicted SO_2 values closely matched those measured *in vivo*, except in the case of the Andeans at high altitude, where *in vivo* SO_2 became progressively greater than predicted SO_2 as hypoxia became more severe. This finding is consistent with the *in vivo* leftward shift of the ODCs in this group.

We then used the Kelman and Severinghaus SO_2 values with the Hill Equation to predict where *in vitro* P_{50} would be under our measurement conditions. Not only was the Hill equation used to

determine P_{50} using measured SO_2 s, it was also used with the Kelman and Severinghaus SO_2 values to determine the P_{50} s that would be predicted by these models. In lowlanders at sea level the predicted P_{50} s were lower than *in vivo* measured P_{50} s, signifying that the *in vivo* affinity of Hb for O_2 was lower than expected despite the fact that the SO_2 values were similar. In native Andeans at high altitude the opposite was true, and *in vivo* P_{50} was significantly lower than those predicted by *in vitro* models.

We used the Kelman and Severinghaus predictions to compare *in vivo* and *in vitro* methods of measurement. The difference between the *in vivo* and *in vitro* results show that *in vitro* methods are not able to fully explore the ODC as it exists in a breathing subject, even in lowlanders at sea level. Further, the discrepancy between the two types of methods demonstrates why the *in vivo* leftward shift of the ODC has not been previously found in humans at high altitude. *In vivo* methods better reflect human physiology and provide new and exciting results.

6.6 Limitations

6.6.1 Samples

Due to the demanding protocol, the samples in this study were relatively small. This meant that many of the statistical tests had low power, such that it was difficult to detect differences between the groups and conditions. Another consequence of this factor is that some of the variables that were used in the analysis were not normally distributed. This would probably have been less of an issue had there been more subjects that provided a more complete spectrum of measurements.

The sample size issue was especially problematic in the cases of Experiments 3 and 4, which were performed at high altitude. Due to logistical problems of running an expedition, the only lowlanders who participated in the study at high altitude were those were researchers in the study. As such, there were only six subjects making up this non-random sample. While the lack of random sampling would not necessarily influence the data, in a sample of six the potential for associated error is much greater.

With regards to the Andean population, the genetic backgrounds of the participants were not known. It is likely that they came from mixed backgrounds, including Native American, European and West African. While there is not yet proof that the ODC of Andeans is influenced

by genetic factors, when dealing with such a population it would be desirable to know the ethnic background of the participants. We also relied on word of mouth in order to get subjects, so the sample was not necessarily representative. Again, because there were only eight subjects in the Andean protocol, this may have influenced the data set. The small sample size also meant that the data was less normally distributed, and the statistical tests had low power.

6.6.2 Methodology

The four groups in these experiments did not go through identical protocols. Experiment 1 entailed progressive hypoxia followed by a return to baseline PO_2 . Experiment 2 included a protocol in which PCO_2 alternated between 35 and 45 mmHg. Experiments 3 and 4, as well as the normocapnia condition of experiment two had normocapnic progressive hypoxia. It was assumed that the protocol would not influence the results. As mentioned above, in the case of Experiment 1, where data from the progressive hypoxia was analyzed together with the return to baseline data in order to get a single P_{50} , a comparison was done to ensure that the two types of data did not differ. There was no statistical difference between the two, so we combined them for statistical analysis. We also assumed that alternation of PCO_2 would not influence study results in Experiment 2.

6.6.3 Measured Variables

One of the major limitations of this study is the lack of analysis for factors affecting P_{50} . Although we did not measure 2,3-DPG, previous studies have consistently shown that its concentration is increased at altitude in both lowlanders and altitude natives. That the two groups of subjects at high altitude showed significant leftward shifts of the *in vivo* ODC despite presumed increases in [2,3-DPG] indicates that one or more powerful factor(s) are overwhelming its effect on the ODC. It is disconcerting that the literature does not provide a hint as to what this/these factor(s) may be. If the ions and molecules in the Hb environment were measured at each PO_2 step, a multiple regression analysis may have allowed for the discovery of new influences on the *in vivo* ODC. However, if the leftward shift was due to gene up-regulation invoking other molecules not mediated by these ions, having more haematological values would not have provided any advantage.

7 Conclusions

This is the first study to investigate the *in vivo* ODC. PO_2 - SO_2 predictions (using the Severinghaus and Kelman models) based on *in vitro* data provided good approximations of the measured SO_2 values in lowlanders at sea level and lowlanders at high altitude, but these predictions failed to describe *in vivo* SO_2 values and left shift of the ODCs of Andean natives at high altitude. Although measured SO_2 was not very different from predicted SO_2 in the case of the lowlanders at sea level, these lowlanders had *in vivo* ODCs that were statistically right shifted as compared to the predicted positions using SO_2 values from the two models. The other significant finding in this study was that, contrary to data from previous *in vitro* studies, the *in vivo* ODCs of the lowlanders and Andeans at high altitude were significantly left-shifted as compared to those of lowlanders at sea level. Additionally, the curves of the Andeans were left-shifted as compared to those of the lowlanders at high altitude. This difference approached significance using all three SO_2 types, and would likely have been significant had the power of the tests been slightly increased. The observed shifts in this study were likely due to a combination of acclimatization, *in vivo* reactions to acute, progressive hypoxia, and, in the case of the Andeans, adaptation to high altitude. Future studies will be able to address the causes of these leftward shifts in the *in vivo* ODC at high altitude.

8 Future Directions

Due to a small sample size, some of our statistical tests had low power and our results may not be representative (especially in comparing lowlanders and Andeans at high altitude). It would therefore be useful to repeat the study in more subjects at high altitude, to better characterize the responses. In order to generalize our findings, similar tests should be carried out in the high altitude natives of the Himalayas and Ethiopia in addition to the Andes. This would allow us to investigate if and how the different populations of high altitude natives have adapted to hypoxia, and whether they are similar in their adaptations. During these studies, more haematological and genetic studies may identify the mechanisms by which the ODC is affected at altitude. Because such studies will be expensive and logistically difficult, it may be useful to first come up with more sea level experiments that would point to the causes of the *in vivo* shifts.

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10 Appendix A: Baseline Haematological Values

10.1 Baseline Haematological Values by Experimental Group

Table 8: Haematological Data for Subjects in Experiment 1

Subject	pH	PCO₂	PO₂	BE	HCO₃⁻	Hct	Hb
21	7.41	41	122	1.2	26	36	12.4
22	7.41	41	99	1.2	26	36	12.5
23	7.37	45	100	0.2	26	43	15.5
24	7.37	40	107	-2	23.1	42	15.3
25	7.45	32	130	-0.9	22.2	44	15.7
26	7.43	39	105	1.5	25.9	38	13.1
27	7.42	35	112	-1.3	22.7	38	13.7
28	7.44	42	111	3.9	28.5	40	13.8
29	7.4	45	99	2.5	27.9	47	14.7
31	7.4	41	106	0.5	25.4	36	12.7
32	7.36	44	104	-0.8	24.9	43	14.7
33	7.41	42	125	1.7	26.6	36	11.7
34	7.42	45	99	4	29.2	36	12.8
Average	7.41	40.9	109.2	0.9	25.7	39.6	13.7
Std Dev	0.03	3.9	10.5	1.9	2.1	3.8	1.3

Table 9: Haematological Data for Subjects in Experiment 2

Subject	pH	PCO ₂	PO ₂	BE	HCO ₃ ⁻	Hct	Hb
1	7.44	42	98	3.8	28.5	44	16.2
3	7.41	40	105	0.7	25.4	38	13.5
5	7.37	40	107	-2	23.1	42	15.3
6	7.42	39	97	0.9	25.3	43	15.8
7	7.37	42	96	-1.1	24.3	46	16.1
8	7.39	40	98	-0.7	24.2	43	15.3
9	7.36	44	103	-0.9	24.9	45	16.7
10	7.4	41	101	0.5	25.4	42	15.1
11	7.39	42	97	0.2	25.4	45	15.9
12	7.43	41	105	2.6	27.2	46	16.6
13	7.34	41	99	-3.5	22.1	36	12.5
14	7.43	40	99	2	26.5	39	12.2
15	7.42	41	99	1.9	26.6	41	14.6
16	7.4	44	100	2	27.3	43	14.4
17	7.4	41	113	0.5	25.4	39	14.1
19	7.42	42	99	2.4	27.2	45	15.2
20	7.43	42	102	3.1	27.9	47	15.4
21	7.4	41	109	0.5	25.4	35	11.7
22	7.38	38	101	-2.3	22.5	39	13.4
Average	7.40	41.1	101.5	0.6	25.5	42.0	14.7
Std Dev	0.03	1.5	4.5	2.0	1.8	3.5	1.5

Table 10: Haematological Data for Subjects in Experiment 3

Subject	pH	PCO ₂	PO ₂	BE	HCO ₃ ⁻	Hct	Hb
1	7.43	27.9	58	-6	18.6	42	14.3
2	7.44	33.9	56	-1	22.8	45	15.3
3	7.47	26.7	57	-4	19.6	40	13.6
4	7.44	26	87	-6	17.9	40	13.6
5	7.42	30.3	74	-5	19.5	48	16.3
6	7.44	30	89	-4	20.3	48	16.3
Average	7.44	29.1	70.2	-4.3	19.8	43.8	14.9
Std Dev	0.02	2.9	15.3	1.9	1.7	3.7	1.2

It should be noted that the pH values for all subjects were relatively alkaline, with the average pH being 7.44 ± 0.02 . This is not surprising given that the average PCO₂ was 29.1 ± 2.9 , which is below the normal range. The increased pH is therefore probably a reflection of a respiratory

alkalosis due to the hypoxic ventilatory response of high altitude. The base excess is below the normal arterial range, which is -2 to +3.

Table 11: Haematological Data for Subjects in Experiment 4

Subject	pH	PCO ₂	PO ₂	BE	HCO ₃ ⁻	Hct	Hb
1	7.46	26	63	-6	18.2	49	16.7
2	7.44	27	57	-6	18.2	47	16
3	7.45	25.8	59	-6	17.8	50	17
4	7.44	28	61	-5	19.1	51	17.3
5	7.41	28.6	57	-7	18	44	15
6	7.43	35.5	50	-1	23.5	44	15
8	7.44	32.7	53	-2	22.2	47	16
9	7.45	27.2	60	-5	18.9	43	14.6
Average	7.44	28.8	57.5	-4.8	19.5	46.9	16.0
Std Dev	0.01	3.4	4.3	2.1	2.2	3.0	1.0

The average control pH value is on the upper range of normal and, like the lowlanders at altitude, is probably reflective of a respiratory alkalosis, as a result of the low PCO₂ of 28.5 ± 3.6 mmHg. The low PCO₂ was also reflected in the low TCO₂, which was 20.2 ± 2.4 mmol/L instead of 23-27 mmol/L, and the low bicarbonate, which was 19.1 ± 2.2 mmol/L instead of the normal range of 22-26 mmol/L. Base excess was outside the normal range of -2 to +3 mmol/L, at an average of -5.2 ± 2.1 . Average hematocrit and hemoglobin levels fell within the normal range, except in the case of subject 4 who was slightly above normal, which is 12-17 g/dL.

10.2 Cross-Experimental Comparison of Baseline Haematological Values

Seven control parameters (pH, PCO₂, PO₂, BE, HCO₃⁻, Hct and Hb) were compared across experimental groups, using a one way ANOVA, with an alpha level of 0.05. All results were significant, so a Tukey post-hoc test was used for pair-wise comparisons. Baseline pH, PCO₂, PO₂, base excess and bicarbonate for lowlanders at sea level were significantly different from those of both groups at high altitude. Those in Experiments 3 and 4 also had significantly different PO₂s from one another. Baseline hematocrit and haemoglobin of the lowlanders at sea level were significantly lower than those of the Andeans at high altitude. Individual p values for each test will be shown below.

Differences in baseline pH values were significant across groups ($F(2,43)=10.529, p<0.001$). pH for the lowlanders at sea level was significantly lower than those of Experiments 3 ($p=0.004$) and 4 ($p=0.002$). There was no significant difference between baseline pH values of the two groups at high altitude.

Differences in baseline PCO₂ values were significant across groups ($F(2,43)=87.916, p<0.001$). PCO₂ for the lowlanders at sea level was significantly higher than those of Experiments 3 ($p<0.001$) and 4 ($p<0.001$). There was no significant difference between baseline PCO₂ values of the two groups at high altitude.

Differences in baseline base excess were significant across groups ($F(2,43)=36.284, p<0.001$). Base excess for the lowlanders at sea level was significantly lower than for those of Experiments 3 ($p<0.001$) and 4 ($p<0.001$). There was no significant difference between baseline base excess values of the two groups at high altitude.

Differences in baseline bicarbonate values were significant across groups ($F(2,43)=47.486, p<0.001$). Bicarbonate for the lowlanders at sea level was significantly higher than those of Experiments 3 ($p<0.001$) and 4 ($p<0.001$). There was no significant difference between baseline Bicarbonate values of the two groups at high altitude.

Differences in baseline PO₂ values were significant across groups ($F(2,43)=109.089, p<0.001$). PO₂ for the lowlanders at sea level was significantly higher than those of Experiments 3

($p<0.001$) and 4 ($p<0.001$). PO_2 for the lowlanders at altitude was also significantly higher than that of the Andeans at high altitude ($p=0.032$).

Differences in baseline hematocrit were significant across groups ($F(2,43)=8.789$, $p<0.001$). Hematocrit for the lowlanders at sea level was significantly lower than for those of Experiment 4 ($p<0.001$). The lowlanders at sea level did not have different hematocrit from the lowlanders at altitude, nor did the lowlanders at altitude have different hematocrit from the Andeans at altitude.

Differences in baseline Hb were significant across groups ($F(2,43)=4.4$, $p=0.018$). Hb for the lowlanders at sea level was significantly lower than for those of Experiment 4 ($p=0.014$). The lowlanders at sea level did not have different Hb from the lowlanders at altitude, nor did the lowlanders at altitude have different Hb from the Andeans at altitude.

11 Appendix B: Experimental Data

11.1 PCO₂, pH and minimum PO₂ during Testing

A summary of the experimental data for each subject is shown below. The tables include the average and standard deviation for PCO₂ and pH, the minimum PO₂ achieved and the number of samples taken.

11.1.1 Experiment 1

Table 12: Experiment 1 Summary Data

Subject	Average PCO ₂ ± Std Dev	Average pH ± Std Dev	Min PO ₂	# of Samples
21	42.9 ± 0.8	7.39 ± 0.01	49	8
22	44.6 ± 2.2	7.37 ± 0.02	48	9
23	44.0 ± 0.7	7.38 ± 0.01	44	9
24	43.0 ± 0.7	7.40 ± 0.01	46	9
25	41.6 ± 1.9	7.37 ± 0.02	45	8
26	45.4 ± 1.2	7.37 ± 0.02	39	9
27	42.7 ± 1.5	7.35 ± 0.02	52	3
28	42.6 ± 1.5	7.41 ± 0.02	36	9
29	42.3 ± 2.2	7.43 ± 0.01	37	9
31	41.9 ± 1.9	7.38 ± 0.01	37	9
32	38.7 ± 0.9	7.40 ± 0.01	44	9
33	40.8 ± 1.2	7.40 ± 0.01	45	9
34	38.9 ± 2.0	7.45 ± 0.00	35	9
Average	42.3 ± 1.46	7.39 ± 0.01	42.8	8.4
Std Dev	2.0 ± 0.55	0.03 ± 0.00	5.3	1.6

11.1.1.1 PCO₂ Summary

In this experiment the target PCO₂ was 40 mmHg, while the average achieved PCO₂ across all subjects was 42.2 mmHg, with only two subjects having PCO₂ averages that fell below the target value, and one subject having a PCO₂ average that was 5 mmHg above the target value.

11.1.1.2 pH Summary

The average pH was 7.39 with an average standard deviation of 0.01 pH units. The pH specification for the device is 0.02 pH units, though the reported accuracy for the measurement device is less than 0.01 in this range pH range. Nine subjects had standard deviations greater than 0.01, while two subjects had standard deviations equal to or greater than 0.02 pH units.

11.1.1.3 PO₂ Summary

The lowest PO₂ achieved in this experiment was 35 mmHg, and the average minimum PO₂ was 42.8 mmHg. Except for one subject who achieved only three PO₂ targets, all subjects had 8 or 9 samples.

11.1.2 Experiment 2

Table 13. Experiment 2 Summary Data: Hypocapnia

Subject	Average PCO₂ ± Std Dev			Average pH ± Std Dev			Min PO₂	# of Samples
1	35.2	±	1.2	7.47	±	0.01	44	6
3	36.4	±	1.6	7.40	±	0.02	37	10
5	39.5	±	4.8	7.35	±	0.05	48	6
6	37.8	±	2.1	7.41	±	0.02	45	6
7	38.2	±	2.6	7.36	±	0.03	62	4
8	36.7	±	1.6	7.40	±	0.02	47	6
9	36.2	±	1.2	7.40	±	0.01	35	6
10	40.3	±	1.0	7.38	±	0.01	48	6
11	38.5	±	0.8	7.40	±	0.01	51	6
12	38.2	±	0.9	7.42	±	0.01	41	10
13	38.3	±	2.2	7.33	±	0.03	51	6
14	39.0	±	1.7	7.42	±	0.01	50	6
15	38.0	±	0.9	7.43	±	0.01	42	6
16	40.0	±	1.1	7.40	±	0.01	50	6
17	40.8	±	2.5	7.40	±	0.01	41	6
19	37.5	±	1.0	7.43	±	0.01	43	6
22	37.20	±	1.6	7.37	±	0.01	43	5
Average	38.11		1.7	7.40		0.02	45.8	6.3
Std Dev	1.53		1.0	0.03		0.01	6.1	1.4

Table 14. Experiment 2 Summary Data: Normocapnia

Subject	Average PCO₂ ± Std Dev		Average pH ± Std Dev		Min PO₂	# of Samples
1	41.4	± 2.0	7.42	± 0.02	36	26
3	40.2	± 1.0	7.39	± 0.01	43	26
5	41.8	± 3.6	7.35	± 0.03	41	22
6	42.9	± 1.5	7.38	± 0.02	44	22
7	45.3	± 1.7	7.31	± 0.02	39	25
8	43.0	± 2.0	7.35	± 0.02	38	26
9	42.2	± 1.9	7.35	± 0.02	34	25
10	44.1	± 1.7	7.36	± 0.02	39	26
11	42.5	± 0.9	7.37	± 0.01	36	24
12	41.5	± 1.2	7.41	± 0.01	43	26
13	44.1	± 1.9	7.29	± 0.02	44	26
14	42.1	± 1.0	7.40	± 0.01	40	26
15	40.5	± 1.0	7.41	± 0.01	36	25
16	44.0	± 1.0	7.37	± 0.01	40	25
17	44.0	± 1.7	7.37	± 0.01	37	26
19	40.8	± 2.2	7.42	± 0.01	35	26
20	41.9	± 1.7	7.42	± 0.01	39	25
21	43.1	± 1.0	7.35	± 0.01	42	26
22	40.7	± 1.5	7.34	± 0.01	35	26
Average	42.6	1.6	7.37	0.01	38.9	25.1
Std Dev	1.4	0.6	0.03	0.01	3.1	1.3

Table 15. Experiment 2 Summary Data: Hypercapnia

Subject	Average PCO₂ ± Std Dev	Average pH ± Std Dev	Min PO₂	# of Samples
1	48.3 ± 1.9	7.37 ± 0.02	47	6
3	45.0 ± 1.8	7.32 ± 0.01	45	10
5	47.8 ± 3.6	7.30 ± 0.03	64	4
6	47.8 ± 2.0	7.34 ± 0.02	41	8
7	49.2 ± 2.0	7.29 ± 0.02	62	4
8	48.0 ± 2.9	7.32 ± 0.01	67	4
9	45.3 ± 2.4	7.33 ± 0.01	38	6
10	47.5 ± 3.2	7.32 ± 0.03	36	8
11	46.3 ± 1.0	7.34 ± 0.01	44	7
12	45.5 ± 1.3	7.36 ± 0.01	47	10
13	47.2 ± 1.9	7.27 ± 0.01	49	6
14	46.7 ± 0.8	7.36 ± 0.01	41	7
15	44.3 ± 1.1	7.38 ± 0.01	38	7
16	48.2 ± 1.0	7.33 ± 0.01	49	6
17	47.8 ± 1.0	7.35 ± 0.01	66	4
19	44.0 ± 1.1	7.38 ± 0.01	41	6
21	48.8 ± 1.0	7.30 ± 0.01	78	4
22	45.5 ± 0.6	7.31 ± 0.01	62	4
Average	46.8 1.7	7.33 0.01	51.1	6.2
Std Dev	1.6 0.9	0.03 0.01	12.4	2.0

11.1.2.1 PCO₂ Summary

The average PCO₂s in each condition were higher than intended. In the hypocapnic condition it was 38.11 ± 1.53 instead of 35 mmHg, in the normocapnic condition it was 42.63 ± 1.38 instead of 40 mmHg, and in the hypercapnic condition it was 46.75 ± 1.57 instead of 45 mmHg. There was greater variability in the hypo and hypercapnic conditions. There was a mean difference between groups of almost 5 mmHg. A 1-way RMANOVA revealed that, as intended, PCO₂ differed significantly across groups ($p < 0.0001$).

11.1.2.2 pH Summary

The three PCO₂ conditions were not surprisingly associated with different pH levels. The hypocapnic condition was associated with “standard” pH of 7.4 ± 0.3 . The normocapnic condition, in which PCO₂ was slightly above “normal”, was associated with slight acidity, having an average pH of 7.37 ± 0.03 . The further PCO₂ increase in the hypercapnic condition

was associated with a more acidic pH of 7.33 ± 0.03 . A repeated measures ANOVA revealed that the pH differences were significant across conditions ($p < 0.0001$).

11.1.2.3 PO₂ Summary

A 1-way repeated measures ANOVA revealed that the minimum PO₂ was significantly different in the three conditions ($p = 0.0004$), with people tending to quit before the final PO₂ target in the hypocapnia and hypercapnia conditions.

11.1.3 Experiment 3

Table 16: Experiment 3 Summary Data

Subject	Average PCO ₂ $\pm$ Std Dev	Average pH $\pm$ Std Dev	Min PO ₂	# of Samples
1	25.7 $\pm$ 0.9	7.49 $\pm$ 0.02	36	5
2	32.0 $\pm$ 0.5	7.45 $\pm$ 0.00	35	5
3	24.6 $\pm$ 0.4	7.50 $\pm$ 0.01	38	6
4	25.4 $\pm$ 0.5	7.46 $\pm$ 0.01	33	5
5	28.4 $\pm$ 0.8	7.42 $\pm$ 0.02	32	5
6	28.0 $\pm$ 0.6	7.47 $\pm$ 0.01	35	6
Average	27.35 $\pm$ 0.6	7.46 $\pm$ 0.01	34.8	5.3
Std Dev	2.74 $\pm$ 0.2	0.02 $\pm$ 0.01	2.0	0.5

11.1.3.1 PCO₂ Summary

While efforts were made to target baseline PCO₂, a paired t-test revealed that experimental PCO₂ differed significantly from control PCO₂, whereby the control values were on average higher than the experimental values $t(5) = 7.44$, $p = 0.0007$. This may be due, in part, to the fact that we focused on maintaining stable PCO₂ rather than attaining the exact PCO₂ target. The average PCO₂ for all subjects during testing was 27.35 ± 2.74 , while the average standard deviation was 0.61 ± 0.16 mmHg.

11.1.3.2 pH Summary

Like PCO₂, pH during testing differed significantly from the control value during testing $t(5) = -3.37$, $p = 0.0199$. pH during the experiment tended to be higher than the control values, which makes sense given that PCO₂ tended to be lower during the experiment than the control value.

The average pH during testing was 7.46 ± 0.02 , while the average standard deviation for pH was 0.012 ± 0.005 .

11.1.3.3 PO₂ Summary

While only 2 of the 6 subjects reached the final PO₂ target, the average minimum PO₂ achieved in this study was 34.83 ± 1.95 mmHg. This is because the main concern regarding PO₂ was that there would be a range of PO₂ targets that fell on the steep part of the ODC. The study was terminated if the subject was too uncomfortable or if SO₂ fell around or below 70%, so the subjects who reached a PO₂ of ~35 on the second last step did not complete the last target. However, we were still able to attain a complete data set for each subject.

11.1.4 Experiment 4

Table 17: Experiment 4 Summary Data

Subject	Average PCO ₂ ± Std Dev		Average pH ± Std Dev		Min PO ₂	# of Samples
1	30.7	± 1.1	7.40	± 0.01	31	6
2	25.4	± 1.1	7.43	± 0.01	35	6
3	21.1	± 1.1	7.47	± 0.02	40	6
4	24.6	± 1.3	7.41	± 0.02	40	6
5	25.7	± 1.5	7.45	± 0.02	40	6
6	31.8	± 1.1	7.43	± 0.02	37	5
8	27.7	± 0.6	7.43	± 0.01	33	6
9	28.2	± 1.3	7.42	± 0.02	31	5
Average	26.6	1.2	7.43	0.02	37.2	5.8
Std Dev	4.0	0.2	0.02	0.00	3.7	0.43

11.1.4.1 PCO₂ Summary

A paired samples t-test did not reveal a significant difference between the mean PCO₂s during testing versus the control values them, $t(7)=1.68$, $p=0.1364$. The average PCO₂ for all subjects during testing was 26.55 ± 4.00 , while the average standard deviation was 1.20 ± 0.25 . Thus, the variation in PCO₂ was within the range of error for the PCO₂ sensors.

11.1.4.2 pH Summary

The PCO₂ difference between control and the experiment did not translate to a pH difference, as revealed by a paired samples t-test, $t(7)=0.67$, $p=0.5263$. The average pH during testing was

7.43 ± 0.02 pH units, while the average standard deviation was 0.019 ± 0.004 . For the most part, such variation falls within the specification of the pH sensor.

11.1.4.3 PO₂ Summary

Only two of the 8 subjects did not reach the final PO₂ target. However, not all of the subjects who reached the final target got to a PO₂ of 35. It is for this reason that the average minimum PO₂ was 37.17 ± 3.71 mmHg. Again, this is because we were not concerned about hitting the specific targets. Rather, we were concerned about having a range of PO₂ targets that fell on the steep part of the ODC. The study was terminated if the subject was too uncomfortable or if SO₂ fell around or below 70%.

12 Appendix C: Hill Parameters

12.1 Data for all Subjects in all Conditions

The P_{50} results for each condition, using Measured SO_2 values, are shown below. The data are first organized by SO_2 type and then by experiment. P_{50} analysis was not conducted on data sets that included fewer than 3 pairs of PO_2/SO_2 . A similar analysis was done using calculated SO_2 using the Severinghaus and Kelman methods. These sets were chosen in order to show where P_{50} was expected to be, based on previous *in vitro* studies.

12.1.1 Measured P_{50} Data

Table 18: P_{50} Data for Measured SO_2 in Experiment 1

Subject	Linearized R^2	Non-Linearized R^2	n	K	P_{50}
21	0.961	0.949	3.32	1.30E-05	29.6
22	0.991	0.982	3.341	1.23E-05	29.5
23	0.990	0.988	3.602	4.54E-06	30.4
24	0.988	0.981	3.61	4.01E-06	31.2
25	0.972	0.943	3.656	3.40E-06	31.3
26	0.985	0.971	3.443	6.43E-06	32.1
28	0.987	0.981	3.362	1.14E-05	29.5
29	0.989	0.984	3.188	2.59E-05	27.4
31	0.977	0.906	2.816	9.46E-05	26.8
32	0.992	0.978	3.598	4.57E-06	30.4
33	0.993	0.996	3.9	1.23E-06	32.7
34	0.980	0.977	3.179	3.05E-05	26.3

Table 19: P_{50} Data for Measured SO_2 in Hypocapnia in Experiment 2

Subject	Linearized R^2	Non-Linearized R^2	n	K	P_{50}
1	0.935	0.856	3.471	7.82E-06	29.6
3	0.996	0.988	3.351	1.29E-05	28.7
5	0.988	0.987	2.606	1.76E-04	27.6
6	0.964	0.976	3.638	3.12E-06	32.5
8	0.998	0.998	3.169	2.04E-05	30.2
9	0.999	0.998	3.16	2.74E-05	27.7
10	0.931	0.958	2.515	3.35E-04	24
11	0.992	0.989	3.275	1.73E-05	28.4
12	0.989	0.978	3.444	9.43E-06	28.7
13	0.995	0.993	3.292	1.20E-05	31.2
14	0.983	0.972	4.394	1.71E-07	34.6
15	1.000	1.000	3.474	8.05E-06	29.2
16	0.999	0.999	3.876	1.38E-06	32.4
17	0.994	0.986	3.453	8.56E-06	29.3
19	0.997	0.999	3.158	2.77E-05	27.7
22	0.998	0.999	3.487	8.60E-06	28.3

Table 20: P_{50} Data for Measured SO_2 in Normocapnia in Experiment 2

Subject	Linearized R^2	Non-Linearized R^2	n	K	P_{50}
1	0.996	0.991	3.528	5.41E-06	31.1
3	0.986	0.988	3.611	4.46E-06	30.3
5	0.988	0.982	3.501	5.22E-06	32.2
6	0.997	0.997	3.494	4.76E-06	33.3
7	0.986	0.985	3.212	1.59E-05	31.1
8	0.988	0.979	3.428	7.42E-06	31.3
9	0.991	0.989	3.193	2.02E-05	29.5
10	0.994	0.990	3.45	7.85E-06	30.1
11	0.985	0.967	3.324	1.28E-05	29.6
12	0.997	0.992	3.891	1.47E-06	31.5
13	0.993	0.991	3.84	1.30E-06	34.1
14	0.994	0.989	3.673	3.07E-06	31.6
15	0.987	0.970	3.627	4.70E-06	29.4
16	0.997	0.992	3.618	4.09E-06	30.8
17	0.993	0.985	3.574	5.08E-06	30.3
19	0.957	0.873	3.131	3.22E-05	27.2
20	0.986	0.970	3.809	1.93E-06	31.6
21	0.988	0.967	3.742	2.07E-06	33
22	0.988	0.967	3.396	1.28E-05	27.5

Table 21: P_{50} Data for Measured SO_2 in Hypercapnia in Experiment 2

Subject	Linearized R^2	Non-Linearized R^2	n	K	P_{50}
1	0.860	0.956	4.946	1.90E-08	36.2
3	0.990	0.975	3.209	1.75E-05	30.3
6	0.946	0.959	3.567	3.48E-06	33.8
9	0.997	0.999	3.022	3.53E-05	29.7
10	0.972	0.966	3.262	1.39E-05	30.8
11	0.994	0.996	3.164	2.21E-05	29.5
12	0.981	0.945	3.542	5.25E-06	30.9
13	0.990	0.994	3.753	1.69E-06	34.5
14	0.995	0.994	3.573	3.93E-06	32.5
15	0.993	0.988	3.815	2.03E-06	31
16	1.000	1.000	3.757	1.91E-06	33.3
19	0.998	0.998	3.278	1.52E-05	29.4

Table 22: P₅₀ Data for Measured SO₂ in Experiment 3

Subject	Linearized R ²	Non-Linearized R ²	n	K	P ₅₀
1	0.996	0.993	3.032	6.19E-05	24.4
2	0.991	0.983	3.366	1.46E-05	27.3
3	0.994	0.995	3.515	7.97E-06	28.2
4	0.959	0.881	3.932	1.72E-06	29.2
5	0.997	0.998	2.437	5.19E-04	22.2
6	0.993	0.983	3.094	4.23E-05	25.9

Table 23: P₅₀ Data for Measured SO₂ in Experiment 4

Subject	Linearized R ²	Non-Linearized R ²	n	K	P ₅₀
1	0.976	0.976	2.830	1.17E-04	24.5
2	0.956	0.969	2.123	1.90E-03	19.1
3	0.974	0.965	3.085	5.83E-05	23.5
4	0.975	0.979	2.785	1.52E-04	23.5
5	0.995	0.985	4.074	1.27E-06	27.9
6	0.958	0.964	2.622	3.24E-04	21.4
8	0.998	0.999	2.817	1.24E-04	24.3
9	0.959	0.966	2.412	6.36E-04	21.1

12.1.2 Severinghaus P₅₀ Data

Table 24: P₅₀ Data for Severinghaus SO₂ in Experiment 1

Subject	Linearized R ²	Non-Linearized R ²	n	K	P ₅₀
21	0.997	0.997	2.892	6.56E-05	27.9
22	0.985	0.987	3.167	2.08E-05	30
23	0.998	0.998	2.933	5.54E-05	28.2
24	0.997	0.996	2.911	6.41E-05	27.5
25	0.991	0.983	3.065	3.14E-05	29.4
26	0.994	0.997	2.997	4.11E-05	29
28	0.999	0.999	2.924	6.18E-05	27.4
29	0.999	0.999	2.968	5.56E-05	27.1
31	0.999	0.997	3.02	3.89E-05	28.8
32	0.999	0.998	2.979	4.88E-05	28
33	0.999	0.999	2.905	6.62E-05	27.4
34	0.999	0.999	2.806	1.15E-04	25.3

Table 25: P₅₀ Data for Severinghaus SO₂ in Hypocapnia in Experiment 2

Subject	Linearized R²	Non-Linearized R²	n	K	P₅₀
1	0.993	0.989	2.889	8.65E-05	25.4
3	0.995	0.993	2.933	5.84E-05	27.7
5	0.984	0.988	2.42	3.52E-04	26.7
6	0.992	0.995	2.533	2.94E-04	24.8
8	0.998	0.996	2.838	8.20E-05	27.5
9	0.999	0.999	2.871	7.54E-05	27.3
10	0.998	0.998	3.133	2.45E-05	29.6
11	0.999	0.998	2.983	4.75E-05	28.1
12	0.999	0.999	2.951	5.88E-05	27.1
13	0.998	0.997	2.898	5.17E-05	30.1
14	0.998	0.997	2.988	4.99E-05	27.5
15	1.000	1.000	2.81	1.04E-04	26.1
16	0.998	0.999	2.699	1.48E-04	26.2
17	1.000	0.999	2.851	7.98E-05	27.3
19	1.000	1.000	2.827	1.00E-04	25.9
22	1.000	1.000	2.906	5.79E-05	28.7

Table 26: P_{50} Data for Severinghaus SO_2 in Normocapnia in Experiment 2

Subject	Linearized R^2	Non-Linearized R^2	n	K	P_{50}
1	0.998	0.996	2.96	5.67E-05	27.2
3	0.999	0.999	3.006	4.29E-05	28.3
5	0.999	0.998	3.112	2.40E-05	30.5
6	0.998	0.996	3.01	3.98E-05	28.9
7	0.998	0.997	3.084	2.47E-05	31.1
8	0.998	0.995	3.103	2.54E-05	30.2
9	0.995	0.996	2.866	6.73E-05	28.5
10	1.000	0.999	2.994	4.14E-05	29.1
11	0.999	0.998	2.923	5.73E-05	28.2
12	0.999	0.998	2.983	4.94E-05	27.7
13	0.997	0.995	3.043	2.73E-05	31.6
14	0.999	0.998	2.928	6.06E-05	27.5
15	0.999	0.999	2.879	7.47E-05	27.1
16	0.999	0.999	2.979	4.49E-05	28.7
17	0.998	0.998	2.966	4.73E-05	28.7
19	0.998	0.997	2.834	9.18E-05	26.5
20	0.998	0.997	2.947	5.95E-05	27.1
21	0.999	0.998	3.03	3.43E-05	29.7
22	0.999	0.998	2.96	4.40E-05	29.6

Table 27: P_{50} Data for Severinghaus SO_2 in Hypercapnia in Experiment 2

Subject	Linearized R^2	Non-Linearized R^2	n	K	P_{50}
1	0.995	0.997	3.068	3.07E-05	29.5
3	0.998	0.997	2.949	4.30E-05	30.2
6	0.993	0.992	2.96	4.32E-05	29.8
9	0.999	1.000	2.946	4.39E-05	30.1
10	0.999	0.999	3.181	1.68E-05	31.7
11	0.999	1.000	2.972	4.22E-05	29.6
12	1.000	1.000	2.962	4.67E-05	28.9
13	0.998	0.998	3.101	1.94E-05	33.1
14	0.999	0.999	3.004	3.95E-05	29.2
15	1.000	1.000	2.958	5.03E-05	28.3
16	0.999	0.999	3.005	3.56E-05	30.2
19	0.999	0.998	2.908	6.14E-05	28

Table 28: P₅₀ Data for Severinghaus SO₂ in Experiment 3

Subject	Linearized R ²	Non-Linearized R ²	n	K	P ₅₀
1	0.997	0.996	2.724	1.86E-04	23.4
2	0.997	0.998	2.907	7.88E-05	25.7
3	0.994	0.992	2.683	2.25E-04	22.8
4	0.998	0.999	2.932	7.50E-05	25.5
5	0.997	0.998	2.902	7.51E-05	26.3
6	0.996	0.997	2.828	1.16E-04	24.6

Table 29: P₅₀ Data for Severinghaus SO₂ in Experiment 4

Subject	Linearized R ²	Non-Linearized R ²	n	K	P ₅₀
1	0.998	0.998	2.924	6.25E-05	27.3
2	0.998	0.998	2.923	6.66E-05	26.8
3	0.994	0.997	2.855	9.94E-05	25.2
4	0.994	0.997	3.000	4.83E-05	27.4
5	0.999	0.998	2.718	1.64E-04	24.7
6	0.999	0.998	3.117	3.21E-05	27.6
8	1.000	1.000	2.825	9.79E-05	26.2
9	0.994	0.994	2.737	1.34E-04	26.0

12.1.3 Kelman P₅₀ Data

Table 30: P₅₀ Data for Kelman SO₂ in Experiment 1

Subject	Linearized R ²	Non-Linearized R ²	n	K	P ₅₀
21	0.996	0.994	2.97	4.94E-05	28.1
22	0.985	0.987	3.326	1.12E-05	30.8
23	0.998	0.998	3.092	2.96E-05	29.1
24	0.997	0.996	3.055	3.65E-05	28.3
25	0.992	0.986	3.200	1.86E-05	30.0
26	0.996	0.998	3.131	2.40E-05	29.8
28	0.999	0.998	3.123	2.81E-05	28.6
29	0.999	0.998	3.215	2.10E-05	28.5
31	0.998	0.997	3.205	1.89E-05	29.7
32	0.999	0.999	3.157	2.47E-05	28.8
33	1.000	1.000	3.101	3.10E-05	28.4
34	0.999	0.998	3.034	4.72E-05	26.6

Table 31: P₅₀ Data for Kelman SO₂ in Hypocapnia in Experiment 2

Subject	Linearized R ²	Non-Linearized R ²	n	K	P ₅₀
1	0.994	0.991	3.125	3.46E-05	26.7
3	0.993	0.991	3.069	3.49E-05	28.3
5	0.984	0.988	2.592	1.84E-04	27.5
6	0.993	0.996	2.765	1.20E-04	26.1
8	0.998	0.996	2.957	5.28E-05	27.9
9	1.000	0.999	3.052	3.79E-05	28.0
10	0.997	0.998	3.347	1.07E-05	30.5
11	0.999	0.999	3.16	2.43E-05	28.8
12	0.999	0.998	3.069	3.68E-05	27.8
13	0.997	0.996	3.11	2.33E-05	30.8
14	0.998	0.997	3.226	1.98E-05	28.7
15	1.000	1.000	3.039	4.27E-05	27.4
16	0.999	0.999	2.906	6.65E-05	27.3
17	1.000	1.000	3.103	2.97E-05	28.7
19	1.000	1.000	3.015	4.84E-05	27.0
22	1.000	1.000	3.157	2.23E-05	29.7

Table 32: P₅₀ Data for Kelman SO₂ in Normocapnia in Experiment 2

Subject	Linearized R ²	Non-Linearized R ²	n	K	P ₅₀
1	0.997	0.995	3.039	4.04E-05	27.9
3	0.998	0.999	3.076	3.28E-05	28.6
5	0.998	0.997	3.224	1.55E-05	31
6	0.996	0.993	3.077	3.07E-05	29.2
7	0.998	0.997	3.218	1.48E-05	31.7
8	0.997	0.995	3.189	1.82E-05	30.6
9	0.993	0.995	2.999	4.01E-05	29.2
10	0.999	0.999	3.162	2.14E-05	29.9
11	0.999	0.998	3.039	3.63E-05	28.9
12	0.997	0.997	3.054	3.72E-05	28.1
13	0.997	0.995	3.206	1.47E-05	32.1
14	0.999	0.998	3.128	2.74E-05	28.7
15	0.999	0.999	3.043	3.90E-05	28.1
16	0.999	0.998	3.125	2.51E-05	29.6
17	0.998	0.998	3.145	2.34E-05	29.6
19	0.998	0.996	2.989	4.96E-05	27.5
20	0.998	0.997	3.08	3.47E-05	28
21	0.998	0.997	3.13	2.34E-05	30.1
22	0.999	0.998	3.114	2.47E-05	30.1

Table 33: P₅₀ Data for Kelman SO₂ in Hypercapnia in Experiment 2

Subject	Linearized R ²	Non-Linearized R ²	n	K	P ₅₀
1	0.992	0.995	3.252	1.46E-05	30.6
3	0.999	0.997	3.044	2.97E-05	30.7
6	0.993	0.992	3.14	2.12E-05	30.8
9	0.999	1	3.054	2.90E-05	30.6
10	0.998	0.998	3.277	1.16E-05	32
11	0.999	1	3.13	2.26E-05	30.5
12	0.999	0.998	3.06	3.16E-05	29.5
13	0.999	0.999	3.32	8.29E-06	33.9
14	0.999	0.999	3.209	1.76E-05	30.3
15	1	1	3.148	2.37E-05	29.4
16	1	1	3.206	1.60E-05	31.3
19	0.999	0.998	3.108	2.78E-05	29.2

Table 34: P₅₀ Data for Kelman SO₂ in Experiment 3

Subject	Linearized R ²	Non-Linearized R ²	n	K	P ₅₀
1	0.997	0.996	2.724	1.86E-04	23.4
2	0.997	0.998	2.907	7.88E-05	25.7
3	0.994	0.992	2.683	2.25E-04	22.8
4	0.998	0.999	2.932	7.50E-05	25.5
5	0.997	0.998	2.902	7.51E-05	26.3
6	0.996	0.997	2.828	1.16E-04	24.6

Table 35: P₅₀ Data for Kelman SO₂ in Experiment 4

Subject	Linearized R ²	Non-Linearized R ²	n	K	P ₅₀
1	0.999	0.999	2.945	5.97E-05	27.1
2	0.999	0.999	3.086	3.79E-05	27
3	0.987	0.993	2.891	9.44E-05	24.6
4	0.991	0.995	3.116	3.35E-05	27.3
5	0.993	0.994	2.742	1.59E-04	24.3
6	0.998	0.998	3.299	1.64E-05	28.2
8	0.999	1	3.03	4.71E-05	26.8
9	0.998	0.998	2.919	7.03E-05	26.4

12.2 Comparison of Hill Parameters (P_{50} , n , K) Across SO_2 Types (Measured, Severinghaus & Kelman) within each Experiment

A one-way repeated-measures ANOVA test was done to compare the P_{50} , n and K values from the measured and calculated data. Since the SO_2 values were different in the case of Experiment 4, we expected to find a difference between these parameters across the data sets. The results for each measured variable are shown below:

12.2.1 Experiment 1

Table 36: Experiment 1 P_{50} Values

Category	SO_2 Type	Mean	Std Dev	SEM
P_{50}	Measured	29.767	2.042	0.589
	Severinghaus	28	1.233	0.356
	Kelman	28.892	1.092	0.315

The analysis of variance revealed a significant difference in the P_{50} s of the three groups, $F(2,22)=7.243$, $p=.004$. A Tukey post-hoc analysis revealed that the measured P_{50} was significantly higher than that of Severinghaus ($p=.003$).

This is surprising, given that measured SO_2 tended to be greater than the calculated SO_2 . While 28 falls on the upper range of “normal” P_{50} s, 29.77 is right-shifted as compared to “normal”. It is not clear why this translated to a right-shifted curve, with a higher average P_{50} .

Table 37: Experiment 1 n Values

Category	SO_2 Type	Mean	Std Dev	SEM
n	Measured	3.418	0.283	0.0818
	Severinghaus	2.964	0.0929	0.0268
	Kelman	3.134	0.0951	0.0275

The analysis of variance revealed a significant difference in the n values of the three groups, $F(2,22)=19.523$, $p<.001$. A Tukey post-hoc analysis revealed that the measured n was significantly higher than that of Severinghaus ($p<.001$) and that of Kelman ($p=.002$). The difference between the Severinghaus n values and the Kelman n values approached significance

($p=.074$). n is dependent upon the medium in which the Hb is found, so it seems likely that some metabolic factor is changing the way in which Hb is binding to O_2 *in vivo*.

Table 38: Experiment 1 K Values

Category	SO ₂ Type	Median	25%	75%
K	Measured	8.92E-06	4.27E-06	1.94E-05
	Severinghaus	5.55E-05	0.00004	6.49E-05
	Kelman	2.64E-05	1.99E-05	3.38E-05

The K value data is not normally distributed ($p<.001$), so a repeated measures ANOVA was performed on ranks instead. There was a significant difference between the median K values among the experimental groups, $X^2(2)=16.167$, $p<.001$. A Tukey test was used for a pairwise comparison of the ranks of the three groups. It revealed a significant difference between the Severinghaus and Measured values ($p<.05$) and between the Severinghaus and Kelman values ($p<.05$).

12.2.2 Experiment 2

Table 39: Experiment 2 P₅₀ Results for Hypocapnia (n=16)

Category	SO ₂ Type	Median	25%	75%
P₅₀	Measured	28.95	28	30.7
	Severinghaus	27.3	26.15	27.9
	Kelman	27.95	27.35	28.75
n	Measured	3.398	3.165	3.481
	Severinghaus	2.88	2.819	2.942
	Kelman	3.069	2.986	3.141
K	Measured	1.07E-05	7.93E-06	2.39E-05
	Severinghaus	7.76E-05	5.48E-05	1.02E-04
	Kelman	3.59E-05	2.38E-05	5.06E-05

Table 40: Experiment 2 P₅₀ Results for Normocapnia (n=19)

Category	SO ₂ Type	Mean	Std Dev	SEM
P₅₀	Measured	30.816	1.765	0.405
	Severinghaus	28.747	1.428	0.328
	Kelman	29.416	1.303	0.299
Category	SO ₂ Type	Median	25%	75%
n	Measured	3.528	3.404	3.662
	Severinghaus	2.979	2.933	3.025
	Kelman	3.114	3.046	3.158
Category	SO ₂ Type	Mean	Std Dev	SEM
K	Measured	8.04E-06	7.81E-06	1.79E-06
	Severinghaus	4.81E-05	1.81E-05	4.14E-06
	Kelman	2.89E-05	1.00E-05	2.30E-06

Table 41: Experiment 2 P₅₀ Results for Hypercapnia (n=12)

Category	SO ₂ Type	Mean	Std Dev	SEM
P₅₀	Measured	31.825	2.206	0.637
	Severinghaus	29.883	1.402	0.405
	Kelman	30.733	1.275	0.368
Category	SO ₂ Type	Median	25%	75%
n	Measured	3.555	3.236	3.755
	Severinghaus	2.967	2.954	3.037
	Kelman	3.144	3.084	3.231
Category	SO ₂ Type	Mean	Std Dev	SEM
K	Measured	1.02E-05	1.08E-05	3.12E-06
	Severinghaus	3.94E-05	1.25E-05	3.60E-06
	Kelman	2.11E-05	7.60E-06	2.19E-06

A two-way repeated measures ANOVA was done on each P₅₀ parameter (P₅₀, n and K) in order to determine the individual and combined effects of PCO₂ and SO₂ type.

12.2.2.1 P₅₀

There is a main effect of PCO₂ on P₅₀ (p < 0.001). A Tukey post-hoc analysis revealed that P₅₀ in the hypercapnia condition was significantly higher than in the hypocapnia condition (q=13.057, p<.001) and the normocapnia condition (q=7.671, p<.001). There is also a main effect of SO₂ type on P₅₀ (p = <0.001). A Tukey post-hoc analysis revealed that P₅₀ was significantly higher when measured SO₂ was used than when Severinghaus (q=6.392, p<0.001) or Kelman was used

($q=4.012$, $p=0.025$). There is no significant interaction between PCO_2 and SO_2 type on P_{50} ($p = 0.789$).

12.2.2.2 n

The effect of PCO_2 on n approached significance ($p=.099$), while there was a significant effect of SO_2 type on the n value ($p<.001$). A Tukey post-hoc analysis revealed that measured n was significantly greater than those of Severinghaus ($q=10.974$, $p<0.001$) and Kelman ($q=7.833$, $p<.001$). There is no significant interaction between PCO_2 and SO_2 type on n ($p=.93$).

12.2.2.3 K

There was a significant effect of PCO_2 on K ($p=.005$). A Tukey post-hoc test showed that the K value in hypocapnia was significantly higher than that in hypercapnia ($q=4.979$, $p=.005$) and normocapnia ($q=3.737$, $p=.038$). There was also a significant effect of SO_2 type on K . A Tukey post-hoc test revealed that Severinghaus K was significantly higher than measured K ($q=5.583$, $p=.002$) and Kelman K ($q=3.909$, $p=0.029$). There was no significant interaction between PCO_2 and SO_2 type on K ($p=0.604$).

12.2.3 Experiment 3

Table 42: Experiment 3 P_{50} Values

Category	SO_2 Type	Mean	Std Dev	SEM
P_{50}	Measured	26.2	2.59	1.057
	Severinghaus	24.833	0.987	0.403
	Kelman	24.717	1.379	0.563

The analysis of variance did not reveal a significant difference in the P_{50} s of the three data sets, $F(2,10)=1.316$, $p=.311$. However, the power of the performed test (0.086) was below the desired power (0.800). With more subjects, they results may have been significant.

Table 43: Experiment 3 n Values

Category	SO ₂ Type	Mean	Std Dev	SEM
n	Measured	3.229	0.506	0.206
	Severinghaus	2.795	0.0709	0.029
	Kelman	2.829	0.104	0.0426

The analysis of variance revealed a significant difference in the n values of the three data sets, $F(2,10)=4.402$, $p=.043$. A Tukey post-hoc pairwise analysis did not detect any significant differences between any of the individual data sets. However, the difference between measured n and Severinghaus n approached significance ($p=.056$) and the difference between Measured n and Kelman n approached significance as well (0.079). Because the power of the Tukey test (0.500) was below the desired power (0.800), these negative findings should be interpreted cautiously.

Table 44: Experiment 3 K Values

Category	SO ₂ Type	Median	25%	75%
K	Measured	2.85E-05	7.97E-06	6.19E-05
	Severinghaus	1.38E-04	9.74E-05	1.53E-04
	Kelman	9.74E-05	7.51E-05	1.86E-04

The K value data is not normally distributed ($p=.002$), so a repeated measures ANOVA was performed on ranks instead. There was no significant difference between the median K values among the experimental groups, $X^2(2)=4.333$, $p=.142$.

12.2.4 Experiment 4

Table 45: Experiment 4 P₅₀ Values

Category	SO ₂ Type	Mean	Std Dev	SEM
P₅₀	Measured	23.163	2.662	0.941
	Severinghaus	26.4	1.065	0.377
	Kelman	26.463	1.346	0.476

The analysis of variance revealed a significant difference in the P₅₀s of the three data sets, $F(2,14)=7.026$, $p=.008$. A Tukey post-hoc pairwise comparison revealed that measured P₅₀ was significantly lower than Severinghaus P₅₀ ($p=.016$) and Kelman P₅₀ ($p=.014$).

Table 46: Experiment 4 n Values

Category	SO ₂ Type	Mean	Std Dev	SEM
n	Measured	2.843	0.577	0.204
	Severinghaus	2.887	0.133	0.0471
	Kelman	3.004	0.169	0.0596

The analysis of variance did not reveal a significant difference between the n values in the three data sets, $F(2,14)=0.36$, $p=.704$. However, because the power of the test (.049) is below the desired power (0.800), there may still be a difference between the n values of the 3 data sets.

Table 47: Experiment 4 K Values

Category	SO ₂ Type	Median	25%	75%
K	Measured	1.38E-04	8.77E-05	4.80E-04
	Severinghaus	8.22E-05	5.54E-05	1.17E-04
	Kelman	5.34E-05	3.57E-05	8.23E-05

The K value data is not normally distributed ($p<.001$), so a repeated measures ANOVA was performed on ranks instead. There was no significant difference between the median K values among the experimental groups, $X^2(2)=7.0$, $p=.03$. A Tukey post-hoc pairwise comparison revealed that the K value for the measured data was significantly greater than that in the Kelman data set ($p<.05$).

12.3 Comparison of Hill Parameters (P₅₀, n, K) Across Normocapnia Conditions within each SO₂ Type

For each experimental group, the Hill parameters were determined in three different ways, using measured SO₂, Severinghaus SO₂ and Kelman SO₂. The comparisons of the results for each parameter within each group were presented above. This section deals only with comparisons across groups, within each SO₂ method. 1-way ANOVA tests were used to compare across groups with an alpha level of .05. We did not expect to find any differences between those in

Experimental Group 1 and Experimental Group 2, since both groups comprised of lowlanders at sea-level. However, the method of testing varied slightly between these two groups, so differences reflecting the method are possible. The results are shown below.

12.3.1 P₅₀ Comparison

12.3.1.1 Measured P₅₀

Table 48: Measured P₅₀ Values for All Groups at Normocapnia

Experiment	N	Mean	Std Dev	SEM
1	12	29.767	2.042	0.589
2 normocapnia	19	30.816	1.765	0.405
3	6	26.2	2.59	1.057
4	8	23.163	2.662	0.941

The analysis of variance showed that measured P₅₀ differed significantly across experimental groups, $F(3,41)=28.108$, $p<0.001$. Post hoc analysis using the Tukey test indicated that P₅₀s in Experiment 1 were significantly higher than those in Experiment 3 ($p=.009$) and those in Experiment 4 ($p<0.001$). Similarly, P₅₀s in Experiment 2 were significantly higher than those in Experiment 3 ($p<0.001$) and those in Experiment 4 ($p<0.001$). The P₅₀ difference between Experiments 3 and 4 approached significance ($p=.054$).

12.3.1.2 Severinghaus P₅₀

Table 49: Severinghaus P₅₀ Values for All Groups at Normocapnia

Experimental Group	N	Mean	Std Dev	SEM
1	12	28	1.233	0.356
2 normocapnia	19	28.747	1.428	0.328
3	6	24.833	0.987	0.403
4	8	26.4	1.065	0.377

The analysis of variance showed that Severinghaus P₅₀ differed significantly across experimental groups, $F(3,41)=17.403$, $p<0.001$. Post hoc analysis using the Tukey test indicated that P₅₀s in Experiment 1 were significantly higher than those in Experiment 3 ($p<0.001$) and those in Experiment 4 ($p=0.042$). Similarly, P₅₀s in Experiment 2 were significantly higher than those in Experiment 3 ($p<0.001$) and those in Experiment 4 ($p<0.001$).

12.3.1.3 Kelman P₅₀

Table 50: Kelman P₅₀ Values for All Groups at Normocapnia

Experimental Group	N	Mean	Std Dev	SEM
1	12	28.892	1.092	0.315
2 normocapnia	19	29.416	1.303	0.299
3	6	24.717	1.379	0.563
4	8	26.463	1.346	0.476

The analysis of variance showed that Kelman P₅₀ differed significantly across experimental groups, $F(3,41)=27.092$, $p<0.001$. Post hoc analysis using the Tukey test indicated that P₅₀s in Experiment 1 were significantly higher than those in Experiment 3 ($p<0.001$) and those in Experiment 4 ($p<0.001$). Similarly, P₅₀s in Experiment 2 were significantly higher than those in Experiment 3 ($p<0.001$) and those in Experiment 4 ($p<0.001$). The P₅₀ difference between Experiments 3 and 4 approached significance ($p=.067$).

12.3.2 n Value Comparison

12.3.2.1 Measured n Values

Table 51: Measured n Values for All Groups at Normocapnia

Experimental Group	N	Mean	Std Dev	SEM
1	12	3.418	0.283	0.0818
2 normocapnia	19	3.529	0.217	0.0498
3	6	3.229	0.506	0.206
4	8	2.843	0.577	0.204

The analysis of variance showed that measured n differed significantly across experimental groups, $F(3,41)=7.146$, $p<0.001$. Post hoc analysis using the Tukey test indicated that n values in Experiment 1 were significantly higher than those in Experiment 4 ($p=.006$). Similarly, n values in Experiment 2 were significantly higher than those in Experiment 4 ($p<0.001$).

12.3.2.2 Severinghaus n Values

Table 52: Severinghaus n Values for All Groups at Normocapnia

Experimental Group	N	Mean	Std Dev	SEM
1	12	2.964	0.0929	0.0268
2 normocapnia	19	2.979	0.0761	0.0175
3	6	2.795	0.0709	0.029
4	8	2.887	0.133	0.0471

The analysis of variance showed that measured n differed significantly across experimental groups, $F(3,41)=7.169$, $p<0.001$. Post hoc analysis using the Tukey test indicated that n values in Experiment 1 were significantly higher than those in Experiment 3 ($p=.004$). Similarly, n values in Experiment 2 were significantly higher than those in Experiment 3 ($p<0.001$). The difference between the n values in Experiments 2 and 4 approached significance ($p=.10$).

12.3.2.3 Kelman n Values

Table 53: Kelman n Values for All Groups at Normocapnia

Experimental Group	N	Mean	Std Dev	SEM
1	12	3.134	0.0951	0.0275
2 normocapnia	19	3.107	0.0718	0.0165
3	6	2.829	0.104	0.0426
4	8	3.004	0.169	0.0596

The analysis of variance showed that measured n differed significantly across experimental groups, $F(3,41)=13.892$, $p<0.001$. Post hoc analysis using the Tukey test indicated that n values in Experiment 1 were significantly higher than those in Experiment 3 ($p<.001$) and Experiment 4 ($p=.043$). Similarly, n values in Experiment 2 were significantly higher than those in Experiment 3 ($p<0.001$). The difference between the n values in Experiments 2 and 4 approached significance ($p=.101$). The difference between the n values in Experiments 3 and 4 was also significant ($p=.018$).

12.3.3 K Value Comparison

12.3.3.1 Measured K Values

These data failed the normality test ($p < .001$) so a Kruskal-Wallis One Way ANOVA was performed on ranks.

Table 54: Measured K Values for All Groups at Normocapnia

Experimental Group	N	Median	25%	75%
1	12	8.92E-06	4.27E-06	1.94E-05
2 normocapnia	19	5.08E-06	3.32E-06	1.16E-05
3	6	2.85E-05	7.97E-06	6.19E-05
4	8	1.38E-04	8.77E-05	4.80E-04

There was a statistically significant difference in the median values among the experimental groups $H(3)=13.205$, $p=.004$. Dunn's Method was used as a pair-wise multiple comparison procedure to determine which groups differed from one another. This revealed a significant difference between the K values in experiments 2 and 4 ($p < .05$).

12.3.3.2 Severinghaus K Values

Table 55: Severinghaus K Values for All Groups at Normocapnia

Experimental Group	N	Mean	Std Dev	SEM
1	12	5.54E-05	2.37E-05	6.84E-06
2 normocapnia	19	4.81E-05	1.81E-05	4.14E-06
3	6	1.30E-04	3.65E-05	1.49E-05
4	8	8.81E-05	4.46E-05	1.58E-05

The analysis of variance showed that Severinghaus K values differed significantly across experimental groups, $F(3,41)=14.981$, $p < 0.001$. Post hoc analysis using the Tukey test indicated that K values in Experiment 1 were significantly lower than those in Experiment 3 ($p < .001$). K values in Experiment 2 were significantly lower than those in Experiment 3 ($p < 0.001$) and higher than those in Experiment 4 ($p=.009$). The difference between the K values in Experiments 1 and 4 approached significance ($p=.069$). The difference between the K values in Experiments 3 and 4 was also significant ($p=.042$).

12.3.3.3 Kelman K Values

This data failed the normality test ($p < .001$) so a Kruskal-Wallis One Way ANOVA was performed on ranks.

Table 56: Kelman K Values for All Groups at Normocapnia

Experimental Group	N	Median	25%	75%
1	12	2.64E-05	1.99E-05	3.38E-05
2 normocapnia	19	2.74E-05	2.19E-05	3.70E-05
3	6	9.74E-05	7.51E-05	1.86E-04
4	8	5.34E-05	3.57E-05	8.23E-05

There was a statistically significant difference in the median values among the experimental groups $H(3)=19.889$, $p < .001$. Dunn's Method was used as a pair-wise multiple comparison procedure to determine which groups differed from one another. This revealed a significant difference between the K values in experiments 1 and 3 ($p < .05$) and between experiments 2 and 3 ($p < .05$).