PALEOPHYSIOLOGY OF OXYGEN DELIVERY IN THE EXTINCT STELLER'S SEA COW, *HYDRODAMALIS GIGAS*

by

Anthony V. Signore

A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Biological Sciences

University of Manitoba

Winnipeg Manitoba

Canada

Copyright © 2016 by Anthony V. Signore

ABSTRACT

The order Sirenia is one of only two mammalian groups to have completely forgone terrestrial life. While extant sirenians are confined to the tropical waters, the recently extinct Steller's sea cow (*Hydrodamalis gigas*) evolved to exploit the frigid waters of the North Pacific Ocean. As limits on O_2 availability during submergence and decreased tissue temperature impose severe constraints on oxygen delivery, the oxygen binding (globin) proteins of sirenians are expected to have evolved under strong selection pressures. My comparative molecular analyses indicate that selection pressures on two globin genes (HBA and HBZ-T1) increased in transitional sirenians. As these genes encode the α-chains of all Hb isoforms throughout sirenian development, the resulting functional consequences to adult sirenian Hbs were tested using recombinant Hbs of Steller's sea cow, the dugong (*Dugong dugon*), their last common ancestor, and the Florida manatee (*Trichechis manatus latirostris*). These tests reveal that high affinity Hbs—exceeding those of other mammals examined to date—arose early in sirenian evolution, presumably to maximize O_2 extraction from the lungs and limit premature O_2 offloading during submergence. Moreover, I demonstrate that the Hb–O_2 affinity of the extinct sub-Arctic Steller's sea cow is less affected by temperature than other sirenians, safeguarding O_2 delivery to cool peripheral tissues. However, while this phenotype has primarily been attributed to the binding of additional allosteric effectors to the Hb moiety, Steller's sea cow Hb binds relatively few of these ligands. Instead, my results suggest the thermodynamic properties of discrete allosteric effector sites are altered by epistatic interactions, a phenomenon that appears to be a critical component to cold adaptation in mammalian Hbs.

As the HBA and HBZ-T1 loci also encode sirenian prenatal Hbs, the functional properties these proteins were tested to reveal their O_2 affinity increased in parallel to maternal

Hb. Notably, Steller's sea cow HbF has the highest reported O_2 affinity of any mammalian Hb tested to date. As the HBA gene encodes the α-subunit of both the prenatally expressed HbF and adult expressed HbA proteins, the molecular remodeling of this locus may have concurrently increased the affinity of each protein.

ACKNOWLEDGEMENTS

First and foremost, I'd like to thank my advisor, Kevin Campbell. His support over the years has provided me with every possible opportunity to succeed. I truly could not have asked for a better advisor and for that I will forever be grateful. While the list of skills, opportunities, and knowledge he has imparted on me is nearly endless, I am perhaps most appreciative for his initial encouragement that started me on my current career path. While I occasionally curse that decision, those scenarios are few and far between, as I am delighted to have found a line of work that I love to do (nearly) every day.

In addition to my advisor, I've been fortunate to have the guidance Jeffery Marcus, Jörg Stetefeld and Dirk Weihrauch, who've served on my advisory committee from the onset of my graduate work. Their expertise, encouragement, and guidance have greatly improved both the quality of this thesis as well as my capabilities as a research scientist. More recently, John Olson joined this committee in the role of external examiner and provided invaluable advice to improve this thesis and my work going forward.

My graduate work began under unusual circumstances, as the building that housed my laboratory suffered a major fire just weeks before the onset of my graduate program. I was displaced for nearly five years, but thanks to lab space provided by Margaret Docker and Steve Whyard, I was able to continue my work with minimal delays. From there our lab moved to semi-permeant space provided by Anne Worley and Bruce Ford, where I stayed for nearly four years until my fire-damaged laboratory was repaired. Without their generosity I could not have completed my graduate work on time.

I was fortunate to have the opportunity to work in a number of other labs outside the University of Manitoba. I was welcomed into the labs of Hendrik Poinar (McMaster University),

Michael Hofreiter (University of York, UK), Roy Weber and Angela Fago (Aarhus University), and Colin Brauner (University of British Columbia). While there I was assisted in the lab by Johanna Paijmans, Elin Ellebæk Petersen, and Phil Morrison. My experiences in these facilities are nothing but positive and I am thankful for the opportunity they've provided. I'd also like to thank my collaborators at the University of Nebraska-Lincoln, Jay Storz and Chandru Natarajan, who assisted me with *in vitro* hemoglobin expression and provided helpful comments to this thesis.

I'd like to express additional thanks to a number of other members of the Department of Biological Sciences. My lab mates, Mason Kulbaba, Mike Gaudry and Diana Hanna, who've provided me with advise, assistance in the lab, and much need escapes from the lab when necessary. Gary Anderson and Michelle Piercey-Normore, who kindly provided the use of various pieces of lab equipment. The departmental support staff, Sylvia Lapointe, Maureen Stopchycki, John Stoyko and Jaime Stringer, who assisted with booking flights, registering me for courses, ordering equipment and a seemingly endless list of other tasks.

Finally, I had a great deal of support from non-academic sources, my family. My parents, Denny and Cathy Signore, and Brittney McCallum have supported me throughout this work in every way imaginable. Even when times were difficult (and I was undoubtedly difficult to live with), their support never faltered and I love them for it.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2xYT	Two times concentrated yeast extract and tryptone culture media
α	alpha hemoglobin chain
aDNA	Ancient deoxyribonucleic acid
β	beta hemoglobin chain
bp	Base pairs
Cl^-	Chloride ion
CO	Carbon monoxide
CYGB	Gene encoding the cytoglobin peptide chain
δ	delta hemoglobin chain
ΔH	Enthalpy change
$\Delta H'$	Overall enthalpy change of hemoglobin oxygenation
ΔH^{Cl-}	Enthalpy change of chloride ion binding to hemoglobin
ΔH^{DPG}	Enthalpy change of 2,3-diphosphoglycerate binding to hemoglobin
ΔH^{H+}	Enthalpy change of proton binding to hemoglobin
dN	Non-synonymous amino acid substitutions
DNA	Deoxyribonucleic acid
DPG	2,3-Diphosphoglycerate
dS	Synonymous amino acid substitutions
ε	epsilon hemoglobin chain
γ	gamma hemoglobin chain
H^+	Proton

Hb(s)	Hemoglobin(s)
HBA	Gene encoding the alpha (a) hemoglobin peptide chain
HBB/HBD	Gene encoding the beta/delta (b/d) chimeric hemoglobin peptide chain
HBE	Gene encoding the epsilon (e) hemoglobin peptide chain
HBG	Gene encoding the gamma (g) hemoglobin peptide chain
HbS	Co-factor free "stripped" hemoglobin solution
HBZ	Gene encoding the zeta (z) hemoglobin peptide chain
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
isoHb	Hemoglobin isoform
LB	Luria-Bertani culture media
Ma	Mega-annum, one million years
MB	Gene encoding the myoglobin peptide chain
Mb(s)	Myoglobin(s)
n_{50}	Hill coefficient
NGB	Gene encoding the neurogliobin peptide chain
ω	Omega, the ratio of non-synonymous:synonymous amino acid substitutions
O_2	Dioxygen
OEC	Oxygen equilibrium curve
P_{50}	Partial pressure of oxygen which saturates 50% of hemoglobin molecules
PAML	Phylogenetic Analysis by Maximum Likelihood
PhyML	Phylogenetics by Maximum Likelihood

pKa	Logarithmic acid dissociation constant
PO_2	Partial pressure of oxygen
R	Universal gas constant
R-state	Relaxed or oxygenated state of hemoglobin
rpm	revolutions per minute
T-state	Tense or deoxy state of hemoglobin
TB	Terrific broth culture media
TN93+G	Tamura-Nei (93) + gamma model of sequence evolution
ζ	zeta hemoglobin chain

CHAPTER 1: GENERAL INTRODUCTION

1.1. Sirenian Life History

In this thesis I explore the molecular aspects underlying physiological adaptations to both aquatic and Arctic environments using the Order Sirenia as a model group. Sirenians, or sea cows, are a group of fully aquatic herbivores which diverged from their closest relatives, elephants and hyraxes, ~65 million years ago (Springer et al., 2015). The fossil record of sirenians demonstrates gradual reductions in limb size, streamlining of the body, and relocation of the nares until the Bartonian (~41 million years ago), at which point this group had completely forgone terrestrial life (Domning, 2000). While dugongs and manatees remain confined to tropical waters–largely due to food availability, poor insulation, and low thermogenic capacity–the ancestors of Steller's sea cows (*Hydrodamalis gigas*) evolved to exploit algal seaweeds (kelp) growing in frigid coastal waters of the North Pacific Ocean (Crerar et al., 2014). Thus, sirenians provide a compelling model to study the evolution of respiratory specializations for both aquatic life and cold tolerance.

1.2. Sirenians as a Model System

In addition to their unique life history (i.e., the only fully aquatic mammalian herbivores), a number of other factors make sirenians a particularly attractive model system. For example, the phylogenetic relationships between sirenians and their relatives (elephants and hyraxes, collectively known as paenungulates) have been examined in detail (Meredith et al., 2011; Springer et al., 2015). This work has been facilitated by the availability of completed genomes from the Florida manatee (*Trichechus manatus latirostris*), African elephant (*Loxodonta*

africana), woolly mammoth (*Mammuthus primigenius*), and Cape rock hyrax (*Procavia capensis*). Sirenians also exhibit a relatively low rate of molecular evolution, small size (four extant species), and relatively recent divergence that should facilitate the identification of amino acid replacements that may have aided their secondary transition to the aquatic and, in the case of Steller's sea cow, the Arctic niche (Campbell and Hofreiter, 2015). Unfortunately, these positives are countered by the fact that Steller's sea cows were hunted to extinction by 1768, a mere 27 years following their discovery (Stejneger, 1887). However, recent advances in the field ancient DNA (aDNA) and biotechnology now allow researchers to recover genomic sequences and 'resurrect' the proteins they encode from recently extinct lineages (Campbell and Hofreiter, 2015).

1.3. Ancient DNA

Ancient DNA refers to DNA isolated from extinct species or from other biological samples that have not been preserved for the purposes of DNA extraction, such as tissues/bones found in Siberian permafrost or museum specimens (Pääbo et al., 2004). This field's humble origins began by sequencing 229 base pairs (bp) of mitochondrial DNA from a 140 year-old muscle sample isolated from the quagga (*Equus quagga quagga*), an extinct subspecies of the zebra (Higuchi et al., 1984). While a sequencing experiment of this size may seem routine, aDNA presents a number of technological hurdles with respect of modern DNA samples. Years of environmental exposure confers a significant amount of damage to aDNA samples, most notably, aDNA is heavily fragmented (fragments typically <200 bp and often less <30 bp) and littered with hydrolytic deamidations (cytosine → uracil) (Pääbo et al., 2004). Damages such as these make the use of traditional polymerase chain reactions (PCR) and Sanger sequencing

unpractical, as amplified fragments generally have to be <120 bp and repeated multiple times to account for damaged bases. For these reasons, the advent of next-generation DNA sequencing technologies (sequencing by synthesis) for use with aDNA has greatly advanced the field, as the short read length (50-200 bp) and high sequence output (millions to billions of sequence reads) of these instruments are ideally suited for aDNA. However, as aDNA samples become heavily contaminated with exogenous DNA (namely microbial and human) following death–which may account for up to 99.9% of the total DNA content–up to 1000 fold more sequencing runs may be required to sequence an ancient genome relative to a modern one, making aDNA sequencing by this method prohibitively expensive for most applications (Orlando et al., 2013). Recently, however, this hurdle has been largely alleviated by the development of targeted sequence capture (Chapter 2; Springer et al., 2015), in which DNA baits (modelled after the desired nucleotide sequence from a closely related, extant organism) are annealed to aDNA samples as a means of enriching the desired sequences. The emergence of next-generation sequencing and DNA enrichment now allow complete genomes to be obtained from specimens that lived hundreds of thousands of years before *Homo sapiens* walked the Earth (Orlando et al., 2013). As 99.9% of species that have ever lived are extinct (Raup, 1992), aDNA allows researchers to regain some of this once lost information, expanding the phylogenetic 'tree of life' to include lineages with no living descendants. This methodology allows for a more comprehensive understanding of the evolution of life on Earth and, perhaps most importantly, when this genetic information is coupled with recombinant protein expression, may lend insights to both the molecular and physiological adaptations to past climate change events and environmental niches (Campbell et al., 2010a; Campbell and Hofreiter, 2015).

1.4. The Globin Family of Proteins

The globin family of proteins arose 2-3 billion years ago and have since diversified both inter- and intraspecifically (via whole genome and tandem duplications) to perform a variety of physiological functions (Burmester and Hankeln, 2014; Hardison, 1999). However, despite striking differences in amino acid composition, globin proteins across all biological Kingdoms contain highly conserved regions (namely heme and intersubunit contacts; Figure 1.1), which maintain a characteristic tertiary structure of 6-8 alpha helices with an embedded heme group (Weber and Fago, 2004). Known as the "globin fold", this distinguishing feature was observed when the crystal structure of sperm whale myoglobin (Mb)—the first protein to be deciphered by X-ray diffraction—was determined (Kendrew et al., 1958). Since this breakthrough, Mb and its blood borne counterpart, hemoglobin (Hb), have acted as models to study protein structure/function relationships. Responsible for O_2 uptake and delivery, these vital molecules have repeatedly been modified to optimize O_2 management in unfavorable environments (such as high altitude, underground, aquatic, and the Arctic) (Campbell et al., 2010a; Campbell et al., 2010b; Mirceta et al., 2013; Nery et al., 2013; Storz and Moriyama, 2008; Weber, 2007). These qualities, coupled with the ease of measuring

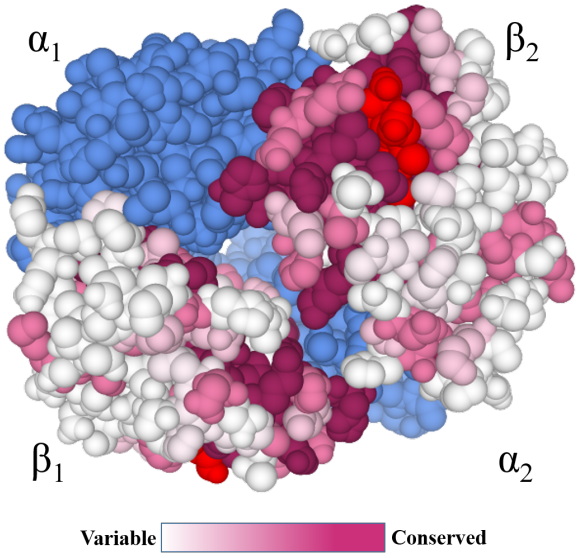


Figure 1.1. Diagram of mammalian hemoglobin. Alpha-type chains and heme groups are represented by blue and red colouration, respectively. The beta-type chains are coloured according to amino acid sequence conservation, as calculated by the ConSurf Server from a subsample of 50 mammalian beta-type hemoglobin chains (http://consurf.tau.ac.il/2016).

O_2 binding properties *in vitro* (Weber, 1992), make this family of proteins ideal candidates to study the molecular and physiological adaptations of sirenians to aquatic and Arctic life.

1.5. Thesis Objectives and Chapter Outline

I employed advanced ancient DNA sequencing technologies, recombinant protein expression, and detailed physiological tests to elucidate the molecular underpinnings of physiological adaptations that may have allowed sirenians to exploit both aquatic and Arctic niches. This work begins in Chapter 2 in which I analyze and describe the complete globin gene repertoire from three sirenians, three elephants, and a hyrax. Briefly, Florida manatee (*Trichechus manatus latirostris*), African elephant (*Loxodonta africana*), Asian elephant (*Elephas maximus*), woolly mammoth (*Mammuthus primigenius*), and Cape rock hyrax (*Procavia capensis*) globin gene sequences were mined and annotated from publically available databases, while corresponding dugong (*Dugong dugong*) and Steller's sea cow sequences were obtained using targeted hybridization capture and Illumina sequencing. Comparative molecular analyses of this data indicates that six of the eight functional globin genes evolved at markedly accelerated rates in the stem sirenian ancestor. Notably, the genes which encode the Hb proteins of adult sirenians (HBA and HBB/HBD) exhibit the highest rates of molecular evolution. In the HBA locus, this is in part due to the emergence of a chimeric pseudogene (HBA/HBQps) in an early ancestral sirenian that presumably accelerated the evolution of HBA via interparalog gene conversion. Moreover, the HBB/HBD genes of the dugong and Steller's sea cow contained a number of amino acid replacements that are known to alter Hb function in other species. However, as the presumed functional effects of these mutations conflict with our current understanding of Hb adaptation to

cold environments, the functional properties of sirenian Hbs were determined and are detailed in Chapter 3.

Recombinant Hbs that exactly match those of the Florida manatee, dugong, Steller's sea cow, and the predicted last common ancestor of the latter two species (ancestral dugongid) were expressed *in vitro*, purified, and their O_2 binding properties measured. The results of these functional tests support the contention that Hb–O_2 affinity increased after the divergence of sirenians with their terrestrial relatives, as manatee, dugong, and ancestral dugongid Hbs all display high O_2 affinity. This phenotype presumably enhances O_2 extraction from the lungs and limits premature O_2 offloading during prolonged dives. Moreover, in contrast to expectations based on amino acid sequences, the Hb–O_2 affinity of Steller's sea cow is less affected by temperature. Evolution of this phenotype is presumed to maintain O_2 delivery in face of decreasing tissue temperatures, allowing species that possess such a trait to take advantage of regional heterothermy (Weber and Campbell, 2011). However, unlike other 'cold-adapted' mammalian Hbs, where this phenotype is predominantly attributed to the binding of additional heterotropic (i.e., non-oxygen) ligands, Steller's sea Hb is weakly responsive to these allosteric effectors, yet their binding seems to contribute disproportionally to lowering the thermal sensitivity of the protein. This unusual observation is explored in greater detail in Chapter 4, where I quantify heterotropic ligand binding to six recombinant Hbs (manatee, dugong, Steller's sea cow, dugongid ancestor, Asian elephant, and woolly mammoth) and their associated thermal contributions. In contrast to the prevailing view, I found that the absolute number of ligands bound to the Hb moiety does not correlate with Hb thermal sensitivity. Rather, the thermal contribution of discrete ligand binding sites varies considerably, which is likely due to amino acid substitutions proximate to the binding sites. This finding challenges the notion that

additional heterotropic ligands bound to the Hb moiety underlie Hb adaptations to cold environments. Instead, the data presented here suggests that epistatic effects, in which amino acids in the vicinity of ligand binding sites alter their physiochemical properties, play an important role in Hb adaptations to cold environments.

While the results presented in Chapters 2, 3, and 4 are focused primarily on O_2 delivery in adult sirenians, the observed differences in adult sirenian Hb function may have physiological consequences that extend to earlier life stages. During mammalian development, a number of Hb isoforms (isoHbs) with high O_2 affinity are expressed in the embryonic/fetal circulatory system to facilitate O_2 transfer from the mother (Storz, 2016). Chapter 2 identified a number of amino acid substitutions in the prenatally expressed gamma Hb chain (γ) of Steller's sea cow that are expected to alter the O_2 binding properties of Hb proteins which contain this polypeptide chain. Thus, my final research chapter (Chapter 5) aimed to characterize the role of isoHbs in sirenian development. Briefly, I expressed recombinant prenatal Hb proteins Gower I ($\zeta_2\varepsilon_2$) and HbF ($\alpha_2\gamma_2$) from Steller's sea cow and HbF from the dugong. I found that each of these Hbs is functionally similar to Hbs expressed in the earliest (embryonic) stages of development in other mammals, which suggests that they are not expressed in fetal blood. Instead, it is likely that the fetal blood of sirenians contains the same Hb component as the maternal circulation, with maternal-fetal O_2 transfer likely being facilitated by reductions to intracellular DPG concentrations, as it is in most other mammals (Weber, 1994). Although, such a mechanism would not have been viable for Steller's sea cows, as Chapters 3 and 4 reveal its adult Hb protein is insensitive to this heterotropic ligand. Without a discrete fetal Hb isoform in Steller's sea cow, this species may be a rare example (felids are another; Novy and Parer, 1969) in which fetal and maternal blood have identical O_2 affinities. However, despite first examining the O_2-binding

properties of fetal blood in the mid-1920s (Huggett, 1927), this field is relatively nascent, as sample collection from unborn mammals is challenging. However, the techniques presented in Chapter 5 not only represent a method to study the role of isoHbs during development of living mammals, but is also the first physiological characterization of prenatal Hbs from an extinct species.

CHAPTER 2: EMERGENCE OF A CHIMERIC GLOBIN PSEUDOGENE AND SIGNIFICANT AMINO ACID REPLACEMENTS IN HEMOGLOBIN UNDERLIE THE EVOLUTION AND AQUATIC SPECIALIZATIONS OF SIRENIA

Anthony V. Signore[1], Johanna L.A. Paijmans[2], Michael Hofreiter[2], Mark S. Springer[3], and Kevin L. Campbell[1]

[1]University of Manitoba, Department of Biological Sciences, Winnipeg, Canada

[2]University of Potsdam, Institute of Biochemistry and Biology, Potsdam, Germany

[3]University of California, Department of Biology, Riverside, USA

Author contributions:

AVS designed the study, performed experiments, analyzed data and drafted the manuscript.

JLAP assisted with data collection.

MH provided laboratory space and research materials

MSS provided research materials and comments on the manuscript

KLC assisted with study design, provided laboratory space, research materials, and revisions to the manuscript.

2.1. Abstract

As limits on O_2 availability during submergence impose severe constraints on aerobic respiration, there are good reasons to expect that the oxygen binding globin proteins evolved under strong evolutionary pressures during the land-to-sea transition of aquatic mammals. Here we address this question for the order Sirenia by retrieving, annotating, and performing detailed selection analyses on the globin repertoire of the extinct Steller's sea cow (*Hydrodamalis gigas*), dugong (*Dugong dugon*), and Florida manatee (*Trichechus manatus latirostris*) in relation to their closest living terrestrial relatives (elephants and hyraxes). Results of our comparative molecular analyses indicate that most globin genes evolved at significantly accelerated rates in the stem lineage of sirenians during the secondary transition from a terrestrial to a fully aquatic lifestyle. While these genes generally evolved under strong purifying selection, the rate of non-synonymous/synonymous amino acid replacements increased in two genes (HBA and HBZ-T1) that encode the α-chains of all hemoglobin isoforms throughout sirenian development. Notably, the altered evolution of HBA appears to be temporally coupled with the emergence of a chimeric pseudogene (HBA/HBQps) that presumably contributes to the tandemly linked HBA of sirenians via interparalog gene conversion. Finally, we demonstrate that the adult-expressed HBB/HBD gene of both the dugong and Steller's sea cow evolved amino acid replacements at sites known to markedly alter the oxygenation properties of hemoglobin in other species. However, these mutations conflict with our current understanding of thermal adaptation and require experimental validation.

2.2. Introduction

Oxygen storage and management are the foundations of successful underwater foraging by aquatic mammals. As such, diving mammals have evolved physiological traits that optimize internal O_2 storage and exchange during periods of submergence apnea when atmospheric gas exchange has ceased (Ponganis, 2011). These specializations were accompanied by a suite of morphological changes, including pronounced changes to limb, tail, and body structures that are apparent in the fossil record of both cetaceans and sirenians (Uhen, 2007; Gatesy et al., 2013; Springer et al., 2015)—the only fully aquatic mammalian groups—which reduced the cost of underwater locomotion (Ponganis, 2011). While substantial work has focused on the molecular evolution of these morphological traits (see McGowen et al., 2014 for review), less attention has been directed to the molecular foundations underlying the evolution of O_2 management strategies that are essential for diving success. As limited O_2 availability during submergence imposes severe constraints on aerobic respiration, such activity presumably exerted strong selection pressures on the oxygen binding proteins (globins) of diving mammals.

The globin family of proteins is an ideal candidate to study the molecular basis of oxygen storage and management in aquatic mammals, as modifications to these proteins are directly linked with oxygen uptake and delivery. The best-known examples of such adaptations occur within vertebrate myoglobin (Mb) and hemoglobin (Hb), the proteins that facilitate O_2 delivery and storage within circulating erythrocytes and muscle, respectively (Mairbäurl and Weber, 2012). Mb is of particular importance to aquatic mammals, who have convergently evolved Mb proteins with an elevated net surface charge that increases protein stability, allowing for markedly increased Mb concentrations, and hence aerobic dive times (Mirceta et al., 2013; Samuel et al., 2015). While the modulation of Hb function with respect to diving is less well understood, modifications that increase O_2 loading in high altitude species, increase CO_2

carrying capacity in fossorial species, and optimize O_2 offloading in regionally endothermic species have been previously demonstrated (Weber, 2007; Campbell et al., 2010a; Campbell et al., 2010b; Storz et al., 2010). While Mb and Hb were long thought to be the only globins present in mammalian genomes, additional, highly distinct globin lineages have been discovered in the past 15 years. These include neuroglobin (Ngb) and cytoglobin (Cygb), which are primarily expressed in neurons and the cytoplasm of all cells, respectively (Burmester and Hankeln, 2014). The amino acid composition of these proteins is highly conserved among vertebrates, suggesting that each serves important physiological functions (Burmester et al., 2004). However, while their primary roles remains elusive, evidence suggests these proteins may be associated with O_2 storage, reactive oxygen species detoxification, NO production/scavenging, cell signaling, and regulation of apoptosis (Burmester and Hankeln, 2014), making them attractive subjects to study the molecular foundations of mammalian diving.

Diving has independently evolved multiple times among mammals, but only two groups, cetaceans and sirenians, have completely forgone terrestrial life. While the cetacean clade encompasses ~90 species (McGowen et al., 2014), the strictly herbivorous sirenian clade is small by comparison in that it consists of only four species divided amongst two living families. The family Trichechidae contains three extant species, the Amazonian manatee (*Trichechus inunguis*), the Florida manatee (*T. manatus latirostris*) and the African manatee (*T. senegalensis*), whereas the dugong (*Dugong dugon*) became the sole living representative of the family Dugongidae following the extinction of Steller's sea cow (*Hydrodamalis gigas*) in 1768 (Stejneger, 1887). Extant species inhabit shallow tropical and subtropical waters along coastlines throughout the Atlantic, western Indian, and southwest Pacific Oceans (Marsh et al., 2012). In the case of manatees, this distribution may reflect their nutritional and physiological restrictions,

as food availability and poor thermogenic abilities confine manatees to shallow tropical waters (Scholander and Irving, 1941; Blessing, 1972). While less is known regarding dugongs, their tropical/subtropical distribution suggests a similarly suppressed metabolic capacity (Marsh et al., 2012). Conversely, the extinct Steller's sea cows last inhabited the frigid waters surrounding the Commander Islands in the Bering Sea, with a recent distribution that extended along the Aleutian archipelago and even north of the Arctic Circle to St. Lawrence Island (Crerar et al., 2014). This ecological shift was presumably initiated by a period of global cooling in the middle Miocene (~15 million years ago; Ma) that reduced sea grass populations along the coast of California in favor of cryophilic kelps, which were adopted as a primary food source by the ancestors of Steller's sea cows (Domning, 1976). Morphological adaptations accompanying this dietary transition included the loss of teeth and the development of cornified pads on the upper palate and lower jaw that aided kelp consumption. These changes coincided with marked reductions in the forelimb elements (including a complete loss of fingers; Domning, 1976), development of a thick bark-like skin and blubber (Steller, 1751), and massive increases in size (up to 10 m in length and over 11,000 kg in mass; Domning, 1978). Nothing, however, is known regarding physiological or molecular adaptations underlying this thermal transition, despite its likely significance for oxygen unloading to cold peripheral tissues (Weber and Campbell, 2011).

To address these shortcomings, we used hybridization capture coupled with Illumina sequencing to retrieve and annotate the complete globin gene repertoire of the extinct Steller's sea cow (*Hydrodamalis gigas*) and dugong (*Dugong dugon*). These loci were combined with those mined from the genome of the Florida manatee (*Trichechus manatus latirostris*) and the molecular evolution of this collection of genes was compared to that of their closest terrestrial relatives (elephants and hyraxes). Briefly, we employed pairwise sequence similarity, synteny

analysis, phylogenetics, and selection analyses to identify both large scale structural changes in the alpha-globin gene cluster and signatures of relaxed selection within two α-type globin genes that coincided with the secondary aquatic transition of Sirenia. These analyses also identified a number of rare amino acid substitutions in the HBB/HBD genes of dugongs and Steller's sea cows, as well as the HBG gene of the latter that are known to markedly alter O_2 binding properties in the pre- and postnatal Hb isoforms of other species.

2.3. Methods and Materials

2.3.1. Genome mining

The complete α- and β-globin clusters, MB, NGB, and CYGB were mined from the draft genomes of the Florida manatee, African elephant, woolly mammoth (*Mammuthus primigenius*), and Cape rock hyrax. Additionally, all available globin gene sequences for these species and the Asiatic elephant (*Elephas maximus*) were mined from the National Center for Biotechnology Information's Sequence Read Archive and Nucleotide Collection (Appendix 1).

2.3.2. Globin Sequencing

We obtained complete coding sequences of MB, NGB, CYGB, and the full complement of α- and β-globin genes from two dugongs and three Steller's sea cow specimens using microarray hybridization enrichment coupled with Illumina sequencing. Three *H. gigas* specimens (ZI 6852, ZI 6853, and ZI 17170) collected in the mid-to late 1800s and housed in the Zoological Institute of the Russian Academy of Sciences (St. Petersburg, Russian Federation) were sampled. Ancient DNA was extracted following the procedure of Rohland et al. (2009) (ZI 6852) or via a DNeasy Blood & Tissue kit (ZI 6853 and ZI 17170). Extraction blanks, serving as negative controls, were

treated in a similar manner throughout. DNA samples from two female dugongs (MD33 and MD118), collected along the coast of Australia in the Torres Strait in 1998 and 1999, were previously extracted by Opazo et al. (2009).

To minimize cross-species contamination, indexed DNA libraries suitable for Illumina sequencing were prepared from the Steller's sea cow extracts in a dedicated ancient DNA clean lab (University of York, UK), while the indexed dugong libraries were prepared at the University of Manitoba (Winnipeg, Canada). The construction of DNA libraries, target enrichment, and Illumina sequencing are detailed elsewhere (Springer et al., 2015). Briefly, DNA libraries were prepared following the protocol of Meyer and Kircher (2010) with the addition of a DNA fragmentation step for modern samples (see Springer et al., 2015 for details). Probes for the targeted capture of complete sirenian globin genes (+500bp upstream and downstream of initiation and termination codons, respectively) were designed from available GenBank sequences for (in order of preference) *Dugong dugon*, *Trichechus manatus*, *Loxodonta africana*, and *Procavia capensis* (Appendix 1). Repetitive elements were identified using RepeatMasker (www.repeatmasker.org) and excised, and Agilent's eArray web application (https://earray.chem.agilent.com) was then used to create a series of overlapping 60 bp oligos (tiled at 1 bp) across all targets. The final microarray design was imprinted on 244K SureSelect microarrays by Agilent Technologies.

Hybridization of DNA libraries was performed following the protocol of Hodges et al. (2009), with the following modifications: (a) species-specific COT-I DNA was omitted from the hybridization mixture, and (b) after elution of the hybridized fragments, the mixture was purified using Qiagen MinElute columns (see Springer et al., 2015 for details). Following this targeted enrichment process, eluted DNA fragments were sequenced on an Illumina GAIIx genome

analyzer using a 55-bp singleton sequencing protocol. Raw Illumina reads were then trimmed of adapter sequences and those <20 bp were removed from the dataset using Trimmomatic (Bolger et al., 2014). These reads were assembled to reference sequences using Geneious R6.1 software (Biomatters Ltd, Auckland, New Zealand). All assemblies were manually checked by eye to remove any remaining unedited adapter sequences and assess the extent DNA damage in ancient samples. The coding sequences of the various globin genes were deduced from these assemblies and used in subsequent analyses.

2.3.3. Substitution Rates

The protein coding regions of each globin gene were aligned separately using MUSCLE (Edgar, 2004) (Appendices 2-10). Using gene specific models of nucleotide substitution (as determined by MODELTEST; Posada and Crandall, 1998) and a user generated timetree adapted from previous studies (Sirenian: Springer et al., 2015; Proboscidea: Rohland et al., 2007) the number of nucleotide substitutions per nucleotide site was calculated for each alignment using baseml, as implemented in the software package PAML 4.7 (Yang, 2007). Substitution rates were calculated both with and without the assumption of a constant molecular clock. Likelihood ratio tests were then used to determine if a constant molecular clock best fit the dataset (Yang, 2007). The relative contribution of non-synonymous and synonymous substitutions to the overall substitution rate was calculated from the above data using codeml, as implemented in the software package PAML 4.7 (Yang, 2007).

2.3.4. Selection Analyses

Selection pressures acting on a gene can be gaged by calculating the rates of non-synonymous (d*N*) and synonymous (d*S*) nucleotide substitutions. If the dN/dS ratio, or ω, at a given branch is less than one, equal to one, or greater than one, said branch has evolved under purifying, neutral or positive selection, respectively. The modes of natural selection acting on sirenian globin genes were determined using codeml, as implemented in the PAML 4.7 software package (Yang, 2007). The same nucleotide alignments and phylogenetic tree as above (see 2.3.3.) were tested under the M2 model using the F3x4 codon frequency model (Yang, 2007). Sirenian lineages (stem Sirenia, manatee, stem Dugongidae, dugong, and Steller's sea cow) were independently designated as the foreground branches and their ω value was estimated for each globin gene. These tests were then repeated with the ω parameter fixed to 1.0, rather than being estimated. Likelihood ratio tests were then used to determine if the estimated ω value for each branch was significantly different than 1.0 ($p < 0.05$). To detect instances of relaxed selection in the above alignments, a RELAX (Werheim et al., 2014) analysis was used, as implemented on the Adaptive Evolution Server (Datamonkey.org). To test for relaxed selection on the HBA gene after the sirenian lineage split from proboscideans (elephants), the stem sirenian branch was designated the "test branch" and the stem proboscidean branch was used as a reference.

Branch-site analyses were then performed in a similar manner using the MA model to test for individual amino acids evolving under positive selection (Yang, 2007). Sites with a posterior probability >0.95 under the Bayes empirical Bayes method were considered to have evolved under positive selection. All tests were run with the parameter "method = 0" which calculates branch length simultaneously, rather than individually.

2.3.5. Phylogenetic analysis

Paenungulate HBA and HBQ genes were aligned using MUSCLE (Edgar, 2004; 848 bp total length). In order to test for independent evolutionary origins of the 5' and 3' ends of the HBA/HBQps chimeric gene, the alignment was divided in two sections: 1) initiation codon to end the of the first intron (bp 1–353) and 2) beginning of the second intron to the termination codon (bp 593–848). Separate maximum likelihood trees were constructed from each of these partial alignments using PhyML under the TN93 + G model of nucleotide substitution (determined by MODELTEST as the best fit for both alignments). Trees were constructed using ten replicate heuristic searches with random number seeds and refined using both nearest neighbor interchange and subtree pruning and regrafting algorithms. The best tree produced by the two competing algorithms was subjected to branch support analysis by performing 500 bootstrap replicates.

2.4. Results and Discussion

2.4.1. Genomic Organization of Paenungulate Hemoglobin Gene Clusters

The α- and β-type Hb genes of mammals are arranged into clusters on (human) chromosomes 16 and 11, respectively. The 5' to 3' order of these genes determines at which life stage they are expressed, whereby genes at the 5' end (HBZ, HBE, and HBG) are expressed prenatally and genes at the 3' end (HBA, HBD, and HBB) are expressed in adults (Hardison, 1999; Hardison, 2012; Storz, 2016). Hemoglobin proteins are tetramers composed of two α-type and two β-type polypeptide chains. In early development, the α-type product of the HBZ (ζ) gene first dimerizes with the β-type product of HBE (ε) prior to forming the $\zeta_2\varepsilon_2$ Hb isoform. As development continues, expression of HBZ is replaced by HBA (α), which dimerizes with the products of the

β-type HBE and HBG (γ) chains prenatally, and then the products of HBD (δ) and HBB (β) after birth (Wood, 1976). Paenungulates exhibit a slight modification to this paradigm, as both the HBB and HBD genes became inactivated following the emergence and ascendancy of an "anti-Lepore" HBB/HBD (β/δ) mutant early in their evolution (Opazo et al., 2009); i.e. they only express a $\alpha_2\beta/\delta_2$ isoform in adulthood (Campbell et al., 2010a).

Orthology between sirenian, elephant, and hyrax Hb genes was identified by synteny analysis and pairwise sequence similarity (Figure 2.1 and 2.2) based on the draft genomes of the Florida manatee, African elephant (*Loxodonta africana*), and Cape rock hyrax (*Procavia capensis*). The arrangement of manatee α- and β-type Hb genes within their respective clusters resembles those of elephants (Figures 2.1 and 2.2), but with one important exception: sirenians do not possess HBA-T2 or HBQ genes, and instead have a non-functional, chimeric fusion hybrid (HBA/HBQps) of the two genes (Figure 2.1). Thus, the adult expressed α-type gene in sirenians (HBA) is orthologous to the HBA-T1ps locus in elephants, rather than to HBA-T2, which is expressed in adult elephant blood (Campbell et al. 2010a). Maximum likelihood phylogenetic trees using available paenungulate HBA, HBQ, and HBA/HBQps genes (Figure 2.3) support this conclusion in that they illustrate that the 5' regions of sirenian HBA/HBQps and HBA genes form a highly supported monophyletic clade (bootstrap value = 100) that is sister to HBQ, whereas the 3' regions of all paenungulate HBA genes form a strongly supported monophyletic clade to the exclusion of sirenian HBA/HBQps and other paenungulate HBQ genes (i.e. the 5' and 3' ends of sirenian HBA/HBQps genes are more similar to HBA and HBQ genes, respectively).

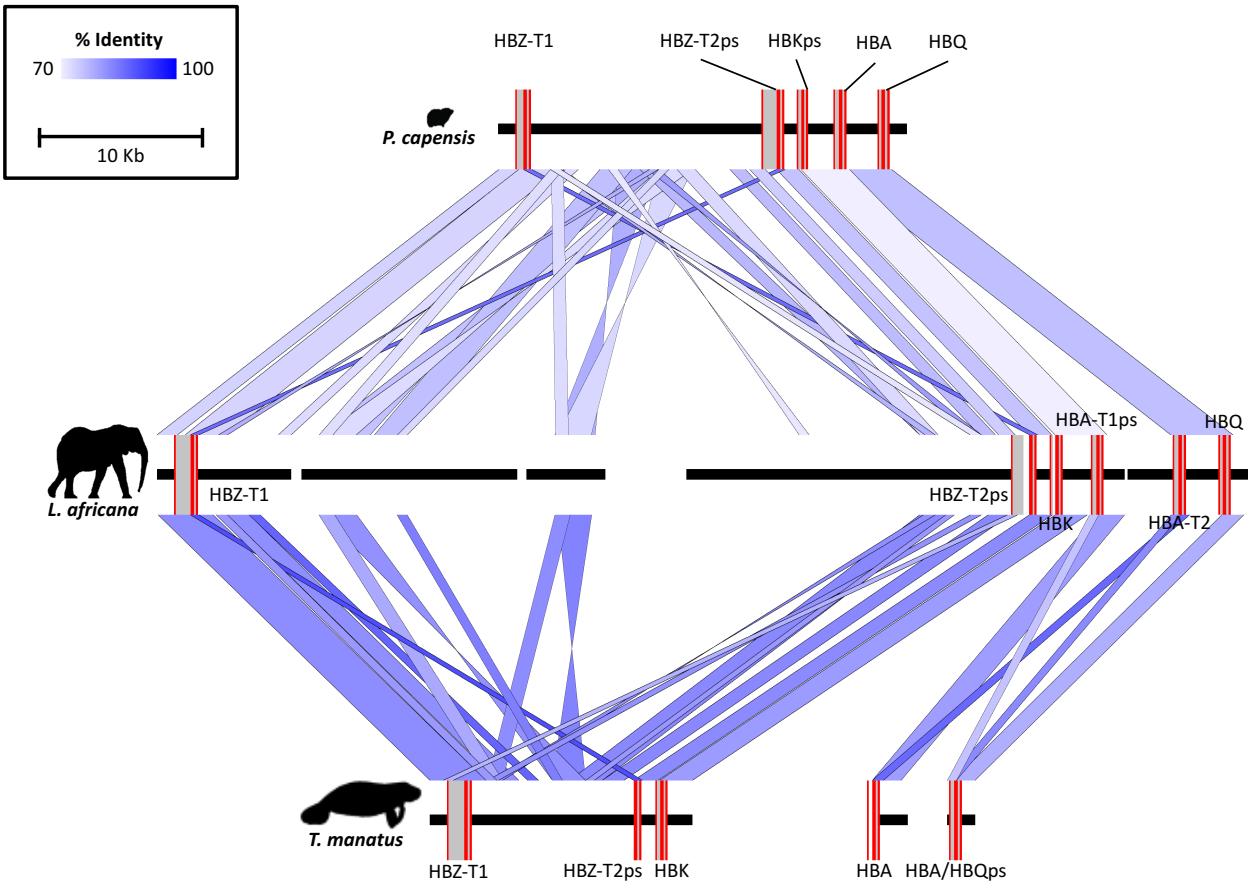


Figure 2.1. Organization and synteny analysis of rock hyrax (*P. capensis*), African elephant (*L. africana*), and Florida manatee (*T. manatus*) α-globin gene clusters. Red and grey boxes represent exonic and intronic sequences, respectively. The "ps" suffix denotes pseudogenes, while "-T1" and "-T2" suffices indicate the 5'-3' linkage order of tandemly duplicated genes. Thick black lines represent intergenic sequences. Gaps in the contiguous sequence denote missing data.

Genomic deletions and duplications are a common occurrence in the evolutionary history of mammalian α- and β-type Hb clusters (Hoffmann et al., 2008; Opazo et al., 2008). These changes in gene copy number often arise via unequal crossing over between mispaired paralogous genes on homologous chromosomes during meiosis or on sister chromatids in mitotic germ cells (Ohta, 1980), and result in a gene duplication on one chromosome and a deletion on the other. However, if the crossover point interrupts the coding region of the mispaired genes, the

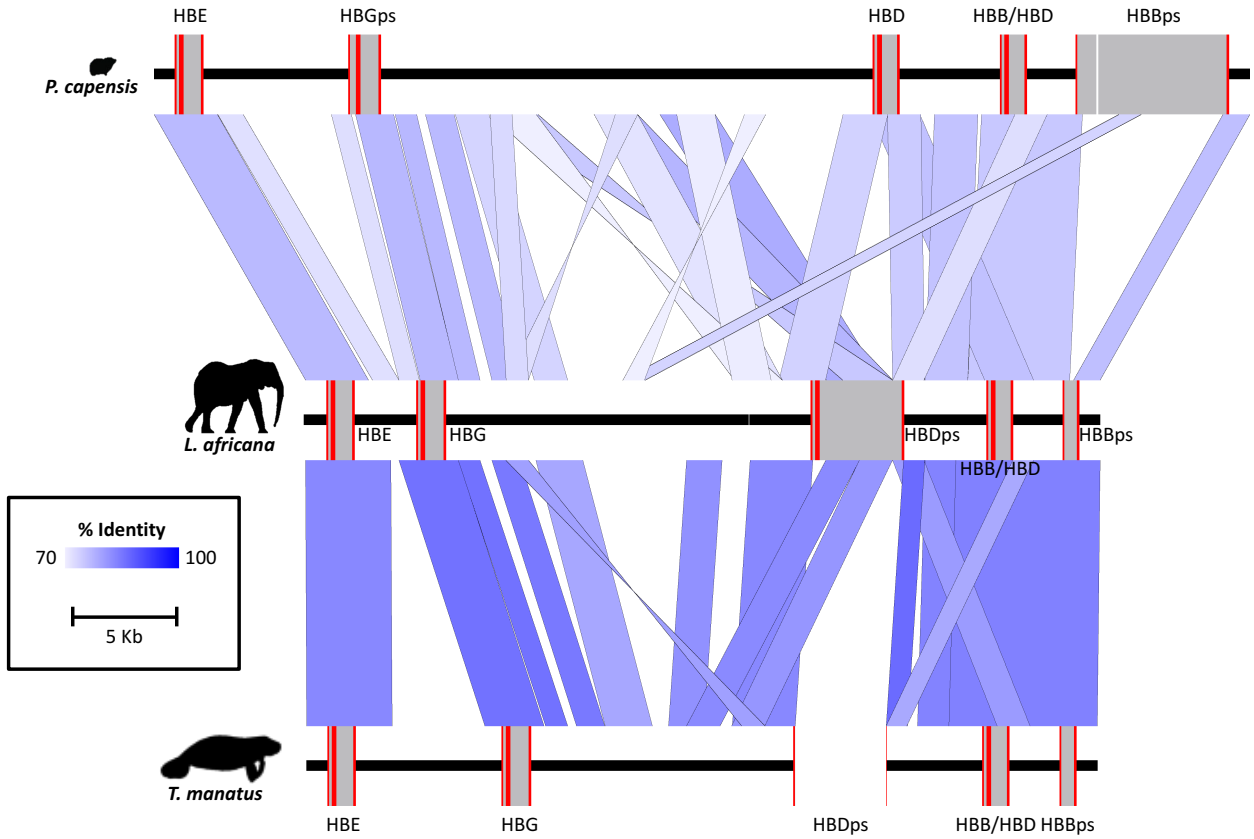


Figure 2.2. Organization and synteny analysis of rock hyrax (*P. capensis*), African elephant (*L. africana*), and Florida manatee (*T. manatus*) β-globin gene clusters. Red and grey boxes represent exonic and intronic sequences, respectively. The "ps" suffix denotes pseudogenes. Thick black lines represent intergenic sequences. Gaps in the contiguous sequence denote missing data.

resulting duplicate (and the surviving gene on the opposite chromosome) will be a hybrid of the two parent genes (Opazo et al., 2009). This mechanism is the underlying cause of hemoglobin Lepore syndrome, where a HBD/HBB hybrid of the HBB and HBD genes supplants the parental HBD and HBB genes on one chromosome, and the corresponding 'anti-Lepore' duplication, where a HBB/HBD mutant is inserted between them on the other (Forget, 2001). The presence of functional anti-Lepore chimeric HBB/HBD genes in the genomes of paenungulates, eulipotyphlans, carnivores, and cetartiodactyls further demonstrates that the resulting fusion genes may become fixed in the genome and even supplant expression of the parental loci

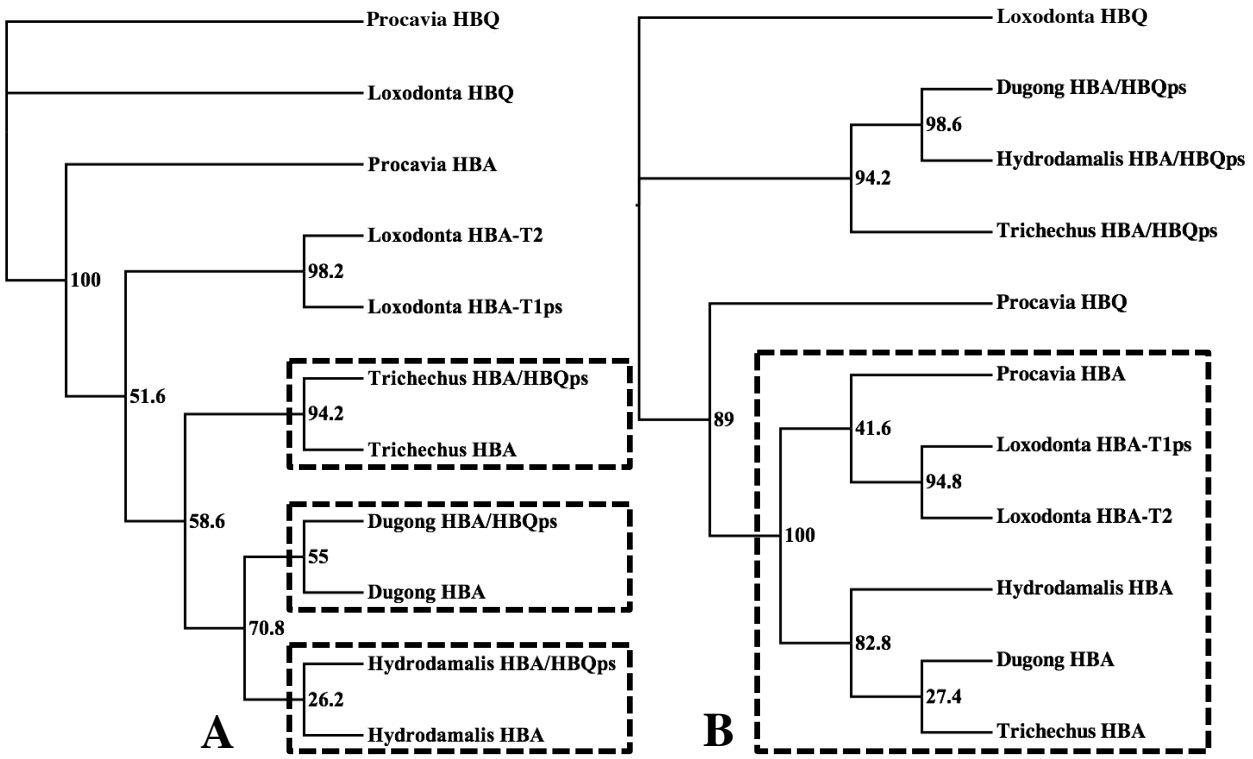


Figure 2.3. Maximum likelihood phylogenies depicting relationships between (A) the first exon and intron (bp 1–353) and (B) the second intron and third exon (bp 593–848) of paenungulate HBA- and HBQ-type genes. Values adjacent to each node represent maximum likelihood bootstrap support. The "ps" suffix denotes pseudogenes, while "-T1" and "-T2" suffices indicate the 5'-3' linkage order of tandemly duplicated genes. Boxes highlight noteworthy gene groupings (see text for details).

(Gaudry et al., 2014). The hybrid HBA/HBQps locus found in the sirenian α-globin cluster is fundamentally similar to the Hb Lepore deletion mutant, as the parental HBA-T2 and HBQ genes are no longer present. Examination of the HBA/HBQps nucleotide sequence reveals that the breakpoint of the unequal crossover event in the sirenian HBA/HBQps locus (between codons 76 and 87) is similar to the breakpoint of both the Hb Lepore Baltimore variant (between codons 66 and 81; Metzenberg et al. 1991) and the paenungulate anti-Lepore HBB/HBD locus (between codons 78 and 87; Opazo et al. 2009). Unlike these previously described chimeric recombinants, however, the sirenian HBA/HBQps locus does not produce a functional product. Nonetheless, this recombination event appears to have contributed to the evolution of the intact

downstream HBA gene–which encodes both pre- and post-natal sirenian Hbs–via gene conversion.

Gene conversion events, whereby a gene copies itself onto a nearby paralogous gene, are quite common among Hb encoding loci (Storz et al., 2007). This type of concerted evolution homogenizes sequence variation among tandemly duplicated genes, resulting in identical sequences in both gene fragments involved. However, if an inactive gene (whose evolution is unconstrained) converts a functional gene, dramatic functional changes to the expressed protein may result (Natarajan et al., 2015). Figure 2.3 reveals a repeated pattern of gene conversion between the 5' ends of sirenian HBA and HBA/HBQps, as these paralogous genes form groups that exclude their orthologous counterparts from other species. However, this is not the case at the 3' ends of these genes, as all paenungulate HBA genes form a monophyletic clade to the exclusion of HBQ and HBA/HBQps genes with 100% bootstrap support (Figure 2.3). Thus, a history of gene conversion events between the 5' ends of sirenian HBA and HBA/HBQps appears to be driving the molecular evolution of this region, with the 5' end of dugong, manatee, and Steller's sea cow *HBA* accordingly containing a disproportionate amount of nucleotide substitutions (55.2-59.0%) compared to the 3' region of this gene.

2.4.2. Molecular Evolution of Paenungulate Globin Genes

We have sequenced the coding regions for five α-type Hb genes (HBZ-T1, HBZ-T2ps, HBK, HBA, and HBA/HBQps), four β-type Hb genes (HBE, HBG, HBB/HBD, and HBBps), and three other globin genes (MB, NGB, and CYGB) encoding non-circulating monomeric proteins from two dugongs and three Steller's sea cow specimens, together with partial coverage of HBDps from both of these species (Appendices 2–9). Complete coverage for these loci was obtained

from both dugong individuals and a single Steller's sea cow. All Steller's sea cow specific mutations were corroborated by at least one additional specimen (Appendix 10). These data were combined with publicly available paenungulate globin sequences (Appendix 1) and the rate of molecular evolution (nucleotide substitutions $site^{-1}$ Ma^{-1}) for each non-pseudogenized gene was calculated. The average rate of nucleotide substitution across all branches in the paenungulate phylogeny was appreciably higher for Mb and the adult-expressed Hb genes (HBA, HBB/HBD) than for Ngb, Cygb, and the prenatal Hbs (HBZ-T1, HBE, and HBG) (Figure 2.4). Evolutionary rate values for paenungulate HBA, HBB/HBD, and MB (0.00101, 0.00093, and 0.00110 substitutions nucleotide $site^{-1}$ Ma^{-1}, respectively) are in close agreement to those previously estimated for adult Hb genes (0.001 substitutions nucleotide $site^{-1}$ Ma^{-1}; Efstratiadis et al., 1980). As the *in utero* environment likely does not vary significantly between mammals, there is generally less selection pressure driving the evolution of prenatal Hb isoforms (Koop and Goodman, 1988). Thus, the genes encoding these polypeptide chains generally evolve at a lower rate with respect to adult Hbs, as is reflected in our rate estimates for HBE, HBG, and HBZ-T1 (0.00025, 0.00035, and 0.00033, respectively; Figure 2.4). However, as at least one gene evolved at a rate significantly higher than the average for all genes, tests of a constant molecular clock were rejected for all loci ($p \leq 0.01$). For example, five of eight genes evolved significantly faster than average in the hyrax lineage, which could be attributed to the much shorter generation time and lifespans of hyraxes (Fairall, 1980) relative to sirenians and elephants. Similarly, six of eight globin genes (all but HBZ-T1 and NGB) evolved significantly faster than average in the stem sirenian branch; i.e. during their secondary aquatic transition.

The earliest known fossil sirenians (prorastomids, e.g. *Prorastomus* and *Pezosiren*) are from the middle Eocene and appear to have been fully capable of terrestrial locomotion

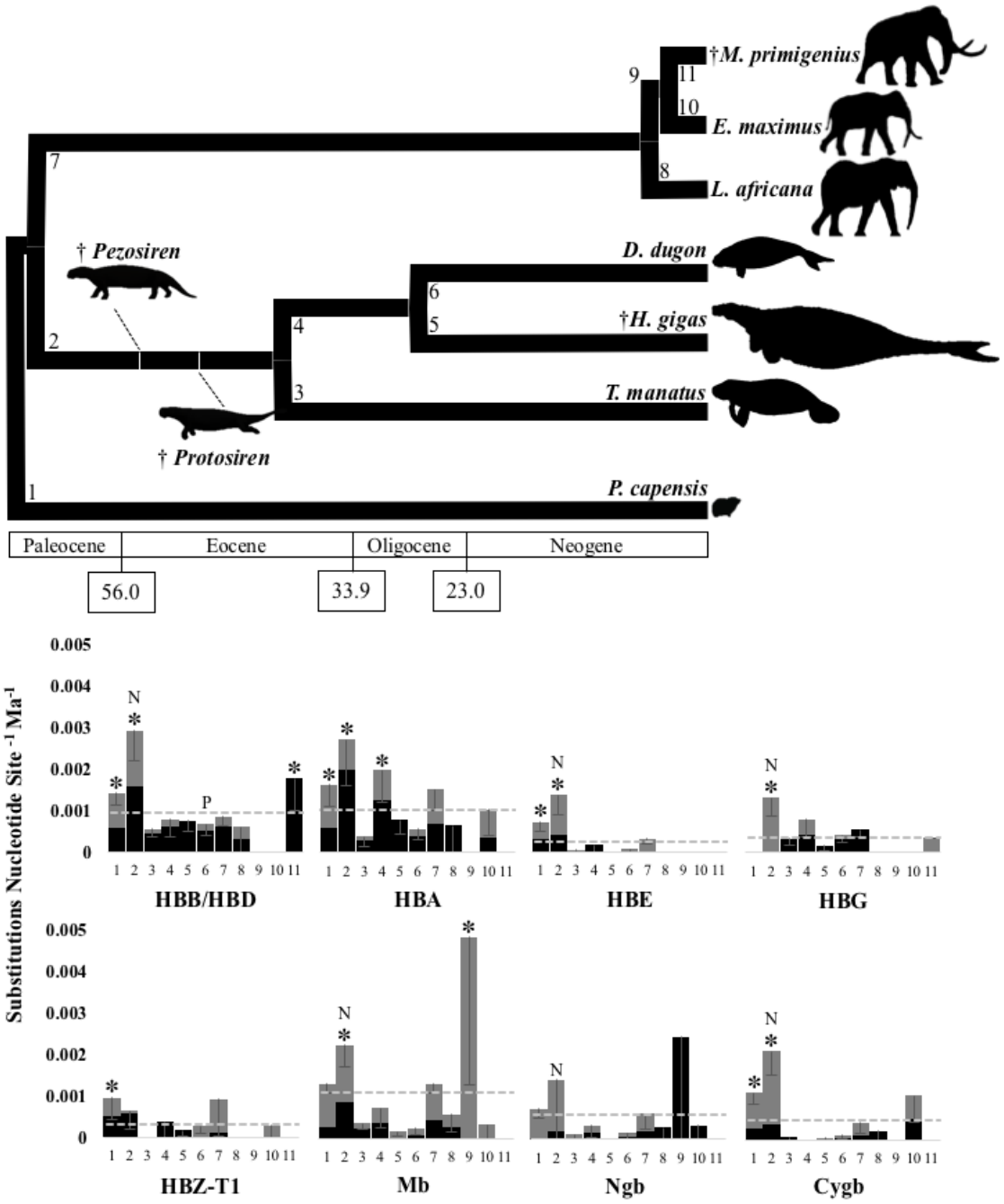


Figure 2.4 Column plots representing the rate of nucleotide substitutions (substitutions per nucleotide site per million years ± SE) among the eight functional globin genes of paenungulate mammals. Black and grey regions of each column represent the contribution of non-synonymous and synonymous substitutions to the overall substitution rate, respectively. Column numbers correspond to specific branches in the above time-calibrated phylogeny (modified from Springer et al. 2015 and Rohland et al. 2007). Asterisks denote sirenian branches that have a rate of nucleotide substitution significantly higher than the locus average (denoted by grey dotted lines). Columns marked "N" or "P" denote negative and positive selection, respectively.

(Domning, 2001). However, they exhibited a number of aquatic specializations that suggested they spent appreciable time foraging underwater, such as retracted nares to aid respiration while breaching water, a jaw modified for bottom feeding, and a slender body with prominent tail that allowed locomotion via dorsoventral spinal undulation combined with hindlimb thrusts (Domning, 2001; Springer et al., 2015). These features are more pronounced in the next phase of sirenian evolution (protosirenids, e.g. *Protosiren*), which show further streamlining of the body, reduced limbs, and an enlarged tail that implies a mostly aquatic lifestyle, but was likely capable of dragging itself short distances on land (Domning and Gingerich, 1994). The eventual loss of hindlimb locomotion in primitive dugongs suggests that aquatic transition was completed by the Bartonian (~41 Ma; Domning, 2000).

The transition of accomplished mammalian divers to the aquatic environment entailed a suite of morphological and physiological specialization to improve O_2 storage and management thereby allowing them to maximize underwater foraging time (Ponganis 2011). However, the limited information available on sirenian diving physiology indicates that this lineage is not typical of mammalian divers. Indeed, cetaceans and pinnipeds greatly enhance O_2 stores by increasing the concentration of Mb in muscle cells and Hb in red blood cells (Ponganis, 2011), whereas these levels in manatees are low and more typical of terrestrial mammals (Blessing, 1972). Cetaceans and pinnipeds generally also have Hb–O_2 affinities equal to or lower than terrestrial mammals (Snyder, 1985), which is presumed to facilitate O_2 transfer to their large Mb stores, while the O_2 affinity of manatee and dugong Hb is greatly increased (Farmer et al., 1979; White et al. 1976; McCabe et al., 1978). Increased Hb–O_2 affinity likely permits more complete depletion of lung O_2 stores during submergence, as was suggested for the Emperor penguin (Meir and Ponganis, 2009), and presumably evolved during the prorastomid/protsirenian stages

of sirenian evolution. Thus, it is not surprising that the most pronounced increases in evolutionary rate on the stem sirenian branch were found for HBB/HBD and HBA (0.00291 and 0.00271 substitutions nucleotide site^{-1} Ma^{-1}, respectively). Of note, the abrupt rate increase in HBA appears to be mechanistically linked with the emergence of the HBA/HBQps chimeric gene that may have contributed to its rapid evolution via interparalog gene conversion (see above). The resulting accumulation of non-synonymous substitutions in HBA may further be expected to drive evolutionary change in other genes, as HBA pairs with HBE, HBG, and HBB/HBD to form tetrameric Hb isoforms at various life stages.

2.4.3. Selection Analysis of Sirenian Globin Genes

To examine the possibility of HBA/HBQps driving the evolution of expressed globin genes we performed selection analyses on all globin loci at each branch of the sirenian lineage. We found that all globin genes of the stem sirenian, save HBA and HBZ-T1, exhibited an ω value significantly lower than one (Appendix 11). The close interactions between amino acid residues along and between globin chains of Hb make it vulnerable to epistatic and pleiotropic effects, which more often than not render substitutions—even those that seem beneficial in isolation—deleterious (Tufts et al., 2015). This is especially prominent for the prenatal globin genes (HBE and HBG), which are strongly conserved at the protein level (Figure 2.4). A similar instance of negative selection was found for the MB gene among all branches of the cetacean lineage, though it was speculated that positive selection occurred during the early stages of their aquatic transition followed by a long history of sequence integrity preservation (Nery et al., 2013). Conversely, the HBA and HBZ-T1 genes of the stem sirenian exhibited increased ω values with respect to both the paralogous globins within this branch and their homologous counterparts

within stem proboscideans. RELAX analysis (Werheim et al., 2014) reveals that selection pressure on both HBA and HBZ-T1 genes of stem sirenians were relaxed following its divergence from proboscideans ($p = 0.024$ and 0.006 for HBA and HBZ-T1, respectively). Interestingly, as HBA and HBZ-T1 encode the only functional α-type globin chains in sirenians, relaxed selection on these genes may have impacted O_2 delivery in all life stages. However, the functional consequences of this evolutionary shift were presumably greater in the HBA gene, as it exhibits 20 non-synonymous nucleotide substitutions (with respect to the paenungulate ancestor) compared to only 5 in the HBZ-T1 gene. This difference is likely attributed to the emergence of a chimeric HBA/HBQps pseudogene in stem sirenians, which has converted the expressed HBA gene throughout sirenian evolution.

Only a single instance of positive selection was found among sirenian globin genes, the HBB/HBD gene of the dugong (Figure 2.4, Appendix 11), which coincided with a shift in diving behavior. While the neutrally buoyant Trichechidae generally remain static, feeding at any point in the water columns of both fresh and marine waters, the strictly marine dugong is an obligate bottom feeder that is capable of much greater dive depths (>30 m; Marsh et al., 2012). Thus, positive selection on the dugong HBB/HBD gene may be reflective of these foraging habits. In light of this finding, we also performed branch-site analyses to identify amino acid residues that may have aided the sirenian transition to the aquatic environment.

Branch-site analyses revealed a number of individual amino acid positions that potentially evolved under positive selection, however, only three had a probability >0.95 under the empirical Bayes method. These included two residues in the Mb protein (141Asp→Ala and 145Lys→Asn) and a single residue in the Cygb protein (155Gly→Ala) of manatees (Appendix 12). While the functional effect (if any) of the Cygb replacement is unknown, the Mb exchanges

are particularly interesting as they involved replacements of charged residues with a neutral residues and thus likely contributed to fine tuning the concentration and stability of the protein (see Mirceta et al., 2013; Samuel et al. 2015). Notably, the branch-site analyses also identified several amino acid substitutions in the HBB/HBD gene of dugong and Steller's sea cow that are known to markedly alter the O_2 affinity of other mammalian Hbs.

2.5. Amino Acid Substitutions of Modern Sirenians

The protein encoded by the HBB/HBD gene (β/δ) of the dugong has a rare amino acid replacement adjacent to the highly conserved sliding interface of the Hb tetramer (β/δ101Glu→Gln). The interactions between amino acids in this region are essential for the tetramer's physical transition between the tense (T or deoxy) and relaxed (R or oxy) states (Perutz, 1970). As such, these amino acids are strongly conserved among vertebrate globin chains, with only a few additional species (two lemurs and the woolly mammoth) known to exhibit substitutions (101Glu→Gln in each case) at this site. The mammoth replacement has been studied extensively and is thought to confer a selective advantage for this Arctic species by lowering the effect of temperature on its O_2 binding properties (Campbell et al., 2010a; Yuan et al., 2011; Noguchi et al., 2012; Yuan et al., 2013). Briefly, the exothermic nature of heme oxygenation dictates that free energy, in the form of heat (i.e. the enthalpy of oxygenation; ΔH), is required to break the Hb–O_2 bond (Atha and Ackers, 1974). The woolly mammoth β/δ101Glu→Gln substitution has been shown to reduce the thermal sensitivity of O_2 binding by creating a proton-linked Cl^- binding site that lowers the ΔH (Campbell et al., 2010a; Yuan et al., 2011; Noguchi et al., 2012; Yuan et al. 2013). It is therefore surprising to find this mutation in the dugong, as there is presumably little selective advantage for a low ΔH in a subtropical

species. However, it is possible that a second substitution at another highly conserved site in the central cavity— β/δ104Arg→Lys (this residue interacts with β/δ101; Noguchi et al. 2012)— eliminates the proton-linked Cl^- binding site, as O_2 binding measurements of dugong hemoglobin show no reductions in thermal sensitivity when compared to other mammalian Hbs (McCabe et al., 1978).

The prenatally expressed peptide chain encoded by the HBG gene (γ) of Steller's sea cow also has a mutation at position 101 (γ101Glu→Asp), with available evidence from mutant human Hbs suggesting that this replacement may also alter Hb–O_2 function (Shih et al., 1985). Indeed, this substitution is homologous to the human mutant Hb Potomac, which displays a normal response to heterotropic ligands but a strongly increased intrinsic O_2 affinity (Charache et al. 1978). Perhaps more significantly, the Steller's sea cow γ-chain has a second internal replacement (38Thr→Ile) at a heme/α2β1 contact (position four of helix C) that is invariable for all known wild-type vertebrate α, β, and Mb proteins. Interestingly, the equivalent human HBB mutant (Hb La Coruña) displays increased O_2 affinity in affected patients (Ropero et al., 2006). Taken together with the γ101Glu→Asp replacement, these findings suggest that selection pressures have operated to alter O_2 uptake by the developing circulation in this recently extinct lineage.

Perhaps not surprisingly then, the protein encoded by the adult expressed β-type gene of Steller's sea cow (β/δ) has accumulated several residue exchanges that likely alter its functional attributes. Chief among these is a β/δ82Lys→Asn mutation within the central cavity of the Hb tetramer. This central cavity is typically lined with positively charged amino acids that repel each other, destabilizing the T-state (Perutz et al., 1994). However, anions present in erythrocytes, such as Cl^- and 2,3-diphosphoglycerate (DPG), may bind and neutralize these amino acids during

the R→T transition, thereby lowering O_2 affinity and reducing thermal sensitivity (Perutz et al. 1994; Weber and Campbell, 2011). Position 82 of β-type Hb chains is of particular importance as this residue forms five concurrent salt bridges with DPG and also constitutes a Cl^- binding site (Richard et al., 1993). As such, the human mutant Hb Providence (β82Lys→Asn) displays a reduced Cl^- effect and a greatly diminished DPG effect (Bonaventura et al., 1976). Presence of this same substitution within a (sub)Arctic aquatic mammal, however, conflicts with expectations as in the absence of compensatory changes, reductions in anion binding are expected to markedly increase the thermal sensitivity of the protein and hinder O_2 delivery to poorly insulated appendages (Weber and Campbell, 2011). In light of the unusual substitutions in the HBB/HBD genes of dugong and Steller's sea cow, we have performed detailed functional analyses on sirenian adult Hb proteins to assess the thermal consequences of the observed residue exchanges (Chapter 3).

2.6. Conclusions

Our analyses revealed that the genes encoding the oxygen-binding globin proteins of sirenians generally evolved at an elevated rate during the early aquatic transition of this lineage. While most of these loci evolved under strong purifying selection, the HBA and HBZ-T1 genes—which encode Hb isoforms at all sirenian life stages—exhibited elevated rates of non-synonymous/synonymous amino acid replacements in the stem sirenian branch. Notably, the rate change in HBA appears to be temporally linked to the emergence of a chimeric HBA/HBQ pseudogene in sirenians, which presumably contributes to the downstream HBA locus via gene conversion and may thus have been responsible for the evolution of a high Hb–O_2 affinity phenotype present within living sirenians. While all Steller's sea cow globin genes exhibited

dN/dS signatures consistent with strong sequence conservation, the β-type chains encoded by the prenatal HBG (γ) and postnatal HBB/HBD (β/δ) loci exhibit rare amino acid replacements that are known to dramatically alter the oxygenation properties of other mammalian Hbs that may in part underlie the adaptation of this recently extinct lineage to the sub-Arctic niche.

CHAPTER 3: FUNCTIONAL MECHANISMS UNDERLYING AN EVOLVED CHANGE IN HEMOGLOBIN THERMAL SENSITIVITY IN THE EXTINCT STELLER'S SEA COW (HYDRODAMALIS GIGAS)

Anthony V. Signore[1], Angela Fago[2], Roy E. Weber[2], and Kevin L. Campbell[1]

[1]University of Manitoba, Department of Biological Sciences, Winnipeg, Canada

[2]Aarhus University, Department of Bioscience, Zoophysiology, Aarhus, Denmark

Author Contributions:

AS designed the study, performed experiments, analyzed data and drafted the manuscript.

AF provided laboratory space, research materials, and comments on the manuscript.

REW provided laboratory space, research materials, and comments on the manuscript.

KC assisted with study design, provided laboratory space, research materials, and revisions to the manuscript.

3.1. Abstract

Key questions in evolutionary genetics revolve around the importance of amino acid replacements that exhibit manifold effects on protein function. Sirenians provide an instructive model to study such questions, as a number of rare amino acid replacements in the adult-expressed hemoglobins (Hbs) of the dugong (*Dugong dugon*) and extinct Steller's sea cow (*Hydrodamalis gigas*) are expected to alter multiple aspects of Hb function in manners that conflict with our current understanding of thermal adaptation. In order to assess the physicochemical consequences of these mutations, we recombinantly synthesized the Hb proteins of these two species, their predicted last common (~28.6 million year old) ancestor, and the Florida manatee (*Trichechus manatus latirostris*). Our detailed functional analyses confirm that Hbs with high O_2 affinity—exceeding those of other mammals examined to date—arose early in sirenian evolution, presumably to maximize O_2 extraction from the lungs and limit premature O_2 offloading during submergence. Moreover, in contrast to expectations based on sequence data, we demonstrate that the Hb–O_2 affinity of the extinct sub-Arctic Steller's sea cow is less affected by temperature than other sirenians, thereby safeguarding O_2 delivery to cool peripheral tissues. While such 'temperature insensitivity' has largely been attributed to an increased number of allosteric effectors bound to the Hb moiety, we find this phenotype arises from temperature induced changes in effector sensitivity together with reductions in the intrinsic temperature sensitivity of *H. gigas* Hb. These findings call for a reassessment of the molecular mechanisms underlying the thermal dependence of O_2 binding to vertebrate Hbs.

3.2. Introduction

Mammalian hemoglobin (Hb) is a tetrameric protein that binds atmospheric O_2 at the lungs and distributes it throughout the body via quaternary structural shifts between a high affinity (predominately oxygenated) relaxed (R-) state and a low affinity (predominately deoxygenated) tense (T-) state (Perutz, 1970). While this mechanism is conserved in all mammalian Hbs, variations in the sequences encoding the α- and β-type polypeptide chains may alter Hb's intrinsic O_2 affinity, response to heterotropic (i.e. non-oxygen) ligands, and temperature (Perutz, 1983; Storz et al., 2010; Campbell et al., 2010a). While amino acid substitutions permit tremendous variability in the structural and functional properties of Hb, the evolution of adaptive phenotypes may be constrained by mutational pleiotropy and by epistasic interactions acting on the tertiary and quaternary structures of the protein (Natarajan et al., 2013; Tufts et al., 2015). A potential case in point relates to the Hb phenotype of heterothermic mammals, whose O_2 uptake and release properties are less affected by temperature than the Hbs of non-cold adapted species thereby maintaining sufficient O_2 offloading in the face of decreasing tissue temperatures (De Rosa et al., 2004; Weber and Campbell, 2011). As breaking the weak covalent bond between O_2 and the heme iron is endothermic (i.e. absorbs heat), this phenotype predominantly arises from the exothermic binding of additional heterotropic ligands to the T-state protein, which 'donate' heat for deoxygenation, and hence reduce the overall enthalpy of oxygenation (ΔH'; Weber and Campbell, 2011). However, these ligands are also critical effectors of O_2 affinity *in vivo* (Antonini and Brunori, 1971), such that mutations that lower O_2 affinity via increased effector binding may also be expected to lower thermal sensitivity and vice versa.

This linkage raises an important question in evolutionary genetics as the co-varying of these traits may lead to either agonistic or antagonistic pleiotropic effects, depending on the ecological setting. For example, aquatic mammals inhabiting shallow tropical waters may benefit

from a high Hb–O_2 affinity and a high thermal sensitivity to maximize O_2 extraction from the lungs and optimize O_2 offloading to warm (active) tissues, respectively. Conversely, these same traits are expected to be antagonistic in an Arctic environment as they would hamper oxygen off-loading in cold appendages. It is, however, possible to circumvent the linkage between Hb–O_2 affinity and thermal sensitivity in some cases as demonstrated by a Glu→Gln residue replacement at position 101 of the β-type chain (β/δ, a chimeric protein derived from unequal crossing over of the parental β and δ genes in the ancestor of paenungulate mammals; Opazo et al., 2009) of woolly mammoth (*Mammuthus primigenius*) Hb (Campbell et al., 2010a). Briefly, this substitution forms a proton-linked Cl^- binding site in the T-state protein that liberates heat needed to break the heme-oxygen bond. The accompanying reduction in O_2 affinity, however, is counterbalanced by a marked increase in the intrinsic Hb–O_2 affinity caused by this same mutation such that the thermal sensitivity of woolly mammoth Hb is reduced without a corresponding effect on blood O_2 affinity at 37°C (Campbell et al., 2010a).

Sirenians are a group of strictly aquatic, (sub)tropical herbivores encompassing only four extant members, three species of manatee (family Trichechidae) and the dugong (*Dugong dugon*; family Dugongidae). However, first-hand accounts and genetic material of a sub-arctic dugongid hunted to extinction in 1768—Steller's sea cow (*Hydrodamalis gigas*)—are also available for study (Chapter 2; Springer et al., 2015; Stejneger, 1887; Steller, 1751). While sirenians are proficient divers, they do not exhibit the strongly elevated body O_2 stores or enhanced dive reflex common to other lineages of marine mammals (Scholander and Irving, 1941; Blessing, 1972). Rather, the sirenian's secondary transition to aquatic life appears to have coincided with a rapid evolution their Hb encoding genes (Chapter 2). Resulting amino acid exchanges may have tailored sirenian Hb to maximize O_2 extraction from the lungs while submerged and limit

premature O_2 offloading during prolonged dives (Synder, 1983), as limited studies conducted on living sirenians suggest that they possess blood with a high O_2 affinity (White et al., 1976; McCabe et al., 1978). Manatee Hb is also less thermally sensitive than human HbA (-50.7 kJ mol^{-1} O_2 at pH 7.4, in the presence of 0.1M Cl^-; Weber et al., 2014), though the heat liberated upon its oxygenation in the presence of Cl^- (-42.7 kJ mol^{-1} O_2; Farmer et al., 1979) is well above the range found for 'cold adapted' mammalian Hbs ($\Delta H'$ = -16 to -20 kJ mol^{-1} O_2; Weber and Campbell, 2011; De Rosa et al., 2004). As the Steller's sea cow lineage colonized sub-arctic waters of the North Pacific during the Miocene (Domning, 1976), we predicted that it evolved a numerically low-$\Delta H'$ Hb phenotype in parallel to its close relative the woolly mammoth. In other words, we hypothesized that Steller's sea cow Hb would possess the same β/δ101Glu→Gln mutation found in the mammoth to facilitate O_2 delivery to cold appendages and reduce respiratory associated heat loss without compromising Hb–O_2 affinity. Surprisingly, however, this substitution was found to be present in dugong Hb but not in Steller's sea cow Hb, despite the former species being confined to tropical and sub-tropical waters where selection pressures favoring reductions in thermal sensitivity should be minimal or lacking (Chapter 2). Steller's sea cow instead exhibits a functionally significant replacement (β/δ82Lys→Asn) that markedly reduces anionic effector binding and hence increases blood–O_2 affinity in humans carrying the Hb Providence (β82Lys→Asn/Asp) variant (Bonaventura et al., 1976). This change conflicts with our understanding of thermal sensitivity as it is predicted to increase the effect of temperature on O_2 binding and release (Chapter 2). However, it is conceivable that these substitutions: (1) do not induce equivalent functional changes in sirenian Hbs due to epistatic effects or (2) that changes in other traits (e.g. Hb–O_2 affinity) are favored over thermal sensitivity within these particular ecological contexts. Thus, this group makes an interesting

model system to test the influence of environmental selection pressures acting on protein biochemistry that controls multiple physiological phenotypes.

To address these questions, we synthesized recombinant Hb proteins that exactly match the Hbs of the Florida manatee (*Trichechus manatus latirostris*), dugong, and extinct Steller's sea cow and tested their O_2 binding properties, responses to allosteric effectors, and thermal sensitivities. Owing to the presence of amino acid substitutions with large presumed phenotypic effects in the latter two dugongid species (see above), we also synthesized the Hb of their last common ancestor (from ~28.6 million years ago; Springer et al., 2015) to map the evolution of key physicochemical changes onto the sirenian phylogeny.

3.3. Methods and Materials

3.3.1. Globin Gene Sequence Collection and Optimization

Previously published nucleotide sequences of Florida manatee (*Trichechus manatus latirostrus*), dugong (*Dugong dugon*), and Steller's sea cow (*Hydrodamalis gigas*) adult-expressed Hb genes (HBA and HBB/HBD) were optimized for expression in *E. coli* and synthesized *in vitro* by GenScript (Piscataway, NJ). The HBA and HBB/HBD gene sequences of the most recent common ancestor shared by Steller's sea cow and the dugong (ancestral Dugongidae) were reconstructed with all available paenungulate HBA and HBB/HBD genes (Chapter 2) using baseml, as implemented in the PAML software package (Yang, 2007). This sequence was optimized for expression in *E. coli* and synthesized as above.

3.3.2. Construction of Recombinant Hb Expression Vectors

Sirenian alpha- and beta-type Hb genes (synthesized in tandem by GenScript, as above) were digested with restriction enzymes and ligated into a custom Hb expression vector (Natarajan et al., 2011), kindly donated by C. Natarajan from the University of Nebraska–Lincoln, using a Quick Ligation Kit (New England BioLabs) as recommended by the manufacturer. Chemically competent JM109 (DE3) *E. coli* (Promega) were prepared using a Z-Competent *E. coli* Transformation Kit and Buffer Set (Zymo Research). Hb expression vectors were co-transformed into JM109 (DE3) chemically competent *E. coli* alongside a plasmid expressing methionine aminopeptidase (Natarajan et al., 2011), plated on LB agar containing ampicillin (50 µg/ml) and kanamycin (50 µg/ml), and incubated for 16 hours at 37°C. A single colony from each transformation was cultured in 50 ml of 2xYT broth for 16 hours at 37°C while shaking at 200 rpm. Post incubation, 5 ml of the culture was pelleted by centrifugation and plasmid DNA was isolated using a GeneJET Plasmid Miniprep Kit (Thermo Scientific). The plasmid DNA sequence was verified using BigDye 3.1 sequencing chemistry, gene specific sequencing primers, and 250 ng of plasmid DNA on an ABI3130 Genetic Analyzer. The remainder of the culture was supplemented with glycerol to a final concentration of 10%, divided into 25 ml aliquots and stored at -80°C until needed for expression.

3.3.3. Expression and Purification of Recombinant Hemoglobin

25 ml of starter culture of JM109 *E. coli* carrying expression vectors with Sirenian alpha- and beta-type Hb inserts (above) was added to 1250 ml of TB media containing ampicillin (100 µg/ul) and kanamycin (50 µg/ul) and distributed evenly amongst five 1 L Erlenmeyer flasks. Cultures were grown at 37°C while shaking at 200 rpm until the absorbance at 600 nm reached

0.6-0.8. Hb expression was induced by supplementing the media with 0.2 mM isopropyl β-D-1-thiogalactopyranoside, 50 μg/ml of hemin and 20 g/L of glucose and the culture was incubated at 28°C for 16 hours while shaking at 200 rpm. Once this induced expression step was complete, dissolved O_2 was removed by adding sodium dithionite (1 mg/ml) to the culture, which was promptly saturated with CO for 15 minutes. Bacterial cells were then pelleted by centrifugation and the Hb was purified by ion exchange chromatography according to Natarajan et al. (2011).

3.3.4. Functional Analyses of Hemoglobins

O_2-equillibrium curves for Hb solutions (0.25 mM heme in 0.1 M HEPES buffer) were measured at 25 and 37°C using the thin film technique described by Weber (1992). The conditions of Hb solutions were manipulated with respect to their pH (6.9, 7.4, and 7.9), chloride concentration (0 or 100 mM KCl), and organic phosphate concentration (0 or 2-fold molar excess of DPG) in order to test the influence of these cofactors on Hb function. Each Hb solution was sequentially equilibrated with three to five different oxygen tensions (PO_2) capable of saturating 30 to 70% of Hb molecules. Hill plots (log[fractional saturation/[1-fractional saturation]] vs. logPO_2) constructed from these measurements were used to determine the PO_2 at half saturation (P_{50}) and the cooperativity coefficient (n_{50}) from the χ-intercept and slope of these plots, respectively. By this method, the R^2 determination coefficients for the fitted curves exceed 0.995 and the standard errors (SEM) are less than 3% of the P_{50} and n_{50} values (Weber et al., 2014). P_{50} values at 25 and 37°C were used to assess the thermal sensitivity of sirenian Hbs by calculating the apparent enthalpy of oxygenation using the van't Hoff isochore:

$$\Delta H = 2.303R \times \Delta \log P_{50} \times (1/T_1 - 1/T_2)^{-1}$$

where R is the universal gas constant and T_1 and T_2 are the absolute temperatures (°K) at which the P_{50} values were measured. All ΔH values were corrected for the heat of O_2 solubilization (12.55 kJ mol^{-1} O_2).

3.4. Results

3.4.1. Ancestral Sequence Reconstruction

The globin gene repertoire of Steller's sea cow, dugong, and the Florida manatee have previously been determined and are detailed elsewhere (Chapter 2). The adult expressed Hb genes (HBA and HBB/HBD) from the most recent common ancestor shared by the dugong and Steller's sea cow ('ancestral dugongid') were reconstructed using a maximum likelihood approach (Yang, 2007). The ancestral HBA (α globin) protein is 94.3% (133 of 141 amino acids) and 97.1% similar to that of the dugong and Steller's sea cow (Figure 3.1), respectively, indicating that the dugong HBA locus evolved more rapidly after this lineage split from Steller's sea cow (see Chapter 2 for details). This difference is less pronounced for the chimeric HBB/HBD locus, as the β/δ chain of the dugongid ancestor is 94.5% (138 of 146 amino acids) and 95.2% similar to that of the dugong and Steller's sea cow, respectively (Figure 3.1).

3.4.2. Oxygen Binding Characteristics

Measured O_2 equilibrium curves of the four examined Hbs revealed marked differences in intrinsic O_2 affinity and sensitivity to heterotropic ligands (Figure 3.2; Table 3.1). In the absence of allosteric effectors (i.e. "stripped") the P_{50} (the O_2 tension resulting in 50% Hb–O_2 saturation)

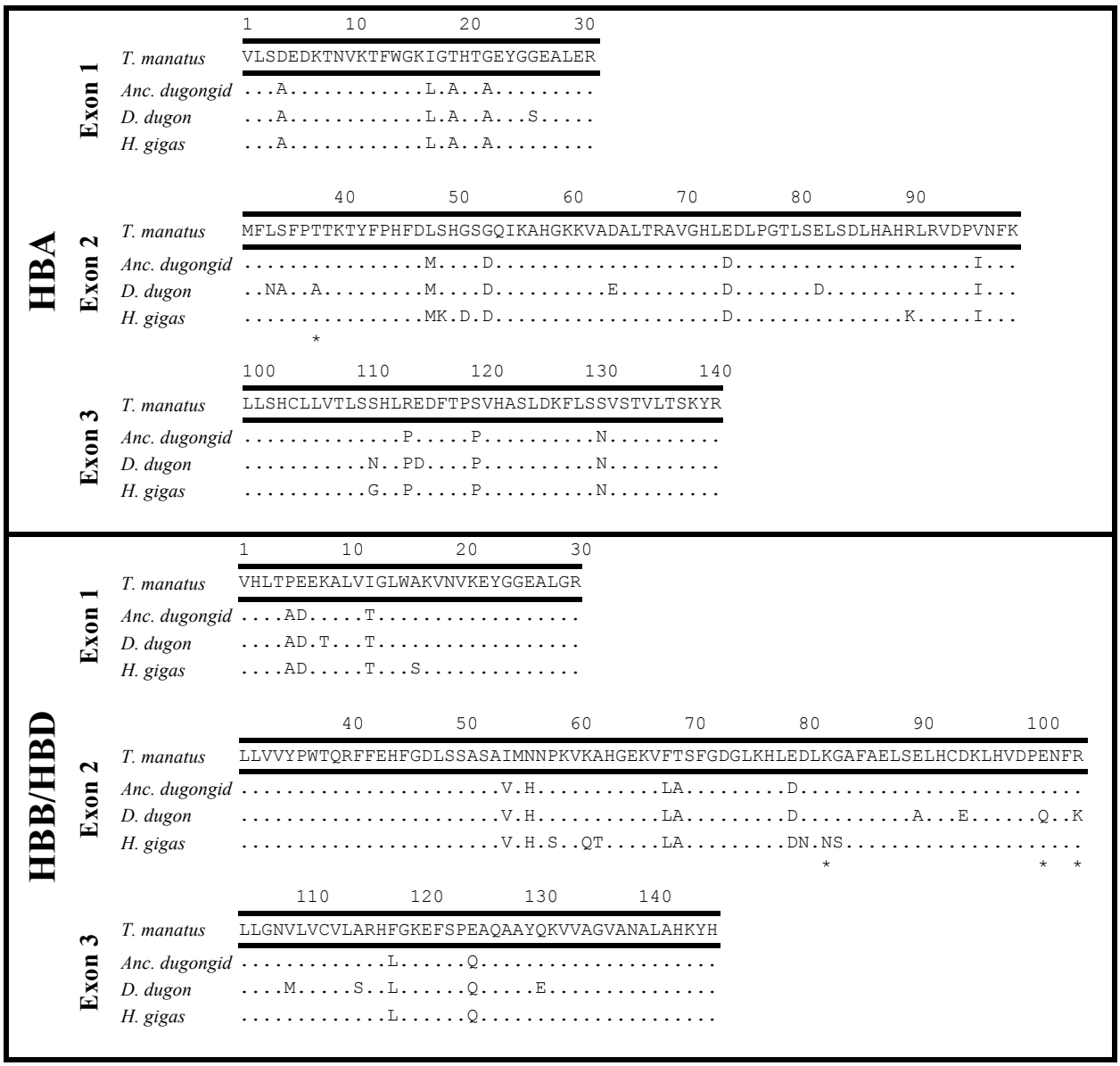


Figure 3.1. Amino acid sequences of sirenian HBA and HBB/HBD genes and the reconstructed sequences of the last common ancestor ('Anc. dugongid') shared by the dugong (*Dugong dugon*) and Steller's sea cow (*Hydrodamalis gigas*). Dots represent sequence identity with *T. manatus*. Amino acid substitutions of potential physiological significance are denoted by asterisks (see text for details).

of Steller's sea cow Hb is ~2-3× higher than that of dugong, ancestral dugongid, and manatee Hbs under the same conditions (Table 3.1). However, Steller's sea cow Hb is generally less responsive to the heterotropic ligands H^+, Cl^-, and 2,3-diphosphoglycerate (DPG) than that of its

relatives (Figure 3.2; Table 3.1). As a result, the O_2 affinity of Steller's sea cow Hb in the presence of these

Table 3.1. Intrinsic oxygen affinities (P_{50}, mmHg) for hemoglobins of the Florida manatee (*Trichechus manatus latirostris*), dugong (*dugong dugon*), Steller's sea cow (*Hydrodamalis gigas*), and the ancestral dugongid ('ancestral dugongid'), and their sensitivity to allosteric effectors at 25 and 37°C in 0.1 M HEPES buffer. All values are corrected to pH 7.2 unless otherwise noted.

Species	Temp	[a]P_{50}	[a]$\log P_{50}$	[b]Cl^- Effect	[c]DPG Effect	[d]Bohr effect ($\Delta\log P_{50}/\Delta$pH)			
						Stripped	+KCl	+DPG	KCl + DPG
T. manatus	37°C	5.42	0.73	0.23	0.13	-0.09	-0.23	-0.44	-0.37
	25°C	1.89	0.28	0.30	0.28	-0.10	-0.18	-0.71	-0.49
	Δ 37→ 25°C	-3.53	-0.46	0.07	0.15	-0.01	0.05	-0.27	-0.12
Ancestral dugongid	37°C	4.12	0.62	0.29	0.24	-0.20	-0.34	-0.61	-0.60
	25°C	1.86	0.27	0.34	0.39	-0.19	-0.42	-0.83	-0.60
	Δ 37→ 25°C	-2.26	-0.35	0.05	0.15	0.01	-0.08	-0.23	0.00
D. dugon	37°C	3.40	0.53	0.40	0.30	-0.18	-0.35	-0.63	-0.47
	25°C	1.25	0.10	0.43	0.40	-0.39	-0.39	-0.73	-0.60
	Δ 37→ 25°C	-2.15	-0.43	0.03	0.10	-0.21	-0.04	-0.10	-0.13
H. gigas	37°C	9.07	0.96	0.15	0.00	-0.06	-0.29	-0.13	-0.36
	25°C	4.28	0.63	0.24	0.03	-0.15	-0.27	-0.27	-0.50
	Δ 37→ 25°C	-4.79	-0.33	0.09	0.03	-0.08	0.02	-0.14	-0.14

[a]PO_2 at half-saturation (mm Hg)
[b]$\Delta\log P_{50}^{(0.1M\ KCl - 0.0M\ KCl)}$
[c]$\Delta\log P_{50}^{(0.2mM\ DPG - 0.0mM\ DPG)}$
[d]pH range 7.2 to 7.4

allosteric effectors (P_{50} = 13.5 mmHg) is only moderately reduced in relation to dugong, ancestral dugongid, and manatee Hbs (P_{50} = 9.3, 9.5, and 10.3 mmHg, respectively) at pH 7.2, 37°C.

At 37°C, under stripped conditions, the Bohr effects ($\Delta\log P_{50}/\Delta$pH) of Steller's sea cow and manatee Hbs (-0.06 and -0.09, respectively) are lower than those of the dugong and dugongid ancestor (-0.18 and -0.20, respectively; Table 3.1), indicating that fewer protons are released by these Hbs upon oxygenation. The Bohr coefficients of all sirenian Hbs are increased by the addition of Cl^- and DPG, though remain lower for Steller's sea cow and manatee Hbs (-0.36 and

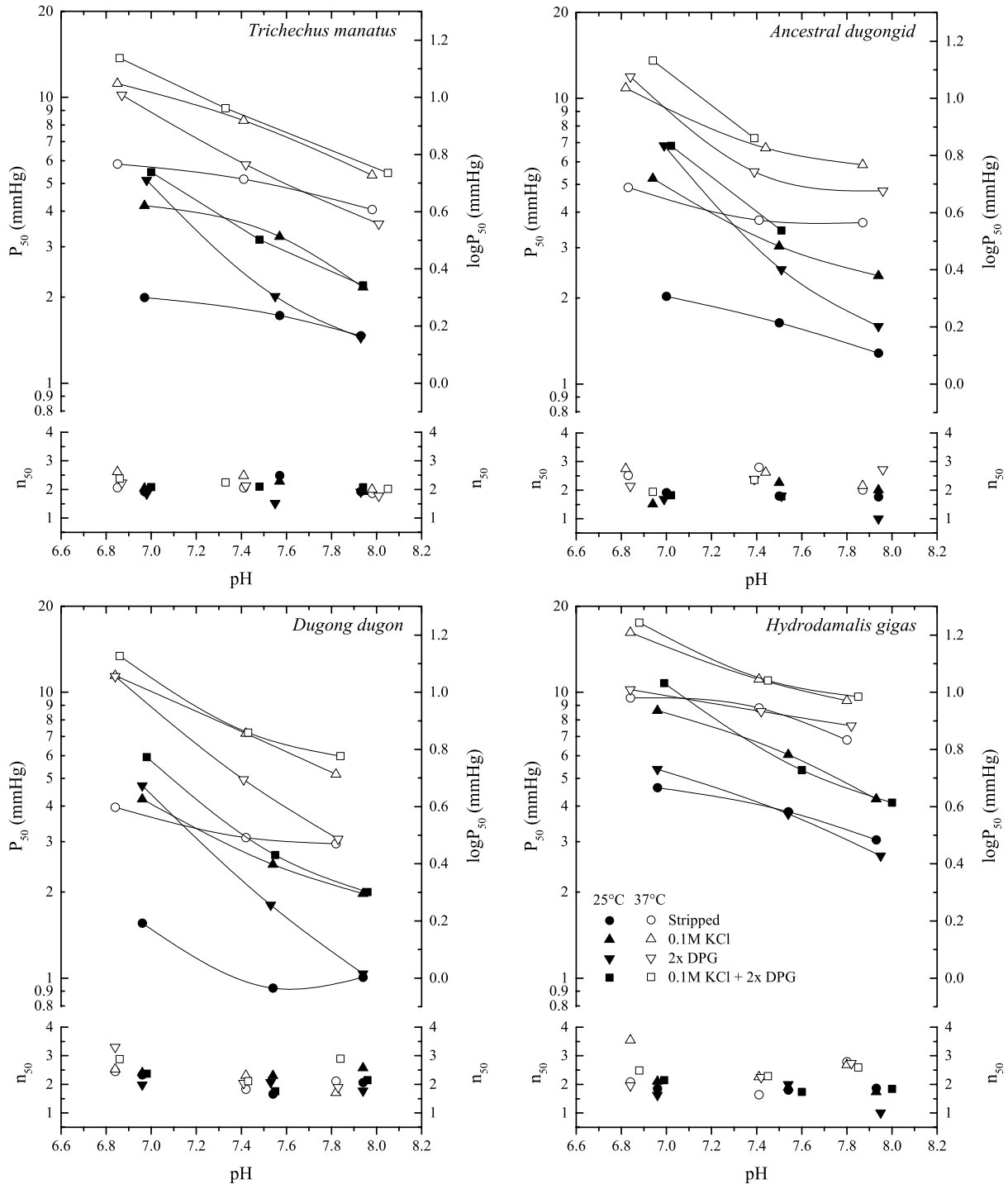


Figure 3.2. The pH dependence of oxygen tensions at half saturation (P_{50}) and the cooperativity coefficients (n_{50}) for hemoglobins of the Florida manatee (*Trichechus manatus latirostris*), dugong (*dugong dugon*), Steller's sea cow (*Hydrodamalis gigas*), and the last common dugonid ancestor ('ancestral dugongid') in stripped Hb (circles), and in the presence of 0.1 M KCl (triangles), of a 2-fold molar excess of 2,3-diphosphoglycerate (DPG; inverted triangles) and of both KCl and DPG (squares), at 25°C (solid symbols) and 37°C (open symbols).

-0.37, respectively) relative to dugong and ancestral dugongid Hbs (-0.47 and -0.60, respectively) when both of these ligands are present. Steller's sea cow and manatee Hbs also have lower Cl^- ($\Delta\log P_{50}^{(0.1M\ KCl\ -\ 0.0M\ KCl)}$) and DPG ($\Delta\log P_{50}^{(0.2mM\ DPG\ -\ 0.0mM\ DPG)}$) effects than dugong and ancestral dugongid Hbs. Steller's sea cow Hb is the least responsive to these ligands, as it has the lowest Cl^- effect (0.15) and no observable DPG effect at 37°C (Table 3.1; Figure 3.2). Moreover, effector binding was found to be temperature dependent, as the effects of H+, Cl^-, and DPG all increased as temperature decreased, indicating that the ionic interactions with these allosteric effectors become stronger at low temperatures, with few exceptions (Table 3.1).

3.4.3. Thermal Sensitivity

The intrinsic thermal sensitivities (indexed as the enthalpy of oxygenation, or ΔH, of stripped Hb) of dugong, ancestral dugongid, and manatee Hbs (-58.6, -50.1, and -53.7 kJ mol^{-1} O_2, respectively) at pH 7.8 (where the Bohr effect is expected to be minimal), are notably higher than that of Steller's sea cow Hb (ΔH = -34.2 kJ mol^{-1} O_2; Figure 3.3). This trend remains at pH 7.2, although the ΔH of dugong and ancestral dugongid Hbs are substantially more affected by protons than are Steller's sea cow and manatee Hbs, mirroring the pattern seen for Hb–O_2 affinity (Table 3.1). The ΔH values of all sirenian Hbs are lowered in the presence of Cl^- or DPG, with Steller's sea cow Hb being notably less affected by the latter heterotropic ligand than are the other Hbs (Figure 3.3). Despite exhibiting a lower sensitivity to Cl^- and DPG than the other sirenian Hbs (Table 3.1), the combined presence of these anions lower the ΔH of Steller's sea cow Hb to a greater degree (a reduction of 18.2 kJ mol^{-1} O_2, pH 7.2) than is found for the other sirenian Hbs (range: 12.9 to 15.9 kJ mol^{-1} O_2). As a consequence, Steller's sea cow Hb has a

lower ΔH' in the presence of both heterotropic ligands (-17.4 kJ mol^{-1} O_2) than dugong, ancestral dugongid, and manatee Hbs (ΔH' = -35.6, -24.4, and -42.2 kJ mol^{-1} O_2, respectively; Figure 3.3).

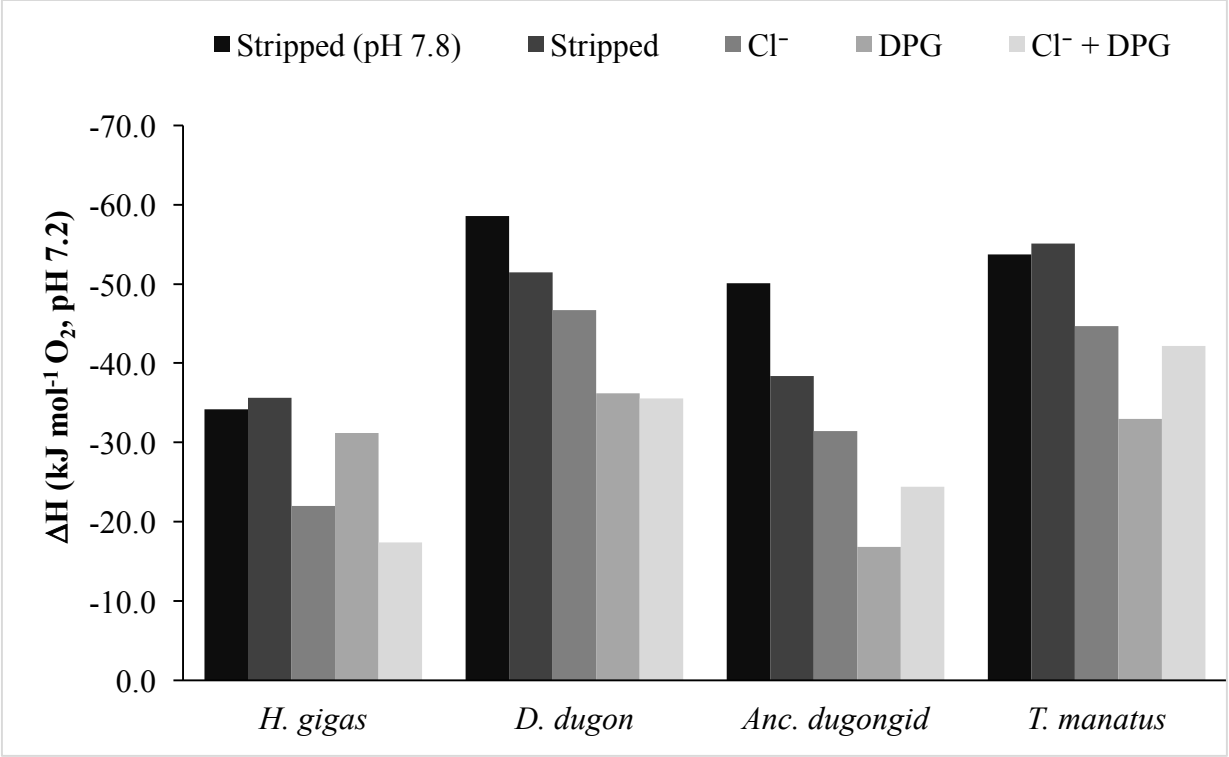


Figure 3.3. The enthalpy of oxygenation (ΔH) of hemoglobins from the Florida manatee (*Trichechus manatus latirostris*), dugong (*dugong dugon*), Steller's sea cow (*Hydrodamalis gigas*), and the ancestral dugongid ('Anc. dugongid') between 25 and 37°C in stripped (pH 7.2 and pH 7.9), the presence of 0.1 M KCl, a 2-fold molar excess of 2,3-diphosphoglycerate (DPG) and of both allosteric ligands. All values are corrected to pH 7.2 unless otherwise noted. The ΔH of *T. manatus* hemoglobin in the presence of 0.1M Cl^- (-44.7 kJ mol^{-1} O_2) closely agrees with the value reported by Farmer et al. (1979) under similar conditions (-42.7 kJ mol^{-1} O_2).

3.5. Discussion

3.5.1. O_2 Affinity of Sirenian Hemoglobins

The oxygenation of Hb coincides with a conformational change to the quaternary structure of the protein. As Hb transitions from the T- (tense or deoxy) to the R-state (relaxed or oxy), the αβ

dimers slide and rotate in relation to one another, resulting in the formation and breakage of numerous intersubunit and intrasubunit bonds (Perutz, 1970). Briefly, four intra-α-chain (α141Arg-α1Val and α141Arg-α126Asp), six α/β chain (α40Lys-β146His, α42Tyr-β99Asp, and α97Asn-β99Asp), and two intra-β-chain salt bridges (β146His-β94Asp) are broken during this transition, while four intersubunit bonds are formed (α94Asp-β102Asn and α41Tyr-β40Arg; Perutz 1970). The relative stability and abundance of bonds such as these have been proposed to dictate the intrinsic O_2 affinity of Hb (Perutz, 1970). However, the O_2 binding properties of whole blood are not solely determined by the intrinsic Hb–O_2 affinity, as Hb's interactions with allosteric effectors (DPG, Cl^-, H^+, and CO_2) in the red cell may also reduce its affinity for O_2. Thus, mutations within the genes encoding mammalian Hb that alter subunit interaction during the T→R transition and/or Hb's sensitivity to allosteric effectors underlie the diversity of whole blood O_2 affinities among mammals (Perutz, 1983). For example, the whole blood O_2 affinity of mammals scales with body mass and generally ranges between 20 and 40 mmHg (at 37°C, pH 7.4), presumably to match O_2 offloading with tissue requirements (Bunn et al., 1974; Lahiri, 1975). It has also been shown that shallow diving and semi-aquatic mammals whose lungs form a significant O_2 depot generally have slightly higher Hb–O_2 affinities than similar sized terrestrial mammals, whereas mammals that collapse their lungs during dives (e.g. deep diving pinnipeds and cetaceans) generally have lower Hb–O_2 affinities (Snyder, 1983). As the lungs of manatees constitute a larger proportion of body oxygen stores (~33%; Gallivan et al., 1986) than most other diving mammals, it has accordingly been suggested that their high blood O_2 affinity (P_{50} = 16.5 mmHg; White et al., 1976) maximizes the amount of O_2 extracted from lung stores while submerged (Gallivan et al., 1986). However, it is notable that manatee blood O_2 affinity is well above that of both closely related elephants (P_{50} = 23.2-25.2 mmHg; Dhindsa et al., 1972)

and other diving mammals (P_{50} = 26-31 mmHg; Snyder, 1983), though comparable to sloths and pangolins (P_{50} = ~19-21 mmHg; Lahiri, 1975; Weber et al., 1986), suggesting this trait may also be associated with the markedly low mass-specific metabolic rate of sirenians (Scholander and Irving, 1941).

We extend previous studies conducted on sirenian Hbs (McCabe et al., 1978; Farmer et al., 1979), and reveal that their high blood O_2 affinity is due to increased intrinsic Hb–O_2 affinity, as their responses to allosteric effectors are roughly equal to or greater than those of elephant Hbs (Campbell et al., 2010a). The high intrinsic Hb–O_2 affinity of ancestral dugongid Hb confirms this is an ancient trait that likely aided the secondary transition of the group to the aquatic environment, and presumably coincided with the rapid evolution of the genes encoding these proteins (HBA and HBB/HBD) in stem sirenians (Chapter 2). Notably, the strictly marine dugong, which dives deeper and longer than manatees (Marsh et al., 2012), has the highest Hb–O_2 affinity among sirenians (Figure 3.2; Table 3.1). We suggest this alteration may in part result from a substitution at α38 (Tyr→Ala), as variants at this position confer an increased O_2 affinity to recombinant human Hbs and Rüppell's griffon (Hashimoto et al., 1993; Hiebl et al., 1988).

In contrast to other sirenians, Steller's sea cow Hb possesses a markedly lower intrinsic O_2 affinity that is relatively unaffected by heterotopic ligands (Figure 3.2; Table 3.1), which we partially attribute to a single charge altering replacement (β/δ82Lys→Asn) in the central cavity of the protein. This region typically contains three pairs of anionic residues and eight pairs of cationic residues, and it has been postulated that the resulting electrostatic repulsion arising from the excess positive charge destabilizes the T-state conformation (Perutz et al., 1994). Removal of two strongly cationic Lys residues from the central axis are thereby expected to minimize this effect and result in a higher intrinsic P_{50}, as has also been shown for Hb Providence

(β82Lys→Asn), Hb Helsinki (β82Lys→Met), and Hb Rahere (β82Lys→Thr) mutants (Bonaventura et al., 1976; Ikkala et al., 1976; Sugihara et al., 1985). The Hb Providence mutation is particularly notable as the β82Asn residue is highly susceptible to deamidation (i.e. Asn→Asp) in vivo (Moo-Penn et al., 1976), thereby further minimizing the charge differential in the central cavity. The markedly elevated P_{50} (~9.1 mm Hg at 37°C and pH 7.2; Table 3.1) of *H. gigas* Hb in the absence of Cl^- and DPG relative to other sirenian Hbs (P_{50} = 3.4-5.4 mm Hg) is consistent with similar post-translational modifications. The β/δ82Lys→Asn exchange in Steller's sea cow predictably also curbs DPG, Cl^-, and H^+ binding at this site.

3.5.2. Effects of Heterotropic Ligands

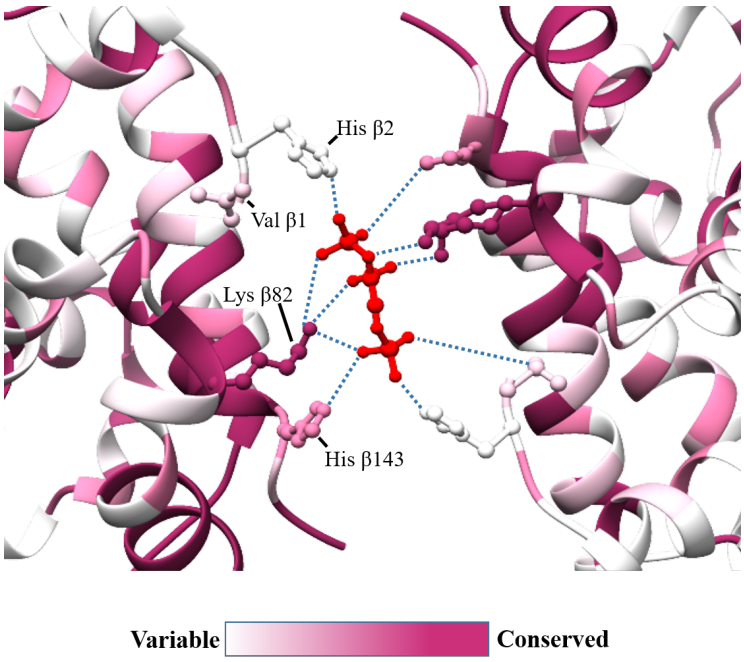


Figure 3.4. Molecular model of DPG (red) binding mammalian hemoglobin. Amino acids are coloured according to sequence conservation, as calculated by the ConSurf Server (http://consurf.tau.ac.il/2016) from a subsample of 50 mammalian beta-type hemoglobin chains. Dotted blue lines represent Hb–DPG bonds, as determined by Richard et al. (1993).

The polyanionic organic phosphate DPG may also strongly influence O_2 affinity in a pH dependent manner via its electrostatic interactions with the α-NH_2 group of 1Val of the β_2 chain and 2His, 82Lys, and 143His of both the β_1 and β_2 chains (Richard et al., 1993). Unlike the other DPG-binding residues, β82Lys is strongly cationic within the physiological pH range and is the principal DPG docking site, as DPG is able to form three bonds with $\beta_1$82Lys and two with $\beta_2$82Lys (thus,

this residue is highly conserved among mammalian Hbs) (Figure 3.4; Richard et al., 1993). Loss of a positive charge at β/δ82 in Steller's sea cow Hb is thus consistent with the negligible effect of DPG on P_{50} (Figure 3.2; Table 3.1), as was also demonstrated for the human HbA mutants with substitutions at this position (Bonaventura et al., 1976; Ikkala et al., 1976; Sugihara et al., 1985). Loss of DPG binding dramatically reduces the *in vivo* plasticity of Hb–O_2 modulation and is uncommon among mammals, with feliformid carnivores, ruminants, the eastern mole, and two species of lemur being the only other species known to exhibit this phenotype (Bunn, 1980; Campbell et al., 2010b).

Mutations that disrupt the discrete Cl^- binding site between β1Val and β82Lys of each β chain have repeatedly been shown to minimize the effect of this anion on P_{50} (Bonaventura et al. 1976; Sugihara et al., 1985; Campbell et al., 2010b). It is thus unsurprising that the Steller's sea cow β/δ82Lys→Asn replacement similarly confers their Hb with a Cl^- sensitivity (0.15) that is about half that of its dugongid ancestor (0.29) at 37°C (Table 3.1). The elevated Cl^- sensitivity of dugong Hb (0.40) relative to its dugongid ancestor aligns nicely with the β/δ101Glu→Gln substitution that creates an additional Cl^- binding site in both human Hb Rush mutants and woolly mammoth Hb (Shih et al., 1985; Campbell et al., 2010a). Detailed crystal structures of mammoth Hb have revealed that the β/δ101Glu→Gln replacement allows nearby 104Arg of the same chain—which normally interacts with 101Glu—to extend within 5 Å of its symmetry mate in the T-state (Noguchi et al., 2012). The heightened electrostatic repulsion confers a ~40% increase in the intrinsic O_2 affinity of the mammoth and Hb Rush proteins (Shih et al., 1985; Campbell et al., 2010a). Conversely, dugong Hb only exhibits a minor decrease in intrinsic P_{50} versus that of its dugongid ancestor (3.40 vs. 4.12 mmHg at 37°C, pH 7.2; Table 3.1), which we

attribute to a β/δ104Arg→Lys replacement that introduces a shorter side chain into the central cavity, thereby reducing conformational perturbations of the T-state protein.

A final substantive effect of the β82Asn/Asp mutants is a moderately reduced Bohr effect (Bonaventura et al. 1976), which is also evident for the Steller's sea cow protein (Figure 3.2; Table 3.1). Because the logarithmic acid dissociation constant (pKa) of specific histidine imidazole side chains within the Hb tetramer are oxygenation-linked and lie within the physiological pH range, it is widely recognized that these residues play a dominant role in mediating the Bohr effect (Berenbrink, 2006). While an underlying mechanism involving resulting alterations in the pKa of β143His allowing it to form a salt-bridge with nearby β82Asp in the R-state—thereby acting in opposition to the Bohr effect—has been proposed (Bonaventura et al., 1976), this explanation is unlikely as β82Met and β82Thr mutants also exhibit depressed Bohr effects (Ikkala et al., 1976; Sugihara et al., 1985). We instead propose that this effect arises from the loss of DPG-dependent H^+ binding (Riggs, 1971), as Steller's sea cow Hb shows a greatly reduced Bohr effect in the presence of DPG (-0.13) with respect to the dugongid ancestor (-0.61; Table 3.1). A reduced Bohr effect is also evident for manatee Hb (Table 3.1) and may arise from the α53Asp→Gly exchange that precludes an electrostatic interaction with nearby α58His.

3.5.3. Thermal Sensitivity

The invariant energy change associated with forming the weak covalent bond between O_2 and the heme iron (i.e. the enthalpy of heme oxygenation; ΔH^{O_2}) is exothermic (-59 kJ mol^{-1} O_2; Atha and Ackers, 1974), and only moderately opposed by the endothermic solubilization of O_2 (ΔH^{H_2O}; 12.55 kJ mol^{-1} O_2), thus dictating an inverse relationship between temperature and

inherent Hb–O_2 affinity. However, a number of factors including the heat of T→R conformational change ($\Delta H^{T\rightarrow R}$), and the oxygenation-linked dissociation of H^+ (ΔH^{H+}), Cl^- (ΔH^{Cl-}), and DPG (ΔH^{DPG}) may influence this relationship, such that the overall enthalpy of oxygenation (ΔH'), can become greatly minimized or even endothermic (Weber et al., 2010; Weber and Campbell, 2011). By facilitating adequate oxygenation of cool peripheral tissues, numerically low ΔH' Hbs are interpreted to be adaptive for cold-tolerant, regionally heterothermic mammals. While evolution of this phenotype has predominantly been attributed to the formation of additional heterotropic ligand binding sites on the protein moiety (Weber and Campbell, 2011), more recent studies have shown that elevated large positive $\Delta H^{T\rightarrow R}$ may also contribute to the low ΔH' of deer mouse, cow, shrew, and mole Hbs (Campbell et al., 2010b; Campbell et al., 2012; Jensen et al., 2016; Signore et al., 2012; Weber et al., 2014). The inherently low ΔH of stripped Steller's sea cow Hb (-34.2 kJ mol^{-1} O_2) relative to dugong, ancestral dugongid, and manatee Hbs (range: -50.1 to -58.6 kJ mol^{-1} O_2) at pH 7.8—where oxygenation-linked binding of protons is expected to be minimal—indicates that structural differences modifying the T→R transition underlie, at least in part, the low thermal sensitivity of *H. gigas* Hb. The observation that all known bonds stabilizing the T- and R-states of human Hb (see above) are conserved among sirenians (Table 3.1), further suggests this difference arises from the formation of additional salt bridges in the T-state of Steller's sea cow Hb. The moderately increased P_{50} of *H. gigas* Hb in the presence of allosteric effectors (13.9 mmHg at pH 7.2, 37°C) relative to other sirenian Hbs (range: 9.5-10.4 mmHg) provides additional evidence for the presence of such stabilizing bonds. It should be emphasized that this difference is unlikely to be attributed to the *H. gigas* β/δ82Lys→Asn replacement, as all known human Hb mutants at this position exhibit elevated oxygen affinities in the presence of allosteric effectors

(Bonaventura et al., 1976; Ikkala et al., 2976; Sugihara et al., 1985). We suggest that α51Asp of *H. gigas* Hb (Gly in other sirenians) may form a bond with nearby α56Lys in the T-state protein, though confirmation of this hypothesis will have to await structural determinations of Steller's sea cow Hb.

The β/δ82Lys→Asn substitution in Steller's sea cow has major functional consequences that include a reduction in the intrinsic O_2 affinity, and reduced proton, Cl^-, and DPG effects that constrain the *in vivo* modulation of Hb–O_2 affinity. Importantly, by reducing the number of binding sites for each of these allosteric effectors, *H. gigas* Hb is also predicted to exhibit diminished ΔH^{H+}, ΔH^{Cl-}, and ΔH^{DPG} values relative to other sirenians. Consistent with this expectation, Steller's sea cow Hb shows no reduction in ΔH as the pH is reduced from 7.8 to 7.2 (nor does manatee Hb, which displays a similarly low Bohr coefficient to that of Steller's sea cow; Table 3.1), and has a reduced enthalpy of DPG binding (ΔH^{DPG} = 4.5 kJ mol^{-1} O_2) relative to other sirenian Hbs (range: 15.3 to 22.1 kJ mol^{-1} O_2; Figure 3.3). However, despite the loss of a Cl^- binding site between β/δ1Val and β/δ82Lys, and consequently having the lowest Cl^- effect of all sirenian Hbs at both test temperatures (Table 3.1), the addition of this anion resulted in a larger ΔH^{Cl-} (13.7 kJ mol^{-1} O_2) than in other sirenian Hbs (range: 4.8 to 10.4 kJ mol^{-1} O_2; Figure 3.3). Conversely, by forming an additional Cl^- binding site not found in other sirenians, the dugong β/δ101Glu→Gln replacement heightens the Cl^- effect, yet its Hb exhibits the lowest ΔH^{Cl-} value. These observations provide compelling evidence that the ΔH' may not be determined by the absolute number of ligands bound to the T-state protein per se. It is interesting in this regard that, with three exceptions, the Bohr, Cl^-, and DPG effects of all sirenian Hbs increased as temperature was lowered from 37→25°C (Table 3.1); i.e., heterotropic ligand binding increased as temperature decreased. Importantly, the magnitude of this effect varied

between species (Table 3.1), with for example, Steller's sea cow Hb exhibiting the largest increase in the Cl^- effect with declining temperature (0.09; Table 3.1), and a correspondingly larger reduction in ΔH' (13.7 kJ mol^{-1}; Figure 3.3). Similarly, dugong Hb displays the lowest ΔH^{Cl-} (4.8 kJ mol^{-1}) and the lowest 37→25°C Cl^- effect differential (0.03; Table 3.1). The low ΔH^{Cl-} value of dugong Hb relative to that of mammoth Hb (4.8 vs. 13.0 kJ mol^{-1} O_2, respectively) also aligns with the higher 37→25°C Cl^- effect differential of mammoth Hb (0.09; Campbell and Weber, unpublished data). Taken together, these observations indicate that the extent to which allosteric effectors reduce thermal sensitivity may not be dictated the absolute number of effector binding sites, but rather by the temperature dependence of effector binding (i.e. the degree to which effector binding increases as temperature decreases). This inference is consistent with previous work on the temperature dependence of the Bohr effect in fishes (Fago et al., 1997; Wyman et al., 1978) and calls for a reassessment of the molecular mechanisms underlying the thermal dependence of O_2 binding to vertebrate Hbs.

3.5.4. Evolution and adaptive significance of numerically low ΔH' Hemoglobins

The ΔH' of Steller's sea cow Hb (-17.4 kJ mol^{-1} O_2) falls within the range found for the Hbs of cold-hardy, regionally heterothermic mammals (-16 to -20 kJ mol^{-1} O_2; Weber and Campbell, 2011; De Rosa et al., 2004). This close accord suggests that evolution of this 'optimal' ΔH' value plays an important role in matching O_2 delivery with O_2 demand in tissues maintained at temperatures different than the body core. However, it also opens questions regarding the fine tuning of Hb–O_2 thermal sensitivity as the addition or loss of discrete chemical bonds are predicted to cause abrupt step-wise changes in ΔH'. For example, the woolly mammoth phenotype arose by small (~4 kJ mol^{-1} O_2) increases in both ΔH^{Cl-} and ΔH^{DPG} relative to its

elephantid ancestors (Campbell et al. 2010a), while that of the Steller's sea cow was predominantly driven by a heightened $\Delta H^{T \rightarrow R}$ (by 15.9 kJ mol^{-1} O_2) with respect to its dugongid ancestor. In the absence of other changes, elevating the $\Delta H^{T \rightarrow R}$ of ancestral dugongid Hb by 15.9 kJ mol^{-1} O_2 would be expected to shift its ΔH' well below that of other 'cold adapted' Hbs (i.e., from -24.4 to -8.5 kJ mol^{-1} O_2), and may thus have been compensated by the β/δ82Lys→Asn replacement, which reduced the ΔH^{H+} and ΔH^{DPG} contributions. The higher ΔH^{Cl-} of Steller's sea cow Hb relative to its dugongid ancestor is particularly interesting in this regard, as it does not appear to be associated with the formation of new stabilizing bonds or an 'additional' (with respect to other sirenians) Cl^- binding site, but is rather dependent on the degree to which ligand binding increases as temperature decreases. This finding implies that substitutions in the immediate vicinity of Cl^-, H^+, and DPG binding sites may play a key role in the evolution of numerically low ΔH' Hbs, as these substitutions may alter the biochemical properties of effector binding sites. The dugong β/δ104Arg→Lys replacement is another potential case in point, as it presumably alters the pKa of α99Lys (the putative proton-linked Cl^- binding site in mammoth Hb; Noguchi et al., 2012) such that it is (nearly) equally protonated at both high and low temperatures (i.e. minimizing the effect of temperature on Cl^- binding). Notably, this mechanism could provide a means of modulating thermal sensitivity without altering blood oxygen affinity at normal core body temperature. By modifying the degree of effector binding without altering the number of potential docking sites, we further suggest amino acid replacements in the close vicinity to ligand binding sites may be a prevalent force driving the fine scale calibration of vertebrate blood affinity and other attributes.

CHAPTER 4: CHARACTERIZATION OF HETEROTROPIC LIGAND BINDING TO PAENUNGULATE HEMOGLOBINS IN RELATION TO THERMAL SENSITIVITY

Anthony V. Signore[1], Phillip R. Morrison[2], Colin J. Brauner[2], and Kevin L. Campbell[1]

[1] University of Manitoba, Department of Biological Sciences, Winnipeg, Canada

[2]University of British Columbia, Department of Zoology, Vancouver, Canada

Author Contributions:

AVS designed the study, performed experiments, analyzed data, and drafted the manuscript.

PRM assisted with data collection, analyses, and revisions to the manusctipt.

CJB provided laboratory space, research materials, and revisions to the manuscript.

KLC assisted with study design, provided laboratory space, research materials, and revisions to the manuscript.

4.1. Abstract

As the inherent affinity of hemoglobin (Hb) for O_2 is inversely related to temperature, regionally heterothermic mammals are expected to experience reduced O_2 offloading to cool, poorly insulated appendages. Presumably to compensate, some such species have evolved Hbs with reduced thermal sensitivity that can maintain adequate O_2 delivery at low temperatures. This reduced thermal sensitivity of Hb has primarily been attributed to the binding of additional allosteric effectors to the Hb moiety relative to those of non-cold adapted species. However, recent evidence suggests that Hb from the extinct Steller's sea cow binds fewer allosteric effectors than those of its tropical relatives (dugongs and manatees), yet surprisingly, this reduced binding is associated with reduced thermal sensitivity contrary to expectations. To further investigate the mechanistic basis for this observation, we measured the O_2 affinity of six paenungulate Hbs (woolly mammoth, Asian elephant, Steller's sea cow, dugong, ancestral dugongid, and Florida manatee) in the presence of serially increasing Cl^- and 2,3-diphosphoglycerate (DPG) concentrations at both 25 and 37°C. Quantitation of effector binding revealed that total effector binding (i.e., number of oxygenation-linked effector molecules bound) is unrelated to the thermal sensitivity of Hb. Instead, the results presented here reveal variable thermodynamic properties of Cl^- and DPG binding to discrete sites. We suggest these differences arise from amino acid differences in close vicinity to the respective binding sites, which in the case of Steller's sea cow decreases the enthalpy change of effector binding.

4.2. Introduction

Embedded within each of the four vertebrate Hb subunits lies an iron-containing heme group that reversibly binds O_2 (Perutz, 1970). Formation of the coordinate covalent bond between O_2 and

the heme iron is an exothermic process, thus dictating an inverse relationship between temperature and the affinity for which Hb binds O_2 (Hb–O_2 affinity; Atha and Ackers, 1974). While this trait promotes O_2 delivery to warm (active) tissues, it may impair O_2 delivery in regionally heterothermic mammals that allow their distal appendages to cool as a means of reducing the gradient for heat loss at poorly insulated sites (Irving and Krog, 1955). Accordingly, select cold-hardy mammals possess Hbs with reduced thermal sensitivity (Brix et al., 1990), which presumably helps match O_2 delivery with the varying O_2 demand of heterothermic tissues (Weber and Campbell, 2011).

The degree to which temperature influences Hb–O_2 affinity is dictated by the overall change in enthalpy upon oxygenation (ΔH'), wherein large negative (exothermic) values confer greater thermal dependence (Atha and Ackers, 1974). ΔH' is the net result of the inherent heat of O_2 binding to the heme iron (-59 kJ mol^{-1} O_2; ΔH^{O_2}; Imai, 1982), which is countered by endothermic contributions from the solubilization of O_2 (ΔH^{H_2O}), the heat of the T- (deoxygenated) to R-state (oxygenated) transition ($\Delta H^{T \rightarrow R}$), and the oxygenation-linked dissociation of heterotropic ligands such as H^+ (ΔH^{H+}), Cl^- (ΔH^{Cl-}), and 2,3-diphosphoglycerate (DPG; ΔH^{DPG}) (Weber and Campbell, 2011). Accordingly, variations in $\Delta H^{T \rightarrow R}$ and $\Delta H^{effector}$ among Hbs can lead to a range of ΔH' values, as the ΔH' of human HbA is -36 kJ mol^{-1} O_2, while that of cold-adapted mammals falls between -16 and -20 kJ mol^{-1} O_2 (De Rosa et al., 2004; Weber and Campbell, 2011; Chapter 3).

The numerically low ΔH' phenotype of regionally heterothermic mammals has primarily been attributed to increased Cl^- binding (Campbell et al., 2010a; De Rosa et al., 2004). For example, in woolly mammoth (*Mammuthus primigenius*) Hb a residue replacement at position 101 of the beta-type (β/δ) chain (Glu→Gln) creates a proton-linked Cl^- binding site that

contributes to reducing the ΔH' below that of tropical elephants (Campbell et al., 2010a). Interestingly, an analysis of sirenian Hbs (sister taxa to elephants) revealed that while this same mutation also increases the Cl^- sensitivity of sub-tropical dugong (*Dugong dugon*) Hb, it has only a minor effect on ΔH^{Cl-} (Chapter 3). Conversely, Hb of the extinct sub-Arctic Steller's sea cow (*Hydrodamalis gigas*) has a mutation (β/δ82Lys→Asn) that reduces Cl^- binding, yet exhibits the highest ΔH^{Cl-} of all sirenian Hbs (Chapter 3). These unusual observations suggest that the ΔH^{Cl-} may not be directly linked to the absolute number of chloride ions dissociating from the protein upon oxygenation.

To better understand the relationship between heterotropic ligand binding and oxygenation enthalpy of paenungulate (elephants, sea cows, and hyraxes) Hbs, we experimented on expressed recombinant Hb proteins from the dugong, Steller's sea cow, their predicted last common ancestor (dugongid ancestor), Florida manatee (*Trichechus manatus latirostris*), Asian elephant (*Elephas maximus*), and the woolly mammoth. To quantify effector binding to these Hbs, their Hb–O_2 affinity was measured in the presence of varied concentrations of Cl^- and DPG at two temperatures (25 and 37°C, pH 7.4). We found no significant correlation between absolute effector binding and thermal sensitivity, but instead we find that discrete ligand binding sites have variable thermodynamic properties.

4.3. Methods and Materials

4.3.1. Expression and Purification of Recombinant Hemoglobin Proteins

Previously obtained nucleotide sequences of the genes encoding adult Hb proteins (HBA and HBB/HBD) from the Florida manatee, dugong, Steller's sea cow, dugongid ancestor (Chapter 3), together with those of the Asian elephant and woolly mammoth (Campbell et al., 2010a) were

synthesized *in vitro*, ligated into custom expression vectors (Natarajan et al., 2011) and expressed in JM109 (DE3) *E. coli* as outlined in Chapter 3. Recombinant Hb proteins were purified from 2.5 L of bacterial culture using ion exchange chromatography according to Natarajan et al. (2011).

4.3.2. Functional Analyses of Hemoglobin Proteins

Hb solutions (0.4 mM heme in 0.1 M HEPES buffer, pH 7.4) for each species were prepared with varied concentrations of KCl (0.01, 0.04, 0.1, 0.5, 1.0, and 1.2 M) and DPG (0.05, 0.1, 0.2, 0.6, 1.0, and 2.0 mM). O_2-equilibria curves for each solution were measured at 25 and 37ºC using a multi-cuvette tonometer cell described by Lilly et al. (2013). A mixture of 4.95% O_2 (95.05% N_2) was diluted with pure N_2 using a Wösthoff DIGAMIX® gas mixing pump to create a range of oxygen tensions (0.4 to 37.6 mmHg). In order to determine the O_2 affinity of each solution (i.e., the PO_2 at half saturation or P_{50}), samples were equilibrated with four to six different oxygen tensions (PO_2) capable of saturating 30 to 80% of Hb molecules. The χ-intercept of Hill plots (log[fractional saturation/[1-fractional saturation]] vs PO_2) constructed from these measurements was used to determine P_{50}. Due to sample constraints, O_2-equilibrium curves of manatee Hb in the presence of DPG could not be completed. The data collected here were combined with similar KCl assays collected previously from the Asian elephant and woolly mammoth (Campbell et al., 2010a; Campbell and Weber, unpublished).

4.3.3. Data Analysis

A four parameter logistic (4PL) equation was fitted by nonlinear least squares curve fitting to plots of $\log P_{50}$ vs log[ligand] (Appendices 13 and 14). The maximum slope of these curves (the

binding or 'Hill coefficient') was used to represent the maximum ligand effect and, when rounded up to the nearest integer, the minimum number of ligands bound (Weiss, 1997). In instances where a 4PL curve could not be fit (e.g. instances with too few data points or when all data points fell within the exponential section of the curve), a linear regression was fit instead. As the slope of these plots represented ligand binding per heme (Amiconi and Giardina, 1981), this value was multiplied by four to obtain ligand binding per tetramer. To ensure consistency between methods, both were applied to the same dataset where possible, the results of which agreed closely (within the standard error of the regression estimate). Values for the physiological chloride effect ($\Delta\log P_{50}^{0.1M\ KCl - 0.0M\ KCl}$), the physiological DPG effect ($\Delta\log P_{50}^{0.2mM\ DPG - 0.0mM\ DPG}$), and enthalpy of ligand binding (ΔH^{Cl-} and ΔH^{DPG}) for paenungulate Hbs were previously obtained (Chapter 3; Campbell et al., 2010a). The enthalpy of ligand binding (ΔH^{Cl-} and ΔH^{DPG}) was plotted against ligand effect (i.e., $\Delta\log P_{50}^{0.1M\ KCl - 0.0M\ KCl}$ and $\Delta\log P_{50}^{0.2mM\ DPG - 0.0mM\ DPG}$) and total ligand binding. A linear regression was fit to these data and the significance of the correlation coefficient was tested using a one-tailed *t*-test.

4.4. Results

4.4.1. Phosphate Binding to Paenungulate Hemoglobins

Hill coefficients derived from dose-response curves of $\log P_{50}$ vs. log[DPG] revealed that all paenungulate Hbs, with the exception of Steller's sea cow Hb, contain a single DPG binding site (i.e., Hill coefficients are all close to 1; Figure 4.1). The DPG effect (i.e. the effect of physiological DPG levels, $\Delta\log P_{50}^{0.2mM\ DPG - 0.0mM\ DPG}$), however, was found to be temperature dependent (Chapter 3), and increased in all cases when the test temperature was reduced to 25°C

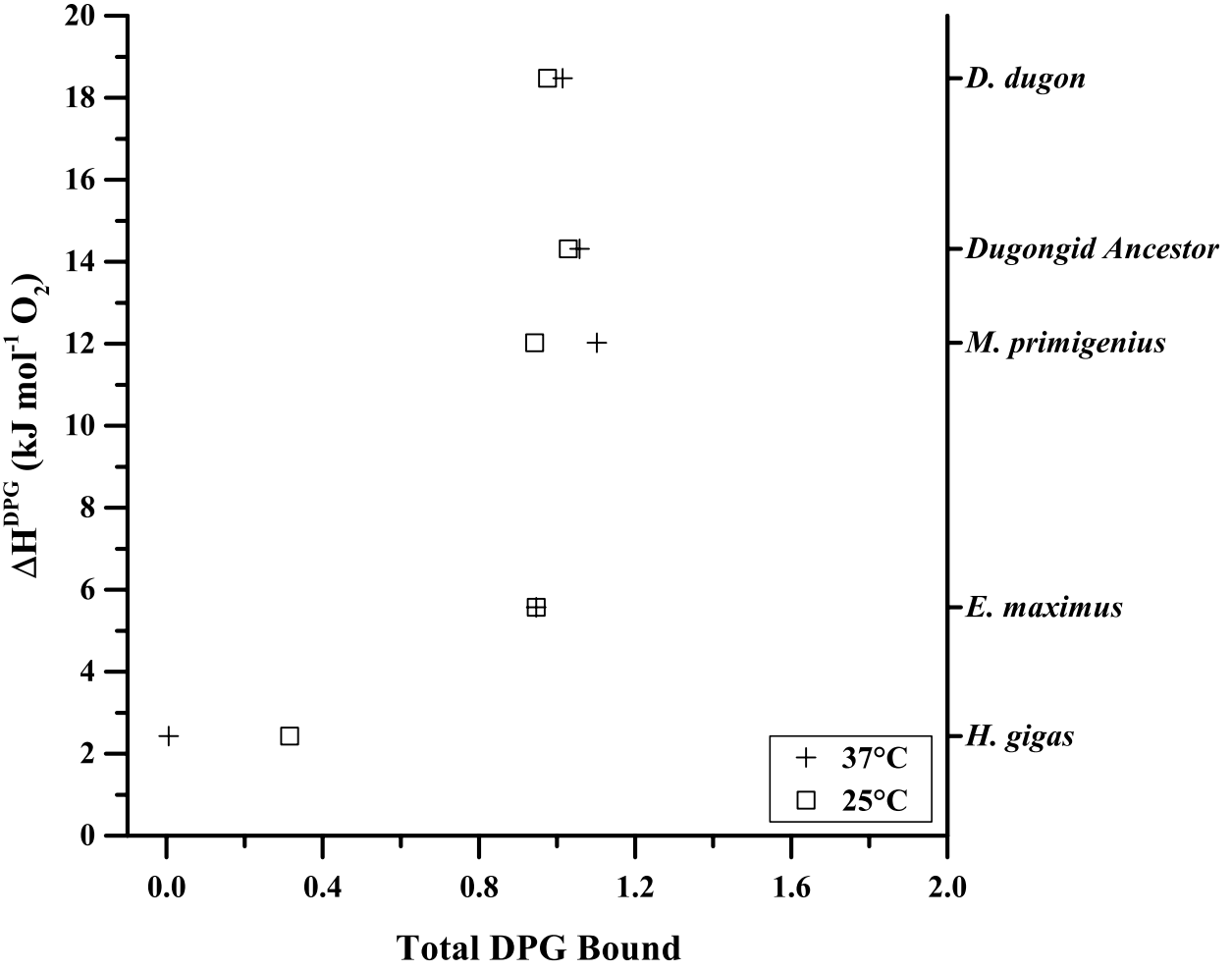


Figure 4.1. The number of 2,3-diphosphoglycerate (DPG) molecules bound to five paenungulate hemoglobins (listed on the right axis) at both 25 and 37°C (as estimated by the Hill coefficient of $logP_{50}$ vs log[DPG] plots, see text for details), plotted against the effect of 0.2mM DPG on the enthalpy of oxygenation (ΔH^{DPG}) at pH 7.4.

(Figure 4.2). Notably, a significant correlation ($p < 0.05$) between the DPG effect and ΔH^{DPG} was found at both test temperatures (R^2 =0.9782 at 25°C, and 0.9921 at 37°C; Figure 4.2).

4.4.2. Chloride Binding to Paenungulate Hemoglobins

The Cl^- effect (i.e., the effect of physiological Cl^- levels, $\Delta logP_{50}^{0.1M\ KCl\ -\ 0.0M\ KCl}$) was not correlated with the enthalpy of Cl^- binding at either test temperature (Figure 4.3). For example,

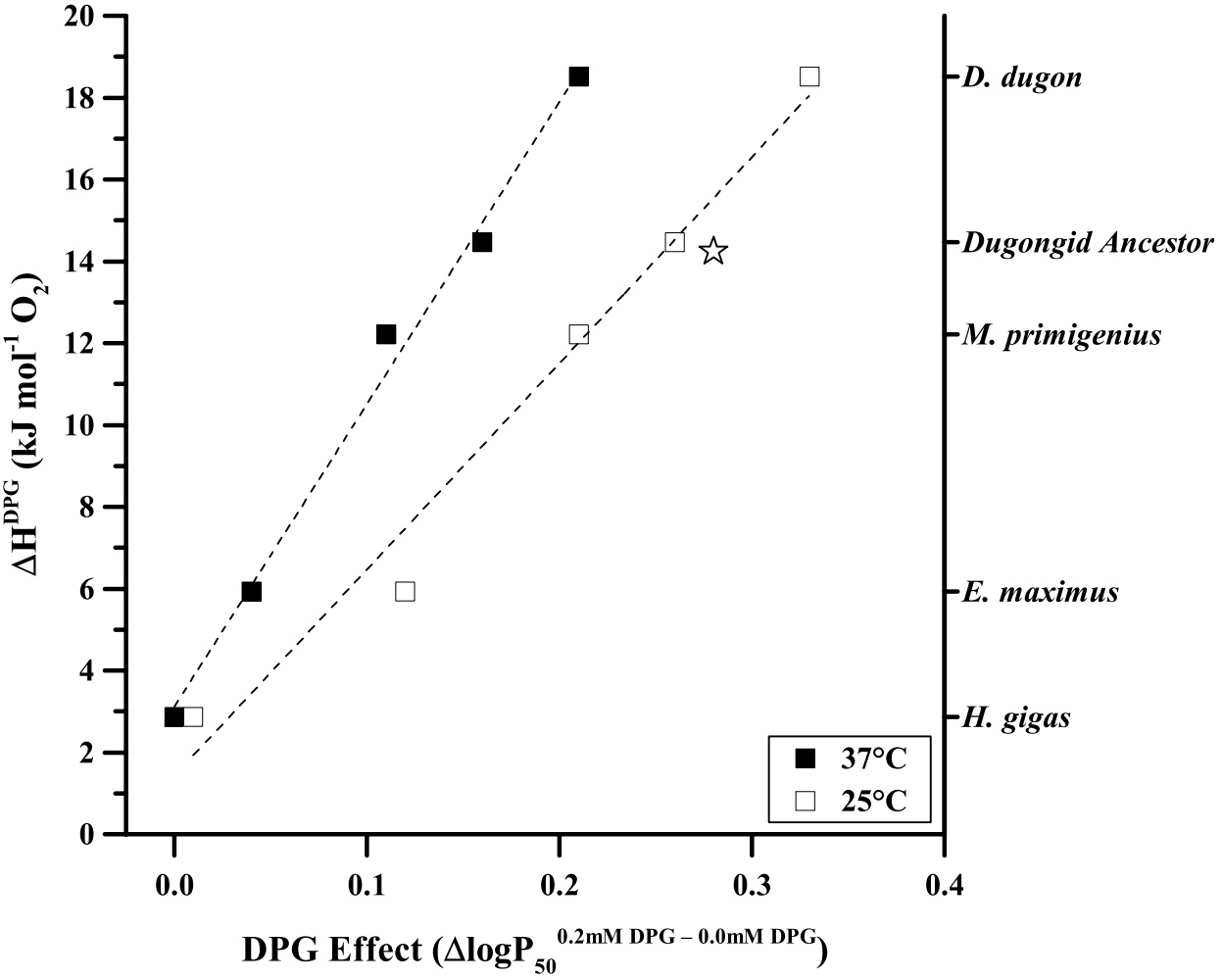


Figure 4.2. The effect of a 2-fold molar excess (0.2 mM) of 2,3-diphosphoglycerate (DPG) on Hb–O_2 affinity ($\Delta\log P_{50}^{0.2mM\ DPG\ -\ 0.0mM\ DPG}$) at both 25 (open symbols) and 37°C (closed symbols), plotted against the effect of 0.2mM DPG on the enthalpy of oxygenation (ΔH^{DPG}) at pH 7.4. These data show strong correlations at both 25 (R^2 = 0.9782) and 37°C (R^2 = 0.9921). Data for human Hb at 25°C (Benesch et al., 1969) is plotted for comparison (star); the R^2 remains relatively unchanged when this value is included in the equation (R^2 = 0.9727).

Steller's sea cow Hb has a markedly lower Cl^- effect than dugong Hb at both test temperatures, yet exhibits a ~1.7-fold higher ΔH^{Cl-} (Figure 4.3). In contrast, the ancestral dugongid Hb exhibits comparable Cl^- sensitivity to that of woolly mammoth and manatee Hbs, but a ~3-fold lower ΔH^{Cl-} (Figure 4.3). No correlation was found between ΔH^{Cl-} and the total number of Cl^- bound at

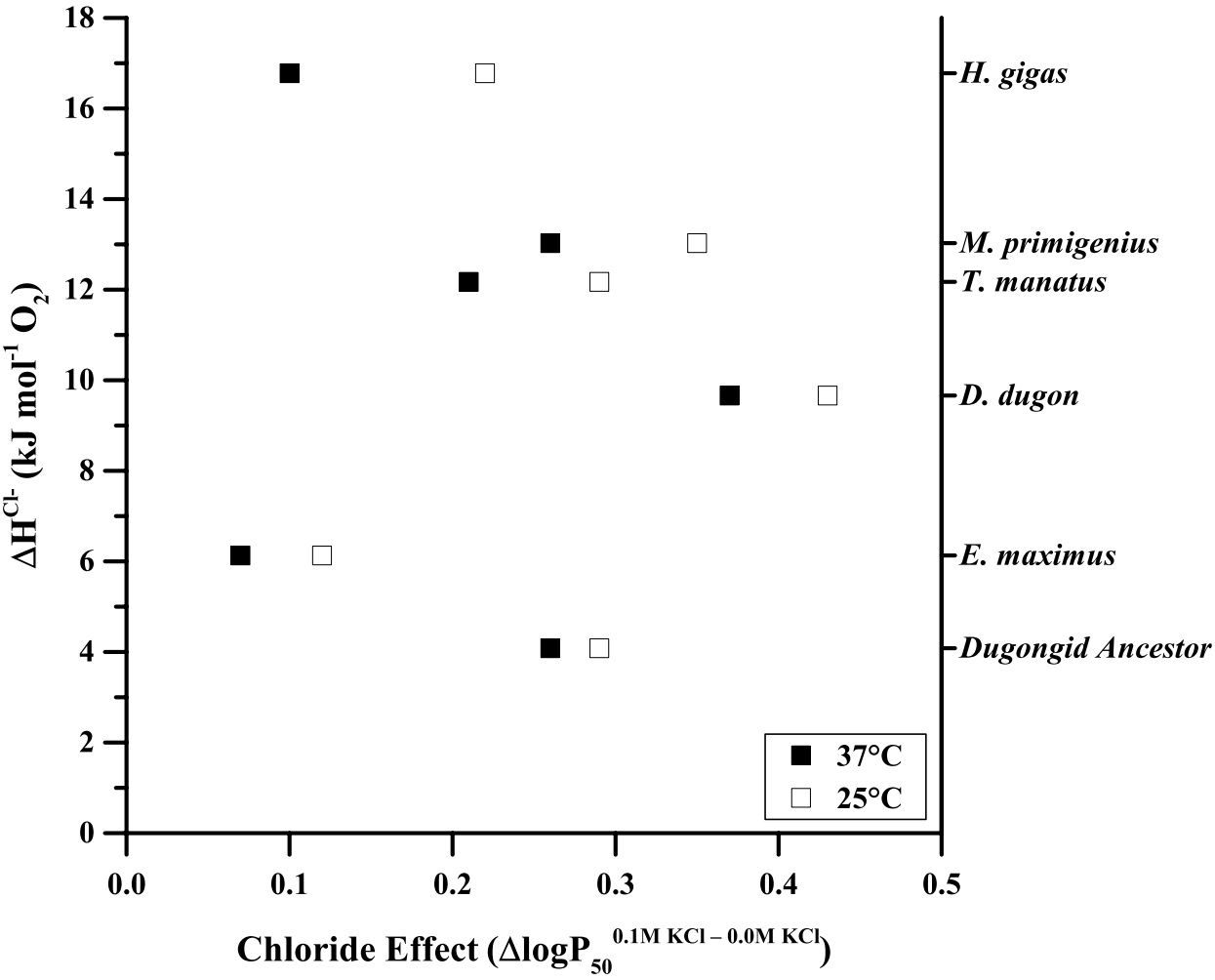


Figure 4.3. The effect of 0.1M KCl on paenungulate Hb–O_2 affinity ($\Delta logP_{50}^{0.1M\ KCl\ -\ 0.0M\ KCl}$) at both 25 and 37°C, plotted against the effect of 0.1M KCl on the enthalpy of oxygenation (ΔH^{Cl-}) at pH 7.4. These data show no significant correlations at either temperature.

either test temperature (Figure 44). Hill coefficients (± S.E. of regression estimate) reveal the presence of at least one Cl^- binding site in Hbs from the dugongid ancestor (0.98±0.42), Steller's sea cow (1.02±0.53), and Asian elephant Hbs (0.41±0.00) at 25°C, while these plots suggest the presence of an additional binding site in dugong (1.51±0.20), woolly mammoth (1.54±0.14), and manatee (1.66±0.39) Hbs (Figure 4.4). As was the case for DPG, the number of Cl^- bound was

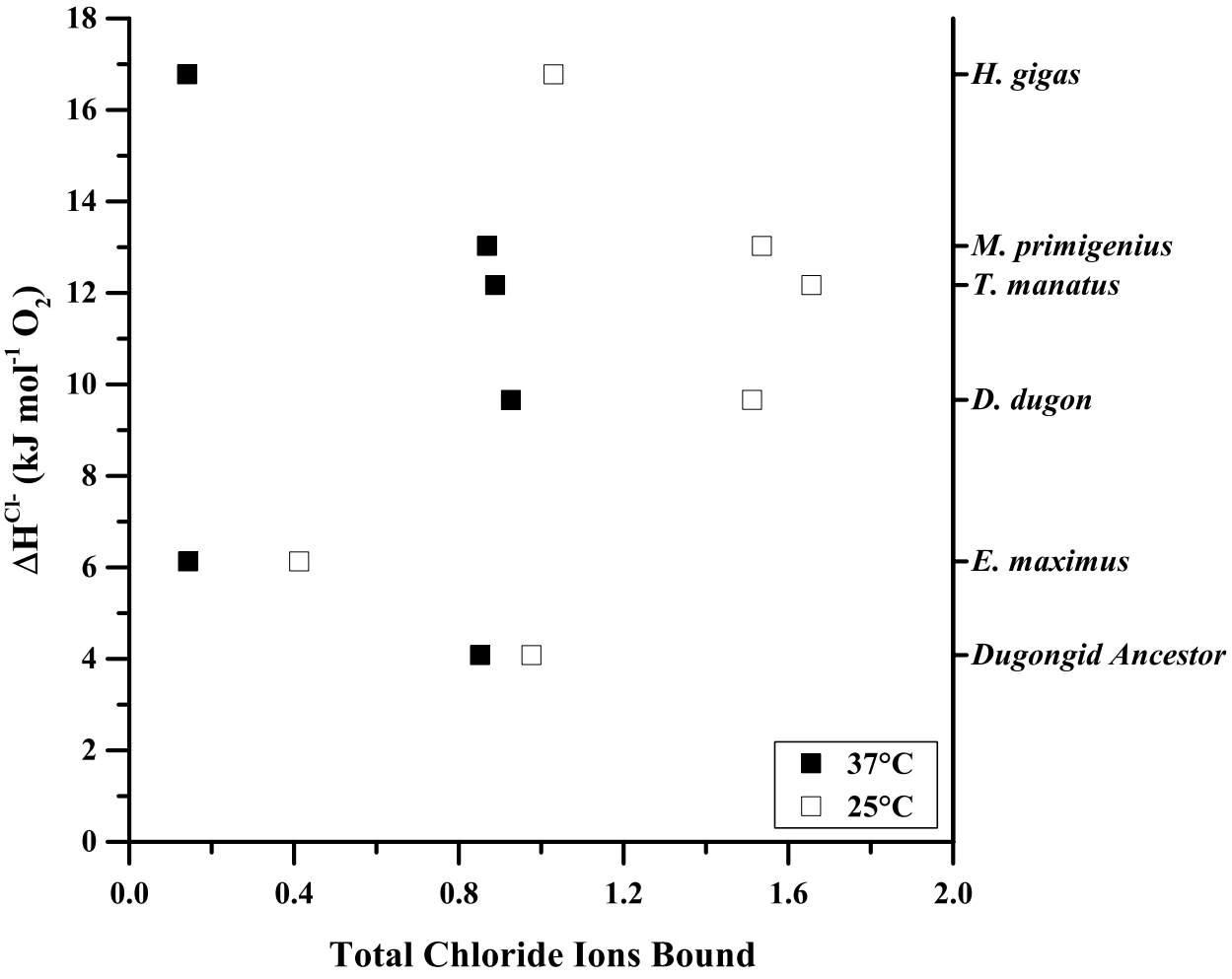


Figure 4.4. The number of chloride ions bound to paenungulate hemoglobins at both 25 and 37°C (as estimated by the Hill coefficient of $logP_{50}$ vs log[KCl] plots, see text for details), plotted against the effect of 0.1M KCl on the enthalpy of oxygenation (ΔH^{Cl-}) at pH 7.4.

invariably higher at the lower test temperature, with Cl^- binding to each Hb increasing proportionately to the ΔH^{Cl-} (Figure 4.5).

4.5. Discussion

Pioneering work by Hill (1910) demonstrated that the maximal slope of sigmoidal dose-response curves–by which a protein's response to increasing ligand concentration is measured–is directly linked to the number of ligand binding sites. However, in practice, the slope (termed the 'Hill

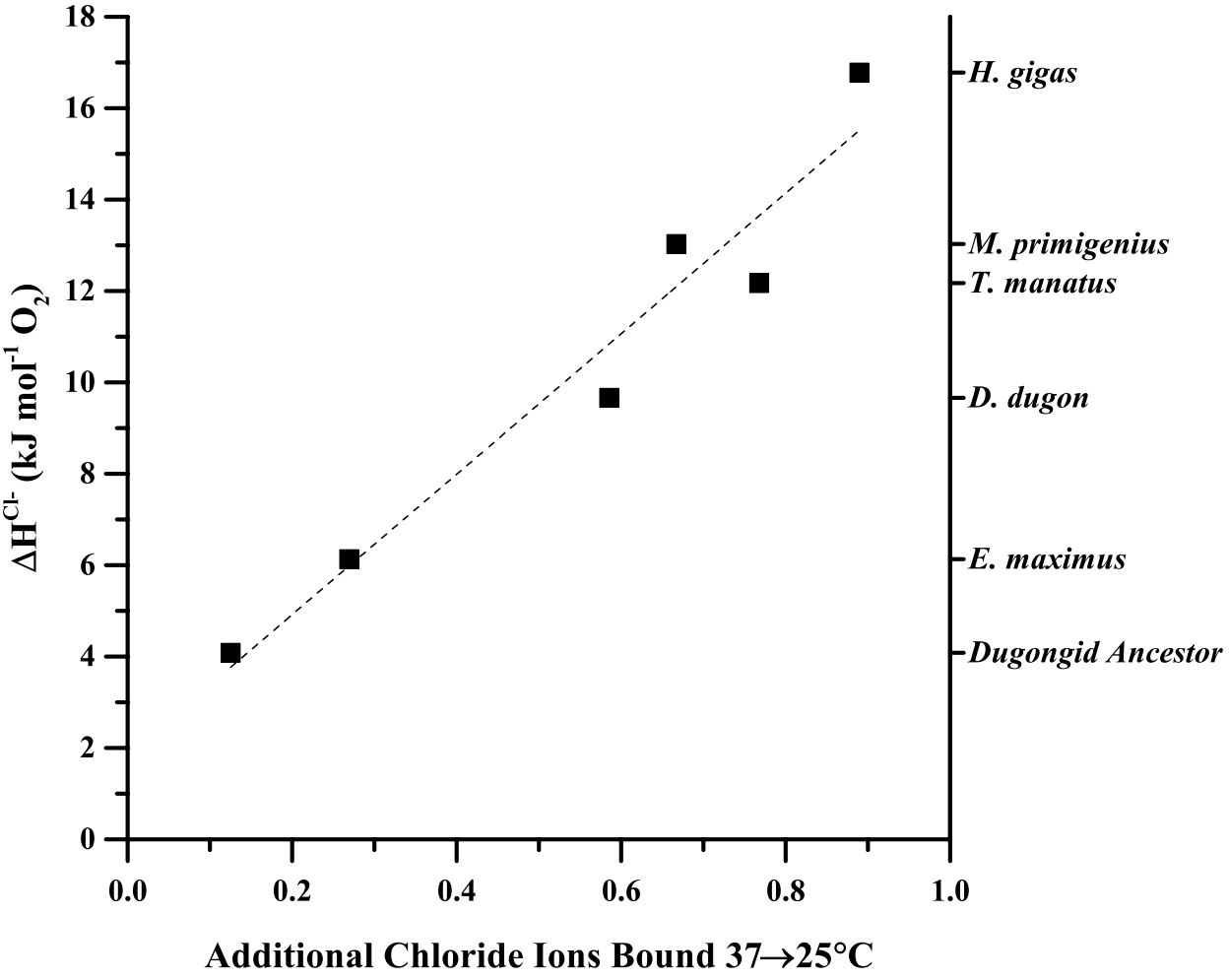


Figure 4.5. The number of additional chloride ions bound to paenungulate hemoglobins when the temperature is reduced from 37 to 25°C (as estimated by the Hill coefficient of $\log P_{50}$ vs log[KCl] plots), plotted against chloride's effect on the enthalpy of oxygenation (ΔH^{Cl-}) at pH 7.4. These data show a strong linear correlation ($R^2 = 0.9436$).

coefficient') is most often lower than the number of binding sites (Prinz, 2010). Indeed, plots of PO_2 vs Hb saturation rarely exceed three, despite the presence of four oxygen binding sites (Hill, 1913). As such, the Hill coefficient is taken to represent (1) the minimum number of binding sites, (2) the maximal effect of the ligand on protein function, and (3) the degree of cooperativity (i.e., the degree to which the binding of a single ligand influences the binding of another; Weiss, 1997). The Hill coefficient derived from dose-response curves of $\log P_{50}$ vs log[DPG] denote the

presence of a single DPG binding site in all paenungulate Hbs, with the exception of Steller's sea cow, whose β/δ82Lys→Asn replacement largely abolishes DPG binding (Chapter 3; Figure 1). Despite sharing the same single DPG binding site, DPG sensitivity varied among Hbs and was strongly correlated with ΔH^{DPG} at both test temperatures (Figure 4.2). Conversely, Hill coefficients derived from plots of $\log P_{50}$ vs log[KCl] reveal that the number of Cl^- binding sites vary interspecifically, as *H. gigas*, *E. maximus*, and ancestral dugongid Hbs release a single Cl^- upon oxygenation, whereas those of *D. dugon*, *T. manatus*, and *M. primigenius* appear to release at least two Cl^-.

4.5.1. Phosphate Binding to Paenungulate Hemoglobins

The organic phosphate DPG preferentially binds and stabilizes the deoxygenated conformation of Hb, reducing Hb–O_2 affinity (Antonini and Brunori, 1971). Physical constraints of the Hb tetramer and the shape of the DPG molecule itself generally limits the binding of DPG to the central cavity of mammalian Hbs, with only dromedary (*Camelus dromedaries*) Hb known to possess a second O_2-linked DPG binding site (Amiconi et al., 1985). With the exception of Steller's sea cow Hb, the data presented here are consistent with the presence of a single DPG binding site in paenungulate Hbs, as Hill coefficients derived from dose response curves of $\log P_{50}$ vs log[DPG]) fall close to 1.0 (range 0.942±0.11 to 1.102±0.40) regardless of temperature. This single docking site occurs within the central cavity between the α-NH_2 group of 1Val of the β_2 chain, and 2His, 82Lys, and 143His of both the β_1 and β_2 chains (Richard et al., 1993). However, as DPG is a polyanion, its binding to each of these sites is contingent on a positive charge (i.e., the respective amino acids being in their acid form). Thus, factors which alter the acid dissociation equilibrium (pKa) of these residues, such as temperature, solvent composition,

and characteristics of nearby amino acids, may affect the number of chemical bonds formed (as a single amino acid residue may form multiple bonds with DPG; Richard et al., 1993) and hence strength of DPG binding. Notably, the pKa of residues 2His, 143His and the α-NH_2 group of β1Val have been shown to vary with both oxygenation state, and solvent composition (Fronticelli et al., 1994; Garner et al., 1975; Lukin and Ho, 2004; Perutz et al., 1980; Riggs, 1971). Conversely, as β82Lys has a pKa well above physiological pH (>9.0 in human HbA), and there is no evidence to suggest that it is reduced by changes to the local protein environment, this residue is strongly cationic in both the T- and R-state proteins (Perutz et al., 1980). The importance of the persistent cationic charge at β82Lys is emphasized by the β/δ82Lys→Asn replacement in Steller's sea cow Hb, as a Hill coefficient of 0.0 at 37°C reveals the complete loss of DPG binding. Notably, however, a small DPG effect (0.3; Chapter 3) and Hill coefficient of 0.32±0.08 at 25°C indicates that the remnants of the DPG docking site (i.e., β/δ1Val, β/δ2His, and β/δ143His) are still able to bind DPG. As the protonation of 2His, 143His and the α-NH_2 group of β1Val are exothermic reactions, removing heat (i.e., decreasing temperature) is expected to shift the equilibrium of these reactions towards protonation, increasing the net positive charge within the central cavity and strengthening DPG binding in all Hbs examined.

4.5.2. Chloride Binding to Paenungulate Hemoglobins

The small size of monoatomic Cl^- relative to DPG confers a greater potential to bind at multiple sites within the Hb moiety. While two discrete Cl^- binding sites per dimer have been identified in human HbA, the relatively low Hill coefficient (1.60 at 25°C, pH 7.4; Colombo and Rau, 1994) suggests that less than half of these are occupied with Cl^- upon deoxygenation in the presence of physiological Cl^- concentrations (100 mM KCl). X-ray diffraction analyses indicate that one of

the Cl^- docking sites on human Hb is formed between the α-amino group of α1Val, the β-hydroxyl group of α131Ser on the same chain, and the guanidinium ion of the C-terminal α141Arg on the other α–chain (O'Donnell et al., 1979). The observation that carbamylation of the α-chain N-terminal valine reduces Cl^- binding supports this assertion (O'Donnell et al., 1979). Manatee Hb is unique among paenungulates, in that it possesses α131Ser and exhibits a similar Hill coefficient (1.66) as human HbA (Figure 4). The α131Ser→Asn replacement found in all other paenungulate Hbs (Chapter 2) presumably deletes this binding site, which aligns well with data presented in Figure 4.4 (e.g., Asian elephant and dugongid ancestor Hbs display half the Cl^- binding of manatee Hb). The heightened Cl^- sensitivity of woolly mammoth and dugong Hb is consistent with their (independently derived) β/δ101Glu→Gln substitutions (Campbell et al. 2010a; Chapter 3). This replacement has been shown to increase Cl^- binding to human, woolly mammoth, and dugong Hb in a pH dependent manner (Shih et al., 1985; Campbell et al. 2010a; Chapter 3), and likely arises from the formation of a single Cl^- binding site (per tetramer) between α99Lys of both chains (Noguchi et al., 2012).

The second Cl^- binding site of human HbA is postulated to be formed between β1Val and β82Lys of each Hb dimer (Nigen et al., 1980). However, as carbamylation of β1Val only results in a small reduction in Cl^- binding (Nigen and Manning, 1975), the latter residue presumably forms the primary docking site, where Cl^- binds competitively with DPG (Imaizumi et al., 1979; Nigen et al., 1980). Experiments on mutant human HbA support the importance of β82Lys as Cl^- binding site since its replacement at β82 causes strong reductions in Cl^- binding (Bonaventura et al., 1976). The Steller's sea cow β/δ82Lys→Asn replacement, in conjunction with α131Asn, should thus be expected to completely block Cl^- binding. However, Steller's sea cow Hb is sensitive to Cl^- in a dose-dependent manner. This observation could potentially be explained by

'delocalized' Cl^- binding—whereby Cl^- diffusion into the widening dyad axis of the protein upon the R→T transition neutralizes the excess positive charge within the central cavity—that stabilizes the T-state conformation (Perutz et al., 1994). However, as this mechanism does not involve the formation of electrostatic bonds it is at odds with the large enthalpy change that accompanies the addition of 0.1 M Cl^- (ΔH^{Cl-}=16.8 kJ mol^{-1} O_2) to Steller's sea cow Hb, which instead argues for the presence of an as of yet unidentified Cl^- binding site.

4.5.3. Enthalpy of Heterotropic Ligand Binding

Hb and its ligands exist in an equilibrium whereby the formation of a Hb–ligand complex results in the production of heat. As dictated by the van't Hoff-Le Chatelier-Braun principle, increasing the temperature at which this exothermic reaction takes place will shift the equilibrium towards dissociation (Posthumus, 1933). Thus, the degree to which Hb–ligand binding changes with temperature is directly related to the heat of reaction (ΔH). This is seen in Figure 4.2, where the linear fits at 25 and 37°C diverge as ΔH^{DPG} increases, and in Figure 4.5 where the difference in Cl^- binding between 25 and 37°C strongly correlates with ΔH^{Cl-}. However, the absolute number of anions bound to paenungulate Hbs shows no correlation with ΔH^{Cl-} or ΔH^{DPG}, which suggests the enthalpy change at individual binding sites can vary substantially. Indeed, Steller's sea cow binds the fewest Cl^- among sirenian Hbs (Hill coefficient of 0.14±0.05 at 37C), yet these Cl^- do so with the highest measured ΔH^{Cl-} (16.8 kJ mol^{-1} O_2; Chapter 3). Conversely, Cl^- binding curves from dugong and woolly mammoth Hbs show nearly identical Hill coefficients (1.51±0.20 and 1.54±0.14 at 25°C, respectively) yet the enthalpy change varies between -9.7 and -13.0 kJ mol^{-1} O_2, respectively. However, despite the additional Cl^- bound to both dugong and woolly mammoth Hbs, the associated enthalpy change is smaller than that of Steller's sea cow Hb.

Similarly, the heat of binding of a single DPG molecule to each paenungulate Hb varies considerably (Figure 1). Thus, these results suggest that the enthalpy change at individual binding sites can vary substantially.

While there is little information regarding the temperature dependence of Cl^- binding specifically, the relationship between temperature and the Bohr effect has been examined (Antonini et al., 1965; Fago et al., 1997; Wyman et al., 1978). Protons bind the imidazole side chains of histidine residues and the α-amino group of α1Val with a pKa that can vary substantially depending on the local environment (i.e., solvent composition, temperature, and the composition of neighboring amino acids; Antonini and Brunori, 1971). For example, Cl^- bound between the α-amino group of α1Val, α131Ser and the C-terminal α141Arg raises the pKa of α1Val, creating a chloride–linked Bohr group (O'Donnell et al., 1979; van Beek et al., 1979). As the ionization of α1Val also includes the generation of heat, removing heat from the system will shift the equilibrium towards ionization, further increasing the pKa of α1Val (Antonini et al., 1965). Finally, changes to the local environment surrounding titratable histidine residues, such as switching between oxy- and deoxygenated states, can alter its likelihood of being protonated at a given pH (Berenbrink, 2006). For example, as β146His of human HbA has a pKa of ~6.5 in the R-state and ~8.0 in the T-state (at 29°C; Lukin and Ho, 2004), this site is likely to be protonated in the T-state but not the R-state when at physiological pH. Conversely, β116His has a pKa between 6.1 and 6.4 regardless of oxygenation state and, thus, is unlikely to ever be protonated (Lukin and Ho, 2004). Such pKa changes are the basis of the Bohr effect, whereby protonation of some histidine residues stabilize the T-state conformation and reduce Hb–O_2 affinity; note however, that the pKa of some histidine residues decrease upon deoxygenation and thereby may contribute to a reverse Bohr effect (Berenbrink, 2006). However, the consequences of these pKa

variations go beyond the Bohr effect, as the protonation state of three DPG docking sites (β1Val, β2His, and β143His) may vary depending on the local environment. Thus, changes to solvent composition, temperature, and the compliment of neighboring amino acids are expected to subtlety alter the charge state of β1Val, β2His, and β143His and in turn alter the strength of DPG binding. This is reflected in Figure 4.2, as the DPG sensitivity of paenungulate Hbs varies considerably (range 0.12 to 0.33 at 25°C, excluding Steller's sea cow), despite the complete conservation of the DPG docking site among these species. These variations are moreover present in the enthalpy of DPG binding, as the ΔH^{DPG} of these paenungulate Hbs range from -5.93 to -18.51 kJ mol^{-1} O_2. Figure 4.2 demonstrates that the intraspecific variation in ΔH^{DPG} strongly correlates with the DPG effect on P_{50} at both test temperatures (R^2 = 0.9782 and 0.9921 at 25 and 37°C, respectively). When similar data for human HbA (Benesch et al., 1969) is plotted in Figure 4.2, the R^2 remains relatively unchanged (R^2 = 0.9727). This close correlation between ΔH^{DPG} and the DPG effect within this sampling of mammalian Hbs suggests that the degree of protonation at β1Val, β2His, and β143His, and hence number of chemical bonds formed between DPG and Hb, dictate both the overall ΔH^{DPG} and the DPG effect.

In contrast to DPG binding, Figure 3 shows no obvious relationship between the Cl^- effect and ΔH^{Cl-}. However, it has been demonstrated that the location of titratable histidine residues within globin chains may affect their heat of ionization, such that the ΔH° for individual histidine residues within horse (*Equus caballus*) myoglobin vary from 16 to 37 kJ mol^{-1}, whereas the ΔH° of free histidine is 29 kJ mol^{-1} (Bhattacharya and Lecomte, 1997). A similar phenomenon may underlie the variability in ΔH^{Cl-} of paenungulate Hbs. For example, ancestral dugongid and Asian elephant Hbs share the same putative Cl^- binding site (between β/δ1Val–β/δ82Lys) yet exhibit different ΔH^{Cl-} values (-4.08 and -6.13 kJ mol^{-1} O_2; Figure 4.5). Similarly,

dugong and woolly mammoth Hbs have both presumably gained the same ('additional') Cl^- binding site, though their ΔH^{Cl-} also vary (-9.66 and -13.03 kJ mol^{-1} O_2). More telling, both of these Cl^- binding sites are absent in Steller's sea cow Hb, yet it exhibits a large ΔH^{Cl-} (-16.78 kJ mol^{-1} O_2) that presumably arises from the formation of a single Cl^- binding site not present in other paenungulate Hbs; i.e. it binds less Cl^- than paenungulate Hbs with two Cl^- binding sites, but does so with a larger enthalpy change. A similar phenomenon may underlie the heightened ΔH^{Cl-} of bovine Hb relative to human HbA (-9.9 vs -4.5 kJ mol^{-1} O_2, respectively at pH 7.4; Weber et al., 2014), as plots of $logP_{50}$ vs log[KCl] yield similar Hill coefficients, and hence Cl^- binding, for each Hb (Perutz et al., 1994). Notably, however, amino acid substitutions near the N-terminus of the bovine β-globin chain delete a Cl^- binding site between β1Val and β82His, while a second replacement (β76Asn→Lys) has been suggested to add a discreet Cl^- binding site, such that bovine and human Hbs each have two Cl^- binding sites per dimer (Fronticelli et al., 1995; Perutz et al., 1994). Thus, the increased ΔH^{Cl-} of bovine Hb (Weber et al., 2014) is not due to an 'additional' Cl^- binding site (i.e., increased Cl^- binding) with respect to human HbA, but instead is presumably due to enthalpic differences at one or both of its Cl^- binding sites. Taken together, these findings demonstrate that the ΔH^{Cl-} of the same Cl^- binding site can vary interspecifically, with each discreet Cl^- binding site further varying within a given Hb molecule. This suggests epistasis, where amino acids in the vicinity of ligand binding sites alter their physiochemical properties and elicit changes to the enthalpy of Cl^- binding independently of its effect of P_{50}.

4.6. Conclusions

In line with previous studies (Mairbäurl and Weber, 2012), paenungulate Hbs contain a single DPG binding site, although that of Steller's sea cow Hb is largely abolished due to a replacement at β/δ82Lys→Asn. The close correspondence between DPG sensitivity and ΔH^{DPG} among the various paenungulate Hbs indicates a mechanistic linkage between the number of bonds formed between polyanionic DPG and the deoxy-state protein. These latter differences presumably arise from amino acid substitutions in the vicinity of the DPG binding site that alter the pKa and hence protonation state of β/δ1Val, β/δ2His, β/δ82Lys, and β/δ143His. In contrast to that found for DPG, ΔH^{Cl-} was not correlated with Cl^- sensitivity. Rather, the ΔH^{Cl-} of a particular binding site (e.g., β1Val–β82Lys) may vary interspecifically and the ΔH^{Cl-} of two different Cl^- binding sites (e.g., β1Val–β82Lys and α1Val–α131Ser–α141Arg) may vary within a single Hb molecule. This variation appears to be shared by other mammalian Hbs, as those of bovine, horse, and bear bind a similar number of or fewer Cl^- than human HbA, but do so with a greater ΔH^{Cl-} (De Rosa et al., 2004; Perutz et al., 1993; Weber et al., 2014). Hence, residue replacements proximate to effector binding sites that alter their thermodynamic properties may be an overlooked contributor to the functional adaptations of Hbs from cold adapted and regionally heterothermic mammals.

CHAPTER 5: FUNCTIONAL CHARACTERIZATION OF SIRENIAN PRENATAL HEMOGLOBIN ISOFORMS

Anthony V. Signore[1], Phillip R. Morrison[2], Colin J. Brauner[2], and Kevin L. Campbell[1]

[1]University of Manitoba, Department of Biological Sciences, Winnipeg, Canada

[2]University of British Columbia, Department of Zoology, Vancouver, Canada

Author Contributions:

AVS designed the study, performed experiments, analyzed data, and drafted the manuscript.

PRM assisted with data collection, analyses, and revisions to the manuscript.

CJB provided laboratory space, research materials, and revisions to the manuscript.

KLC assisted with study design, provided laboratory space, research materials, and revisions to the manuscript.

5.1. Abstract

The genes encoding the adult-expressed hemoglobins (Hb) of the Order Sirenia underwent major molecular changes in association with their secondary transition to aquatic life which resulted in Hbs with high O_2 affinity. While this trait presumably maximizes O_2 extraction from the lungs and limits premature tissue O_2 unloading during a dive, high Hb-O_2 affinity may hinder maternal-prenatal O_2 transfer in the absence of parallel changes to Hb isoforms in the developing circulation. Anecdotal evidence also suggests sirenians may express a specific fetal Hb isoform, which contrasts with the majority of mammalian species that express the adult Hb protein in the fetal stage and instead achieve an increased fetal blood O_2 affinity by lowering red blood cell organic phosphate concentrations. As a first step in characterizing the prenatal Hb isoforms of sirenians, we expressed a recombinant embryonic Hb (Gower I) and a putative fetal Hb protein (HbF) from Steller's sea cow (*Hydrodamalis gigas*), and HbF from the dugong (*Dugong dugon*). Functional tests reveal these Hbs to have extremely high O_2 affinities, reduced cooperativity and attenuated Bohr effects. Notably, Steller's sea cow HbF has the highest reported O_2 affinity of any mammalian Hb tested to date. Such functional properties (O_2 affinity, cooperativity, and Bohr effect) are consistent with Hb proteins expressed during the embryonic stages of development. Thus, the primary Hb component expressed during sirenian fetal development is likely the adult Hb isoform. As the Steller's sea cow fetal/adult Hb component is insensitive to organic phosphates, this species may thus represent a rare example in which fetal and maternal blood have the same O_2 affinity.

5.2. Introduction

Mammalian hemoglobin (Hb) is a tetrameric protein composed of two α-type and two β-type polypeptide chains (Perutz, 1970). Duplication of the proto-Hb gene in the ancestor of gnathostomes (~450 Mya) gave rise to the progenitor α- and β-type Hb genes (Storz, 2016). Subsequent duplications of these progenitor loci resulted in multiple copies of both α- and β-type genes (generally between four and twelve) within mammalian genomes that are organized into clusters on (human) chromosomes 16 and 11, respectively (Hoffmann et al., 2008; Opazo et al., 2008). The 5'–3' linkage order of these genes and their distance from the upstream locus control region (LCR; Peterson and Stamatoyannopoulos, 1993), dictate their expression pattern throughout development. For example, at two weeks post-conception, developing human embryos begin expressing genes at the 5' end of α-type (HBZ) and β-type (HBE) Hb clusters, which are translated to ζ and ε globin chains, respectively, and form Hb Gower I (Fantoni et al., 1981; Table 1). At week four, expression of the downstream HBA and HBG loci add α- and γ-chains to the erythrocytes of the developing circulatory system, which combine with the preexisting ε- and ζ-chains in various combinations to form Hbs Gower II ($\alpha_2\varepsilon_2$) and Portland I ($\zeta_2\gamma_2$) (Hecht et al., 1966; Table 1). As the fetus continues to mature, expression of the HBA and HBG loci increases while HBZ and HBE expression decreases until Hbs Gower I, Gower II, and Portland I are replaced with HbF ($\alpha_2\gamma_2$) (Wood, 1976) within the circulatory system. Shortly before birth, the expression of HBG is gradually replaced by the 3' HBB locus resulting in hemoglobin A (HbA; $\alpha_2\beta_2$) becoming the primary O_2 transporter (Pataryas and Stamatoyannopoulos, 1972). This pattern of gene expression switching during development results in the temporal production of Hb isoforms with successively lower O_2 affinities (i.e., each Hb has a lower O_2 affinity than the protein it replaced), which facilitates O_2 transfer from maternal to embryonic and fetal blood (Weber, 1994). While this same basic expression pattern

is present in all vertebrates examined to date, changes to the organization of the α- and β-globin gene clusters may alter the timing and the number of Hb genes expressed (Brittain, 2002; Peterson and Stamatoyannopoulos, 1993).

While all mammals express discrete Hb isoforms (isoHbs) during embryonic development, the O_2 affinity of fetal blood is generally increased relative to the maternal circulation by three different mechanisms (Ingermann, 1997). The fetal blood of most mammals contains the same Hb protein as is expressed postnatally ($\alpha_2\beta_2$), with Hb–O_2 affinity being increased in the former via reduced 2,3-diphosphoglycerate (DPG) concentrations in the red cell (strategy 1; Weber, 1994). This organic phosphate lowers whole blood Hb—O_2 affinity by preferentially binding to and stabilizing the low affinity ('Tense state') conformation of the protein (Benesch and Benesch, 1967; Brittain, 2002; Richard et al., 1993), and is formed as a side product of glycolysis via the Rapoport-Leubering shunt (Rapoport and Luebering, 1950). Fetal erythrocytes expressing 'adult' HbA proteins exhibit high pyruvate kinase activity (>10 fold higher than in adults) which reduces intracellular DPG levels and increases O_2 affinity with respect to the maternal circulation (Caire and Haydari, 1990; Jelkmann and Bauer, 1978; Qvist et al., 1981; Tweeddale, 1973).

Simian primates have forgone this mechanism in favor of the expression of a distinct fetal Hb isoform (HbF), in which a substitution on the γ–chain (γ143His→Ser) removes a pair of DPG docking sites (Brittain, 2002; Johnson et al., 2000; Takenaka and Morimoto, 1976; Tomita, 1981). As a result, the Hb—O_2 affinity of anthropoid fetal Hbs are less affected by DPG, resulting in a higher affinity in the fetal circulation (strategy 2; Bauer et al., 1969). Ruminants also express a distinct fetal Hb isoform, but in contrast to primates, these Hbs are encoded by a duplicated 'adult type' β^F chain that is prenatally expressed (Schimenti and Duncan, 1985). As

both fetal and adult ruminant Hbs are largely insensitive to DPG, maternal-fetal O_2 exchange is facilitated by the fetal protein ($\alpha_2\beta^F{}_2$) having a higher intrinsic O_2 affinity (strategy 3; Blunt et al., 1971; Clementi et al., 1996; Weber et al., 1988).

In regards to strategies 2 and 3 above, it is notable that anecdotal evidence suggests that paenungulates also express distinct fetal isoHbs. For example, blood from a 5-month old elephant fetus was shown to contain two distinct Hb components, although only a single (adult) Hb component is present in fetal blood during the last 10 months of gestation (Riegel et al., 1967). Likewise, cursory evidence suggests that the blood of newborn manatees contains a second isoHb (comprising ~5% of total Hb; Farmer et al., 1979), which moreover appears to confer fetal blood with O_2 binding properties distinct from that of maternal blood (White et al., 1976). As the genomes of both manatees and African elephants contain a single functional HBA locus and a single HBB-like (HBB/HBD) locus (Chapter 2), the presence of a second Hb component in newborn sirenian blood may thus be due to the delayed expression of an existing gene (likely HBG, which expresses the γ-chain). As the timing of globin gene expression is determined by its distance from the LCR (Peterson and Stamatoyannopoulos, 1993), the delayed expression of sirenian HBG is supported by synteny comparisons of the β-globin gene cluster of paenungulates, as the HBG locus of sirenians is further downstream than the same locus is in the elephant cluster (Figure 2.1). Moreover, the sirenian HBG locus appears to be missing a transcriptional control motif that is crucial for suppression of gene expression during fetal development of mice (Perez-Stable and Costantini, 1990; Sloan, 2005).

Although the presence of distinct fetal isoHbs is uncommon among mammals, the emergence of this phenotype in sirenians may have occurred in response to physiological specializations of the adult Hb for diving (Chapter 3). Briefly, the O_2 affinity of adult sirenian Hb

increased during the groups secondary transition to aquatic life, presumably in order to maximize O_2 extraction from the lungs and limit premature O_2 offloading while submerged (Chapter 2 and Chapter 3). Notably, however, elevated O_2 affinity in adult blood is also expected to negatively impact maternal-to-embryonic/fetal gas exchange, as it may reduce the PO_2 at which O_2 is offloaded. Moreover, Steller's sea cow (*Hydrodamalis gigas*) Hb is insensitive to organic phosphates, which eliminates the viability of strategy 1 (above) for maternal–fetal O_2 transfer. Thus, sirenians provide an interesting model to study the functional properties of pre-natal Hb isoforms. Therefore, we hypothesized that the functional properties of sirenian HbF proteins would be similar to those of fetal isoHbs in other mammals, rather than embryonic isoHbs. As a first step in investigating the properties of sirenian prenatal isoHbs, we have expressed recombinant Hbs corresponding to Steller's sea cow Gower I and HbF, and dugong (*Dugong dugon*) HbF and measured their O_2 binding properties and response to allosteric effectors.

5.3. Methods and Materials

5.3.1. Expression and Purification of Recombinant Hb Proteins

The genome of Steller's sea cow contains two intact α-type (HBZ-T1, HBA) and two intact β-type (HBE and HBG) genes that are known to form embryonic and fetal Hbs of other mammals (Hardison, 2012). These α- and β-type genes were paired in all possible combinations (Table 1) within custom Hb expression vectors (Natarajan et al., 2011) and expressed in JM109 (DE3) *E. coli* (see Chapter 2) to produce the recombinant Hb proteins Gower I (HBZ-T1/HBE), Gower II (HBA/HBE), Portland I (HBZ-T1/HBG), and HbF (HBA/HBG) (Table 5.1). Multiple attempts to express Steller's sea cow Gower II and Portland I Hb proteins were unsuccessful as these products were predominantly insoluble; expression of dugong Gower II and Portland Hbs was

thus not attempted. Dugong HbF was expressed in a similar manner as above, however, dugong Gower I was excluded from this study as it differs from Steller's sea cow Gower I at only two amino acid positions on the α-type chain (ζ17Val→Ile and ζ131Ile→Val); both Val and Ile are hydrophobic, branched chain amino acids, and their replacements within the interior of the molecule were thus expected to be functionally neutral. Recombinant Hb proteins were purified from 2.5 L of bacterial culture using ion exchange chromatography according to Natarajan et al. (2011).

Table 5.1. Sirenian α- and β-type hemoglobin chains that comprise prenatally expressed hemoglobin tetramers. Hb isoforms expressed and investigated in this study are indicated in italics.

	α-Type Chain	
β-type Chain	HBZ-T2	HBA
HBE	*Gower I*	Gower II
HBG	Portland I	*HbF*

5.3.2. Functional Analyses of Hb Proteins

O_2-equilibrium curves (OEC) of Hb solutions (0.4 mM heme in 0.1 M HEPES buffer) were measured at 37°C using a multi-cuvette tonometer cell described by Lilly et al. (2013). Hb solutions varied in their pH (6.8 and 7.2), chloride ion concentration (0 or 100 mM KCl), and organic phosphate concentration (0 or 2-fold molar excess of DPG) in order to test the influence of these cofactors on Hb function. The cooperativity (the Hill coefficient; n_{50}) and the PO_2 at half saturation (P_{50}) were inferred from the slope and χ-intercept of Hill plots (log[fractional saturation/[1-fractional saturation]] vs PO_2) as described in section 4.3.2. Pilot studies using the same set-up as described in section 4.3.2 revealed that the prenatal Hb components exhibited very high Hb–O_2 affinities. Consequently, each OEC was repeated in triplicate using two gas-mixing pumps connected in series in order to produce lower oxygen tensions than possible with

the single gas-mixing pump setup (see 4.3.2). P_{50} and n_{50} values are given as mean±S.D., in which significant differences between mean values for each treatment was determined by two-tailed T-tests (Figure 5.2). The Bohr effect was determined by fitting a linear regression to $\log P_{50}$ values for each treatment at two pHs (approximately 6.8 and 7.2), the slope of which represented the Bohr effect ($\Delta \log P_{50}/\Delta pH$), presented as slope±S.E. of the regression estimate (Figure 5.3).

5.4. Results

5.4.1. Functional Properties of Gower I

In the absence of allosteric effectors ('stripped') the P_{50} of Steller's sea cow Gower I ($\zeta_2\varepsilon_2$; 1.8 mmHg) is well below that of the adult ($\alpha_2\beta/\delta_2$) Hb (9.3 mmHg, pH 7.0; Figure 5.1). The Hb–O_2 affinity of Gower I Hb is reduced by both Cl^- and DPG, with DPG having the larger effect (Figure 5.2). In the combined presence of these allosteric effectors, the P_{50} of Gower I remains markedly less than adult Steller's sea cow Hb (3.0 vs. 15.9 mmHg, respectively) under the same conditions (Figure 5.1). The Bohr effect of Gower I Hb in the presence of DPG was similar to that of the adult protein (-0.207±0.131). In the presence of KCl the Bohr effect was low (-0.05±0.228), relative to the adult protein (-0.287), and was reversed in the other treatments (stripped: 0.176±0.181; KCl + DPG: 0.201±0.070) (Figure 5.3). Under all conditions, the Hill coefficient of Gower I (average n_{50} = 1.25) was lower than that of adult Steller's sea cow Hb (averag e n_{50} = 2.12; Figure 5.1).

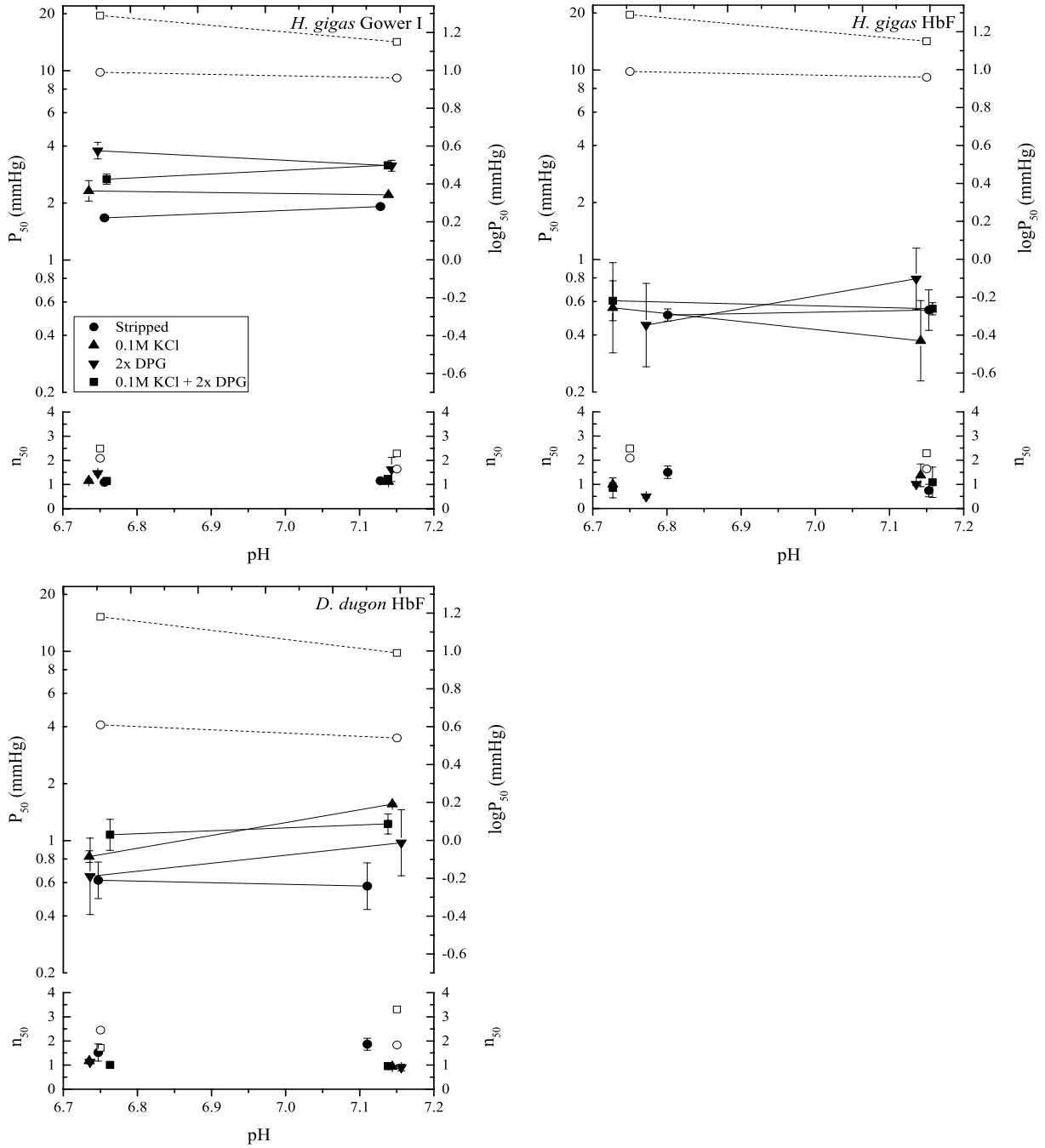


Figure 5.1. Oxygen tensions at half saturation (P_{50}) and the cooperativity coefficient (n_{50}) for sirenian prenatal hemoglobins (± S.D. n=3) and their pH dependence. Measurements were performed on stripped (circles) Hb, and in the presence of 0.1 M KCl (triangles), 2-fold molar excess of 2,3-diphosphoglycerate (DPG; inverted triangles) and both allosteric effectors (squares) at 37°C. Values for maternal Hb under similar conditions (open symbols; taken from Figure 2.2) are included for reference.

5.4.2. Functional Properties of HbF

The Hb–O_2 affinity of Steller's sea cow and dugong HbF are also higher than that of their respective adult counterparts, and that of Steller's sea cow Gower I (Figures 5.1 and 5.2). Under stripped conditions, the P_{50} of both HbF isoforms are similar to one another (0.53 and 0.58 mmHg for Steller's sea cow and dugong, respectively; Figure 5.1). However, the Hb–O_2 affinity of dugong HbF is more strongly affected by Cl^- and DPG, such that the affinity Steller's sea cow HbF (0.57 mmHg) in the combined presence of these effectors is double that of dugong HbF (1.2 mmHg)

(Figure 5.1 and 5.2). Similar to that found for Steller's sea cow Gower I, the stripped HbF isoforms of the dugong and Steller's sea cow exhibit low Bohr coefficients (Figure 5.3). Steller's sea cow HbF does not display a noticeable Bohr effect across all treatments, as each value is smaller than the associated margin of error. Conversely, dugong HbF shows relatively large reverse Bohr effects in treatments containing KCl and DPG (0.677±0.075 and 0.559±0.415, respectively; Figure 5.3). Both dugong and Steller's sea cow HbF display low cooperativity coefficients (average n_{50} of 1.18 and 1.00, respectively) with respect to their adult counterparts (average n_{50} = 2.32 and 2.12, respectively; Figure 5.1).

5.5. Discussion

5.5.1. Functional Properties of Gower I

As mammalian genomes typically possess functional HBZ and HBE genes at the 5' ends of the α- and β-globin gene clusters, respectively (except sheep, whose HBE is pseudogenized; Gaudry et al., 2014; Hoffmann et al., 2008; Opazo et al., 2008), the first Hb expressed in mammalian development is generally Gower I (Peterson and Stamatoyannopoulos, 1993). However, given the difficulty in obtaining blood samples from embryos, functional studies on the Hbs expressed

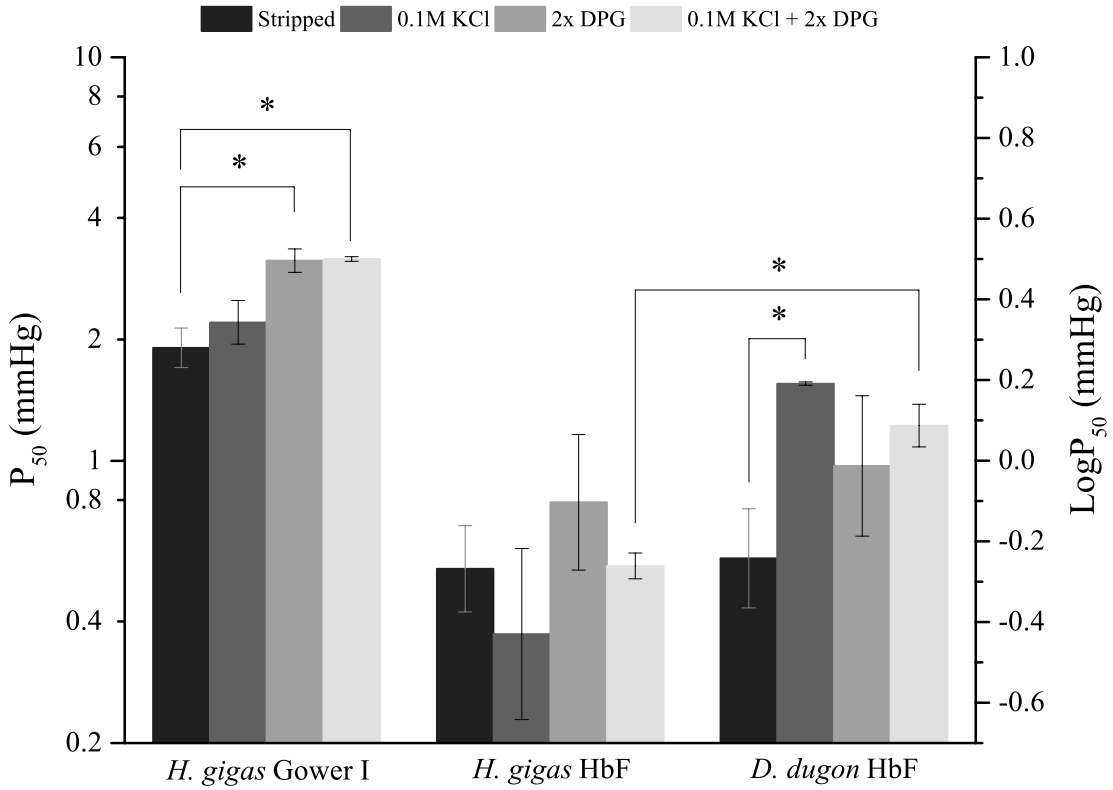


Figure 5.2. Oxygen tensions at half saturation (P_{50}) for sirenian prenatal Hbs in the absence and presence of KCl and 2,3-diphosphoglycerate (DPG) at 37°C, pH ~7.1. Values are presented as mean P_{50} ± S.D. Asterisks denote significant differences ($p < 0.05$).

during this period have generally been limited to model organisms (He and Russell, 2001; Jelkmann and Bauer, 1978; Purdie et al., 1983; Takenaka and Morimoto, 1976; Tomita, 1981) and livestock (Clementi et al., 1996; Moraga et al., 1996; Weber et al., 1988; Weber et al., 1987). Although variations in experimental conditions (temperature, heterotropic ligand concentration, and pH) between studies make interspecific comparisons challenging, it is clear that the O_2 affinity of mammalian Gower I Hbs are much higher than that of maternal Hbs, with the P_{50} value of rabbit, mouse, pig, and human Gower I falling between 0.8 and 3.9 mmHg in the absence of heterotropic ligands at 37°C, pH 7.0 (He and Russell, 2001; Jelkmann and Bauer, 1978; Purdie et al., 1983; Weber et al., 1987). The O_2 affinity of Steller's sea cow Gower I under

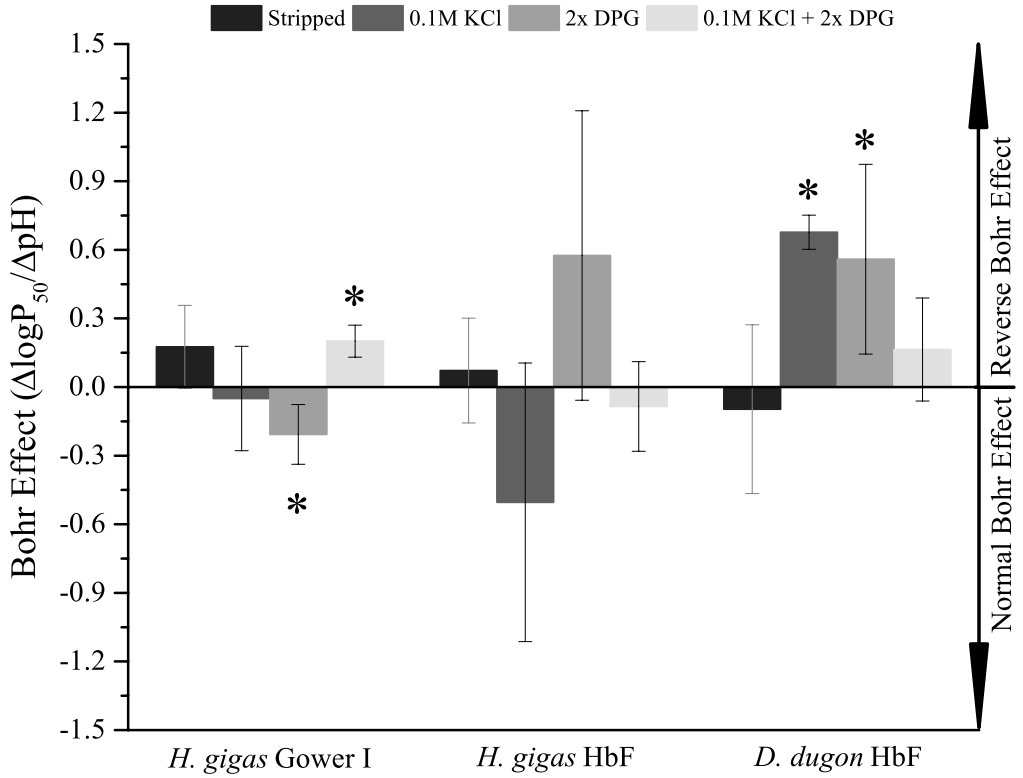


Figure 5.3. The Bohr effect of sirenian prenatal Hbs in the absence and presence of KCl and 2,3-diphosphoglycerate (DPG) at 37°C. Values are derived from the slope of linear regressions from the data in Figure 5.1 (ΔlogP50/ΔpH), presented ± S.E. of the slope. Asterisks denote values greater than the margin of error.

similar conditions (P_{50} = 1.82 mmHg; Figure 5.2) falls within this range, and is also substantially higher than its adult counterpart (P_{50} = 9.34 mmHg). The presence of DPG reduces the Hb–O_2 affinity of Steller's sea cow Gower I (Figure 5.2), similar to other Gower I proteins (He and Russell, 2001; Jelkmann and Bauer, 1978; Weber et al., 1987). Conversely, Steller's sea cow Gower I is relatively insensitive to chloride, as the P_{50} in 100 mM KCl media is not significantly different than that under stripped conditions (Figure 5.2). Human Hb Gower I is substantially less sensitive to Cl^- than is human HbA, which is has been attributed to amino acid differences between embryonic and adult alpha- (ζ and α, respectively) and beta-type (ε and β, respectively) chains. The α1Val→ζ1Ser and β77His→ε77Asn differences have been suggested

to inhibit Cl^- binding at the N-terminal Val and within the central cavity, respectively (Zheng et al., 1999). The ζ- and ε-chains of sirenians also possess ζ1Ser and ε77Asn, thus accounting for the low Cl^- sensitivity of Steller's sea cow Gower I. As the α-amino group of N-terminal Val also constitutes a chloride-dependent Bohr group, species which possess the ζ1Ser are expected to exhibit reduced Bohr effects (see below; O'Donnell et al., 1979).

As Hb–O_2 affinity decreases as pH declines, O_2 delivery to working (acidic) tissues is enhanced (Bohr et al., 1904). Known as the Bohr effect, this phenomenon is underpinned by differential H^+ binding to the R- and T-state Hb conformations (Lukin and Ho, 2004). Mammalian Gower I Hbs display a Bohr effect that is lower than maternal Hb (Jelkmann and Bauer, 1978; He and Russell, 2001; Weber et al., 1987) and may even lack a Bohr effect entirely (Purdie et al., 1983). This also appears to be true of Steller's sea cow Gower I, as the Bohr effect of stripped Gower I Hb and that in the presence of KCl are not significantly different from one another. The addition of DPG to Steller's sea cow Gower I produces a small Bohr effect (-0.21; Figure 5.3), which is reversed when both DPG and KCl are present. As the Cl^- dependent Bohr group at α1Val accounts for ~30% of the Bohr effect in human HbA (O'Donnell et al., 1979), the reduction of the Bohr effect displayed by Steller's sea cow Gower I is consistent with the presence of ζ1Ser. As the imidazole side chains of histidine residues have an acid dissociation constant (pKa) near physiological pH, these sites generally play the largest role in the Bohr effect (Lukin and Ho, 2004). Notably, the ε- and ζ-chains that comprise Steller's sea cow Gower I also have reduced histidine contents, with respect to their adult counterparts (Appendix 2-6). The ζ-chain of Steller's sea cow Gower I has four fewer histidine residues with respect to the adult α-chain, three of which (ζ50, ζ72, and ζ89) are contributors to the normal Bohr effect in humans (Lukin and Ho, 2004). The Steller's sea cow ε-chain also lacks histidine residues at positions 44,

56, and 77, with β77His being a known contributor to the normal Bohr effect of human HbA (Lukin and Ho, 2004). The Gower I Hbs of mice, rabbits, pigs, and humans also exhibit reduced Bohr effects and histidine contents with respect to their adult isoHbs (Jelkmann and Bauer, 1978; He and Russell, 2001; Weber et al., 1987). As reductions in pH should confer a greater reduction to the O_2 affinity of maternal HbA over the Gower I protein, attenuated Bohr effects in the developing circulation presumably enhance O_2 transfer to the embryo under acidic conditions (Weber et al., 1987). This effect is further enhanced in scenarios where a reverse Bohr effect is displayed (Figure 5.3), as reductions in pH are expected to decrease maternal O_2 affinity and subsequently increase embryonic affinity.

In addition to increased O_2 affinity and reduced Bohr effects, mammalian Gower I proteins are also characterized by reduced cooperativity of O_2 binding (Brittain, 1987; Brittain, 2002; Manning et al., 2007). Cooperative binding of O_2, where the binding of a single ligand increases its affinity for additional O_2, results in the characteristic sigmoidal shape of OECs (PO_2 vs Hb saturation; Hill, 1913). The slope of these curves (i.e. the Hill coefficient) is used to quantify cooperativity, which for human HbA falls between 2.8 and 3.0 (Imai, 1989). In contrast, OECs for monomeric myoglobin (Mb) proteins are hyperbolic (n_{50} = 1), as this O_2 storage protein does not display cooperative binding (Rossi-Fanelli and Antonini, 1958). The high cooperativity of Hb allows it to switch between the high affinity (predominantly oxygenated) relaxed (or R-) state and the low affinity (predominately deoxygenated) tense (or T-) state, which confers Hb with greater O_2 offloading within the midrange PO_2 values typically found in systemic and pulmonary circulations, respectively (40-100 mmHg; Imai, 1982). Conversely, the non-cooperative O_2 binding of Mb results in a consistently high O_2 affinity, which facilitates efficient O_2 transfer from the blood and in turn offloads O_2 at lower O_2 partial pressures (<5

mmHg), typically found in muscle (Richardson and Newcomer, 2001). Gower I Hbs of human, pig, mouse, and rabbit tend to bind O_2 with a cooperativity coefficient <2.0 (He and Russell, 2001; Jelkmann and Bauer, 1978; Purdie et al., 1973; Weber et al., 1987), which aligns with the data presented here for Steller's sea cow Gower I (n_{50}=1.1 to 1.6). The low cooperativity of mammalian Gower I proteins may help maintain a high maternal-embryonic O_2 affinity gradient to promote O_2 diffusion through the amniotic fluid (prior to placental development) and buffer the embryo against fluctuations in O_2 supply and demand, similar to vertebrate Mb in muscles (Brittain, 1987; Brittain, 2002; Richardson and Newcomer, 2001; Wells, 1981; Figure 5.1).

5.5.2. Functional Properties of HbF

While the fetal blood of most mammals contains the same $\alpha_2\beta_2$ isoform as maternal blood, two lineages (simian primates and bovid artiodactyls) are known to express fetal-specific Hb isoforms. In stem simian primates, the HBG locus was duplicated forming two daughter paralogs, HBG-T1 (γ^1) and HBG-T2 (γ^2) (Johnson et al., 2002). In New World monkeys (plathyrrhine), the γ^1-chain remains expressed in the embryonic yolk sack, while expression of the HBG-T2 locus is developmentally delayed and its product (γ^2) is subsequently found in erythroid cells derived from the fetal liver (Johnson et al., 1996; Johnson et al., 2002). In catarrhine primates (Old World monkeys, apes, and humans), expression of the γ^1-chain is also developmentally delayed, resulting in the presence of both γ^1- and γ^2-chains in fetal blood (Johnson et al., 2000; Johnson et al., 2002). The delayed expression of HBG loci in catarrhine primates was moreover accompanied by a delayed expression of the adult HBB locus (Johnson et al., 2000). While bovid artiodactyls (cattle, antelope, and goats) also express a fetal Hb isoform, this protein is not encoded by a γ-globin (HBG). Rather, an *en bloc* duplication of the entire β-

globin cluster in an ancestral bovid resulted in two copies of each β-type locus. Expression of the 3' copy of HBB (β^F) subsequently was shifted to the fetal stage, adding a distinct fetal Hb isoform ($\alpha_2\beta^F{}_2$) to the repertoire of cattle, antelope, and goats (Schimenti and Duncan, 1985).

The pattern of gene expression switching displayed by Hbs during development results in the temporal production of Hb proteins with successively lower O_2 affinities (i.e. each Hb isoform has a higher O_2 affinity than the protein it replaced; Weber, 1994). Thus, the O_2 affinity of fetal specific isoHbs in primates and bovids are lower than those of embryonic Hbs, but higher than HbA. For example, human Gower I, HbF, and HbA proteins have O_2 affinities of 4.8, 6.6 and, 7.8 mmHg, respectively (0.1M KCl, 3mM DPG, pH 7.4 at 20°C; Clementi et al., 1996; He and Russell, 2001). This same pattern is observed with the cooperativity of O_2 binding to mammalian fetal Hbs, as those of human, bovine, and sheep display cooperativity coefficients greater than embryonic isoHbs but reduced with respect to HbA (Clementi et al., 1996; Hlastala et al., 1978; Tomita et al., 1981; Weber et al., 1988). However, fetal isoHbs display similar Bohr effects to HbA (Antonini et al., 1964; Clementi et al., 1996; Weber et al., 1988). Thus, if HbF were expressed in the fetal stages of sirenian development, it might be expected to display P_{50} and n_{50} values that fall between Gower I and HbA, and a response to pH that is similar to the latter. However, Figures 5.1 and 5.2 illustrate that both dugong and Steller's sea cow HbF have P_{50}, n_{50}, and Bohr coefficients similar to or below that of Steller's sea cow Gower I.

At 37°C, pH 7.0, the P_{50} of dugong HbF is 0.58 mmHg under stripped conditions (Figures 5.1 and 5.2), which is lower than Steller's sea cow Gower I and all other mammalian Gower I Hbs measured to date (P_{50} range: 0.8 to 3.9 mmHg; He and Russell, 2001; Jelkmann and Bauer, 1978; Purdie et al., 1983; Weber et al., 1987). Steller's sea cow HbF displays similar O_2 affinity under the same conditions (P_{50} = 0.53 mmHg), but is not significantly reduced in the

presence of allosteric effectors, whereas dugong HbF P_{50} is reduced to 1.16 mmHg in the presence of KCl and DPG (Figure 5.2). While this suggests Steller' sea cow HbF is insensitive to allosteric effectors, the large margin of error (relative to the P_{50} values) associated with these measurements suggests these values are too low to measure reliably with the methods employed in this study. The reduced Hb–O_2 affinity of Steller' sea cow HbF (compared to that of the dugong) is moreover accompanied by reduced cooperativity of O_2 binding (n_{50} range of 0.5 to 1.5, compared to 0.9 and 1.9 for dugong HbF; Figure 5.1). Both dugong and Steller's sea cow HbF proteins display Bohr effects that are similar to that of Steller's sea cow Gower I and reduced with respect to adult sirenian Hbs (Figure 5.2 and Table 3.1).

The high O_2 affinity and reduced cooperativity of Steller's sea cow HbF (relative to dugong HbF) are likely attributed to two amino acid replacements in the Steller's sea cow γ-chain (γ38Thr→Ile and γ101Glu→Asp). The γ101Glu→Asp replacement (see Appendix 10) has been shown to increase the intrinsic affinity of human Hb Potomac (β101Glu→Asp) (Charache et al., 1978; Shih et al., 1985). In addition, the γ38Thr→Ile substitution is at a heme/$\alpha_2\beta_1$ sliding contact (position four of helix C) that appears to be invariable for all known wild-type vertebrate α, β, and Mb proteins (Campbell et al., 2010a, Dickerson and Geis, 1983; Mirceta et al., 2013). This extraordinary level of conservation suggests that this residue is a key structural component of globin chains. Indeed, β38Thr forms a bond with the C-terminal α141Arg and α94Asp in the T-state, stabilizing this conformation. Thus, the β38Thr→Ile is expected to disrupt these bonds, destabilize the T-state, and ultimately increase Hb–O_2 affinity, as is observed in patients affected by the human mutant Hb La Coruña (Ropero et al., 2006). As the $\alpha_2\beta_1$ sliding contact region is largely responsible for the cooperative O_2 binding displayed by vertebrate Hbs, this substitution may also underlie the reduced cooperativity of Steller's sea cow HbF (n_{50} between 0.5 and 1.5),

with respect to that of the dugong (n_{50} between 0.9 and 1.9). While the rarity of this substitution limits the availability of functional data regarding its physiological effects, the $\alpha_2\beta_1$ interface is highly conserved across vertebrate Hbs in order to maintain either the T- or R-state conformations (depending on oxygenation state; Baudin et al., 1993). Thus, any substitution in this region may disrupt both cooperativity and Hb–O_2 affinity (Baudin et al., 1993). Moreover, as β101 is near the $\alpha_2\beta_1$ sliding interface, replacements at this position also reduce cooperative O_2 binding, as the human mutant Hb Potomac (β101Glu→Asp) displays a cooperativity coefficient of 1.9, compared to 2.8 for HbA (Baudin et al., 1993; Shih et al., 1985). Thus, both γ38Thr→Ile and γ101Glu→Asp substitutions likely increase the O_2 affinity and reduce the cooperativity of Steller's sea cow HbF, with respect to that of the dugong. As the O_2 affinity of adult Steller's sea cow Hb cannot be reduced by DPG, the substitutions which increase the O_2 affinity of Steller's sea cow HbF may have evolved as a compensatory mechanism.

If expression of the sirenian HBG locus had been developmentally delayed in order to constitute a discrete isoHb in fetal blood, it might be expected to display P_{50} and n_{50} values that fall between Gower I and HbA, and a response to pH that is similar to the latter. However, sireinian HbF proteins have lower P_{50} values than Steller's sea cow Gower I and similar cooperativity and Bohr effects. Thus, sirenian HbF proteins exhibit none of the functional properties that are common to fetal isoHbs in other mammals. Therefore, the primary Hb component of sirenian fetal blood is likely adult HbA, in which the O_2 affinity is increased via reduced DPG levels in fetal erythrocytes, as it is in most other mammals (Weber, 1994). This is supported by the observation of reduced DPG levels in the erythrocytes of newborn manatees (White et al., 1976). However, as Steller's sea cow HbA is insensitive to organic phosphates (Chapter 3), intracellular DPG concentration would have no bearing on Hb function. Thus,

Steller's sea cow may have been a rare example in which maternal and fetal blood O_2 affinity are identical (as it is in felids; Novy and Parer, 1969) and O_2 transfer is facilitated by other means (e.g. increased Hb/erythrocyte concentration or increased blood flow). However, as Steller's sea cow HbA has a lower O_2 affinity (P_{50} = 15.9 mmHg, in the presence of KCl, DPG, pH 7.0 at 37°C; Chapter 3) than other sirenian HbA proteins (P_{50} range: 11.51 to 12.48 mmHg; Chapter 3), it releases O_2 at a higher PO_2 than its relatives, even in the absence of allosteric modulation by DPG.

5.6. Conclusions

Sirenian prenatal Hbs (Gower I and HbF) have markedly elevated O_2 affinities relative to their adult counterparts in order to facilitate O_2 uptake by the developing circulation. While the O_2 affinity of Steller's sea cow Gower I Hb is similar to that found for other mammals, the O_2 affinity of its HbF isoform is surprisingly high. This finding contradicts earlier reports of a fetal isoHb component in the blood of newborn manatees, as the functional properties of sirenian HbF suggest it is best suited for the embryonic stage of development. Thus, the primary Hb component of sirenian fetal blood is likely the adult HbA isoform (as it is in most mammalian species), where reduced DPG concentrations within fetal erythrocytes cause a higher Hb–O_2 affinity (i.e. strategy 1, see section 5.2). However, as Steller's sea cow HbA is unaffected by DPG–possibly as a consequence of 'fine-tuning' its Hb function for cold tolerance (Chapter 3)–reduced intracellular DPG concentrations would have no effect on Hb–O_2 affinity. Thus, Steller's sea cow may have been a rare example where fetal and maternal blood have the same O_2 affinity, in which adequate oxygenation of fetal blood is maintained by the reduced O_2 affinity of Steller's sea cow HbA relative to other sirenians.

CHAPTER 6: FINAL DISCUSSION AND CONCLUSIONS

Despite being one of the most studied proteins in biology, both in terms of its physiological function and molecular structure, there is still a great deal of information that can be gleaned from vertebrate Hb with respect to environmental adaptation and its molecular basis. This assertion is supported by the research put forward in this thesis, as my interdisciplinary approach has yielded new insights to the concerted evolution of two functionally linked gene families, and the molecular underpinnings of respiratory adaptations to both aquatic and Arctic environments.

6.1. Molecular Evolution of Sirenian Globin Genes

The fossil record of sirenians documents gradual morphological changes to their body, limb, and tail structures during their secondary transition to aquatic life (Domning, 2000). While the molecular underpinnings of adaptations such as these have been studied (McGowen et al., 2014), less attention has been directed to the molecular foundations of attendant physiological adaptations that improve O_2 storage and management while submerged (Mirceta et al., 2013). This shortcoming is addressed in Chapter 2, where I have retrieved, annotated, and performed detailed selection analyses on the globin repertoire of the extinct Steller's sea cow (*Hydrodamalis gigas*), dugong (*Dugong dugon*), and Florida manatee (*Trichechus manatus latirostris*) in relation to their closest living terrestrial relatives (elephants and hyraxes). These analyses revealed that six of eight globin genes evolved at substantially accelerated rates (relative to background rates for these loci) as sirenians transitioned to aquatic life. These rates were highest in the genes encoding adult sirenian Hb (HBA and HBB/HBD). In particular, the rapid evolutionary rate of the HBA locus is temporally coupled with the emergence of a chimeric

pseudogene (HBA/HBQps) that contributes to the tandemly linked HBA of recent sirenians via interparalog gene conversion. Moreover, these analyses identified a number of amino acid substitutions in the globin chains of dugongs (β/δ101Glu→Gln) and Steller's sea cows (β/δ82Lys→Asn, γ101Glu→Asp, and γ38Thr→Ile) that are known to alter O_2 binding properties in other species. However, as some of these substitutions contradict with our current understanding of Hb adaptation to cold environments, they were functionally characterized.

6.2. Functional Evolution of Adult Sirenian Hbs

The O_2–binding properties of recombinant Hbs that precisely match those of manatee, dugong, Steller's sea cow, and the last common ancestor of the latter two species were measured in Chapter 3. I found that the rapid evolution of HBA and HBB/HBD genes in the stem sirenian produced Hb proteins with markedly higher O_2 affinity than that of their living terrestrial relatives (elephants and hyraxes). As the lungs of sirenians are a significant O_2 store (Gallivan et al., 1986), high affinity Hbs are presumed to maximize O_2 extraction while submerged. However, this trait may also play a role in facilitating the low mass-specific metabolic rate of sirenians (Gallivan et al., 1983), by limiting O_2 offloading. Thus, a high blood O_2 affinity may optimize the underwater foraging behavior of sirenians by allowing onboard lung O_2 stores to be better exploited while attenuating its consumption. Interestingly, the O_2 affinity of Steller's sea cow Hb appears to have independently undergone a secondary reduction relative to the ancestral state, as it displays P_{50} values similar to those of elephants. As first-hand accounts suggest Steller's sea cows could not fully submerge (Steller, 1751), unobstructed access to atmospheric air may have relaxed selection pressures that favored the high O_2 affinity phenotype of other sirenians.

While selection pressures favoring a high Hb–O_2 affinity phenotype were presumably relaxed in Steller's sea cow, its exploitation of North Pacific waters in the Miocene likely imposed selection pressures on other aspects of Hb function. As Hb–O_2 affinity is inversely related to temperature, the Hbs of Arctic species are often modified to maintain O_2 delivery in the face of variable tissue temperatures (Weber and Campbell, 2011). While this phenotype has historically been attributed to the binding of additional allosteric effectors to the Hb moiety relative to that of non-cold adapted species, I found that Steller's sea cow Hb binds fewer of these ligands, as predicted by the β/δ82Lys→Asn substitution (see Chapter 2). However, despite the near complete abolishment of DPG binding and reductions in the Bohr effect and Cl^- binding, Steller's sea cow possesses a 'cold adapted' Hb phenotype similar to that of other Arctic mammals (De Rosa et al., 2004). I revealed that this is largely achieved by a heightened enthalpy change associated with the T→R transition ($\Delta H^{T\rightarrow R}$). However, the presence of 0.1 M chloride (the allosteric effector often responsible for lowering the thermal sensitivity of Hb–O_2 affinity in many other Arctic mammals) reduced the thermal sensitivity of Steller's sea cow Hb to a greater degree than was found for other sirenian Hbs, despite the loss of a Cl^- binding site (β/δ82Lys→Asn). Conversely, dugongs evolved an 'additional' Cl^- binding site arising from its β/δ101Glu→Gln replacement, yet this ligand has a reduced effect on the thermal sensitivity, with respect to Steller's sea cow.

These perplexing observations were explored further in Chapter 4, in which I measured the O_2 affinity of six paenungulate Hbs (woolly mammoth, Asian elephant, Steller's sea cow, dugong, ancestral dugongid, and Florida manatee) in the presence of serially increasing Cl^- and DPG concentrations at both 25 and 37°C. From these data, the number of oxygenation-linked effector molecules bound to each Hb and their response to temperature was estimated. I found

that the number of discrete Cl^- and DPG binding sites on the Hb moiety is unrelated to its thermal sensitivity. This suggests that individual heterotropic ligand binding sites have differing enthalpy changes, in which their effect on Hb–O_2 affinity and thermal sensitivity vary independently of one another. These differences presumably arise from amino acid replacements in close vicinity to ligand binding sites that alter enthalpy change of effector binding. Moreover, I suggest such epistatic interactions are likely also responsible for reduced thermal sensitivity of other mammalian Hbs, as those of bovine ungulates, horses, and bears bind as many or fewer heterotropic ligands than human HbA, but do so with a greater enthalpy change (De Rosa et al., 2004). Thus, the work presented in this chapter appears to have identified an overlooked mechanism of Hb thermal adaptation.

Much of the early work on 'cold adapted' Hbs employed ungulates as a model system. While Arctic members of this group, such as reindeer (*Rangifer tarandus*) and musk ox (*Ovibos moschatus*), possess 'cold adapted' Hbs (Coletta et al., 1992), this phenotype is also present in beef cattle (*Bos taurus;* Weber et al., 2014), which casts uncertainty on the evolutionary origins of this phenotype (Campbell et al., 2010a). However, recent studies, up to and including the work presented herein, have identified multiple evolutionary origins for this phenotype in other Arctic mammals (Campbell et al., 2010a), small mammals (Campbell et al., 2010b; Campbell et al., 2012; Jensen et al., 2016; Signore et al., 2012), and heterothermic fishes (Ikeda-Saito et al., 1983; Weber et al., 2010). Thus, the repeated evolution of this phenotype suggests it is a key component of temperature adaptation and the exploitation of cold habitats. While each of the studies listed above suggest additional heterotropic ligand binding underlies this phenotype, this thesis highlights the role of epistasis in the thermal sensitivity of sirenian Hb and those of other mammals. However, the precise molecular mechanisms that underlie these epistatic effects

remain unknown. Future work should focus on mapping Hb thermodynamics across the mammalian geophylogeny, which will lend clarity to the evolutionary origins and molecular underpinnings of this phenotype. In the future, this work may then serve as the foundation for artificial blood substitutes or genetically modified erythrocytes tailored for applications involving therapeutic hypothermia (So, 2010).

6.3. Functional Evolution of Prenatal Sirenian Hbs

The β/δ82Lys→Asn substitution that abolishes DPG binding in Steller's sea cow Hb was expected to have consequences that reach beyond O_2 delivery in adult individuals, as DPG plays a large role in maternal-fetal O_2 transport of most mammalian species (Weber, 1994). In order to facilitate O_2 transfer to the developing circulation, the O_2 affinity of fetal blood is typically increased relative to that of the mother (felids being the only known exception). In most mammals, fetal blood contains the same Hb component as the mother, but O_2 affinity is increased via reductions to intracellular DPG concentrations in fetal erythrocytes (Caire and Haydari, 1990; Dhindsa et al., 1972; Jelkmann and Bauer, 1978; Tweeddale, 1973; Qvist et al., 1981). However, such a mechanism would not have been viable in Steller's sea cow. Rather, anecdotal evidence alluded to the presence of a specific fetal-type Hb isoform in the circulation of newborn manatees (White et al, 1976) and <10 month old elephant fetuses (Riegel et al., 1967), which to date is only known to be present in simian primates and bovine ungulates (Clementi et al., 1996; Tomita, 1981). The role of isoHbs in sirenians development was explored further in Chapter 5, in which the functional properties of prenatal sirenian Hb isoforms (Gower I and HbF from Steller's sea cow and HbF from the dugong) were measured. I found that the three prenatal expressed Hbs tested in this study were functionally similar (in terms of their O_2

affinity, cooperativity, and Bohr coefficients) to Hbs expressed in the earliest (embryonic) stages of development in other mammals. This suggests that extant sirenians facilitate maternal-fetal O_2 transfer by reductions to intracellular DPG concentrations, as is common to most other mammals. However, in the absence of a discrete fetal Hb, Steller's sea cow may have been one of the rare examples in which fetal and maternal blood share identical O_2 affinity. This may have been compensated for by the low overall affinity of Steller's sea cow HbA (compared to its relatives), which offloads O_2 at a higher PO_2, thus, maintaining a higher PO_2 in the fetal circulation, relative to other sirenians.

Despite the importance of maternal–fetal O_2 transfer for eutherian mammals and other viviparous species, the risk of compromising a pregnancy has generally restricted studies on this subject to model organisms and livestock. While the idea of using recombinant Hb production to study maternal–fetal O_2 transfer is not a novel concept, this is the first such study to incorporate aDNA in order to examine the prenatal physiology of an extinct mammal. Future studies should expand on these nascent developments in hope of unravelling the role of discrete Hb proteins in the evolution of mammalian viviparity and radiation of the group.

LITERATURE CITED

Antonini, E. and Brunori, M. (1971). Hemoglobin and myoglobin in their interactions with ligands. Amsterdam, Netherlands. Elsevier Science Publishing Company.

Amiconi, G. and Giardina, B. (1981). Measurement of binding of nonheme ligands to hemoglobins. *Meth. Enzymol.* **76**, 533–552.

Amiconi, G., Bertollini, A., Bellelli, A., Coletta, M., Condo, S. G. and Brunori, M. (1985). Evidence for two oxygen-linked binding sites for polyanions in dromedary hemoglobin. *Eur. J. Biochem.* **150**, 387–393.

Antonini, E., Wyman, J., Brunori, M., Fronticelli, C., Bucci, E. and Rossi-Fanelli, A. (1965). Studies on the relations between molecular and functional properties of hemoglobin V. The influence of temperature on the Bohr effect in human and in horse hemoglobin. *J. Biol. Chem.* **240**, 1096–1103.

Atha, D. H. and Ackers, G. K. (1974). Calorimetric determination of the heat of oxygenation of human hemoglobin as a function of pH and the extent of reaction. *Biochemistry* **13**, 2376–2382.

Baudin, V., Pagnier, J., Kiger, L. and Kister, J. (1993). Functional consequences of mutations at the allosteric interface in hetero-and homo-hemoglobin tetramers. *Prot. Sci.* **2**, 1320–1330.

Bauer, C., Ludwig, M., Ludwig, I. and Bartels, H. (1969). Factors governing the oxygen affinity of human adult and foetal blood. *Respir. Physiol.* **7**, 271–277.

Benesch, R. and Benesch, R. E. (1967). The effect of organic phosphates from the human erythrocyte on the allosteric properties of hemoglobin. *Biochem. Biophys. Res. Com.* **26**, 162–167.

Berenbrink, M. (2006). Evolution of vertebrate haemoglobins: Histidine side chains, specific buffer value and Bohr effect. *Resp. Physiol, Neurobiol,* **154**, 165–184.

Bhattacharya, S. and Lecomte, J. T. (1997). Temperature dependence of histidine ionization constants in myoglobin. *Biophys. J.* **73**, 3241–3256.

Blessing, M. H. (1972). Studies on the concentration of myoglobin in the sea-cow and porpoise. *Comp. Biochem. Physiol. A.* **41**, 475–480.

Blunt, M. H., Kitchens, J. L., Mayson, S. M. and Huisman, T. H. (1971). Red cell 2,3-diphosphoglycerate and oxygen affinity in newborn goats and sheep. *Proc. Soc. Exp. Biol. Med.* **138**, 800–803.

Bohr, C., Hasselbalch, K. and Krogh, A. (1904). Ueber einen in biologischer Beziehung wichtigen Einfluss, den die Kohlensäurespannung des Blutes auf dessen Sauerstoffbindung übt1. *Skandinavisches. Archiv. für. Physiologie.* **16**, 402-412.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina Sequence Data. *Bioinformatics* btu170.

Bonaventura, J., Bonaventura, C., Sullivan, B., Ferruzzi, G., McCurdy, P. R., Fox, J. and Moo-Penn, W. F. (1976). Hemoglobin providence. Functional consequences of two alterations of the 2,3-diphosphoglycerate binding site at position beta 82. *J. Biol. Chem.* **251**,

7563–7571.

Brittain, T. (1987). A possible role for protein dissociation in the functioning of embryonic haemoglobins. *J. Exp. Biol.* **127**, 443–447.

Brittain, T. (2002). Molecular aspects of embryonic hemoglobin function. *Mol. Aspects. Med.* **23**, 293–342.

Brix, O., Bårdgard, A., Mathisen, S., Tyler, N., Nuutinen, M., Condo, S. G. and Giardina, B. (1990). Oxygen transport in the blood of arctic mammals: adaptation to local heterothermia. *J. Comp. Physiol. B.* **159**, 655–660.

Bunn, H. F. (1980). Regulation of Hemoglobin Function in Mammals. *Amer. Zool.* **20**, 199–211.

Bunn, H. F., Seal, U. S. and Scott, A. F. (1974). The role of 2,3-diphosphoglycerate in mediating hemoglobin function of mammalian red cells. *Ann. N. Y. Acad. Sci.* **241**, 498–512.

Burmester, T. and Hankeln, T. (2014). Function and evolution of vertebrate globins. *Acta. Physiol.* **211**, 501–514.

Burmester, T., Haberkamp, M., Mitz, S., Roesner, A., Schmidt, M., Ebner, B., Gerlach, F., Fuchs, C. and Hankeln, T. (2004). Neuroglobin and Cytoglobin: Genes, Proteins and Evolution. *TBMB* **56**, 703–707.

Caire, W. and Haydari, K. (1990). Absence of electrophoretically distinct fetal hemoglobins in the bats Tadarida brasiliensis and Lasiurus borealis. *J. mammal*. **71**, 695-697.

Campbell, K. L. and Hofreiter, M. (2015). Resurrecting phenotypes from ancient DNA sequences: promises and perspectives. *Can. J. Zool.* **93**, 701-710.

Campbell, K. L., Roberts, J. E. E., Watson, L. N., Stetefeld, J., Sloan, A. M., Signore, A. V., Howatt, J. W., Tame, J. R. H., Rohland, N., Shen, T.-J., et al. (2010a). Substitutions in woolly mammoth hemoglobin confer biochemical properties adaptive for cold tolerance. *Nat. Genet.* **42**, 536–540.

Campbell, K. L., Signore, A. V., Harada, M. and Weber, R. E. (2012). Molecular and physicochemical characterization of hemoglobin from the high-altitude Taiwanese brown-toothed shrew (Episoriculus fumidus). *J Comp Physiol B* **182**, 821–829.

Campbell, K. L., Storz, J. F., Signore, A. V., Moriyama, H., Catania, K. C., Payson, A. P., Bonaventura, J., Stetefeld, J. and Weber, R. E. (2010b). Molecular basis of a novel adaptation to hypoxic-hypercapnia in a strictly fossorial mole. *BMC Evol. Biol.* **10**, 214.

Charache, S., Jacobson, R., Brimhall, B., Murphy, E. A., Hathaway, P., Winslow, R., Jones, R., Rath, C. and Simkovich, J. (1978). Hb Potomac (101 Glu replaced by Asp): speculations on placental oxygen transport in carriers of high-affinity hemoglobins. *Blood* **51**, 331–338.

Cheviron, Z. A., Natarajan, C., Projecto-Garcia, J., Eddy, D. K., Jones, J., Carling, M. D., Witt, C. C., Moriyama, H., Weber, R. E., Fago, A., et al. (2014). Integrating Evolutionary and Functional Tests of Adaptive Hypotheses: A Case Study of Altitudinal Differentiation in Hemoglobin Function in an Andean Sparrow, Zonotrichia capensis. *Mol. Biol. Evol.* **31**, 2948–2962.

Clementi, M. E., Scatena, R. and Mordente, A. (1996). Oxygen transport by fetal bovine hemoglobin. *J. Mol. Biol.* **255**, 229-234.

Coletta, M., Clementi, M. E., Ascenzi, P., Petruzzelli, R., Condo, S. G. and Giardina, B. (1992). A comparative study of the temperature dependence of the oxygen-binding properties of mammalian hemoglobins. *Eur. J. Biochem.* **204**, 1155–1157.

Colombo, M. F. and Rau, D. C. (1994). Reevaluation of chloride's regulation of hemoglobin oxygen uptake: the neglected contribution of protein hydration in allosterism. *Proc. Nat. Acad. Sci.* **91**, 10519-10520.

Crerar, L. D., Crerar, A. P., Domning, D. P. and Parsons, E. C. M. (2014). Rewriting the history of an extinction--was a population of Steller's sea cows (Hydrodamalis gigas) at St Lawrence Island also driven to extinction? *Biol. Let.* **10**, 20140878–20140878.

De Rosa, M. C., Castagnola, M., Bertonati, C., Galtieri, A. and Giardina, B. (2004). From the Arctic to fetal life: physiological importance and structural basis of an "additional" chloride-binding site in haemoglobin. *Biochem. J.* **380**, 889–896.

Dhindsa, D.S., Sedgwick, C.J. and Metcalfe, J. (1972). Comparative studies of the respiratory functions of mammalian blood. VIII. Asian elephant (*Elephas maximus*) and African elephant (*Loxodonta africana africana*). *Resp. Physiol.* **14**, 332–342.

Dickerson, R. E. amd Geis, I. (1983). Hemoglobin: structure, function, evolution, and pathology. San Francisco, USA. Benjamin-Cummings Publishing Company.

Domning, D. P. (2001). The earliest known fully quadrupedal sirenian. *Nature*. **413**, 625–627.

Domning, D. P. (1976). An Ecological Model for Late Tertiary Sirenian Evolution in the North Pacific Ocean. *Syst. Biol.* **25**, 352–362.

Domning, D. P. (2000). The readaptation of Eocene sirenians to life in water. *Hist. Biol.* **14**, 115–119.

Domning, D. P. and Gingerich, P. D. (1994). *Protosiren smithae, new species (Mammalia, Sirenia), from the late middle Eocene of Wadi Hitan, Egypt No. 56 UNI*. Museum of Paleontology, University of Michigan.

Edgar, R,C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput." *Nuc. Acid. Res.* **32**, 1792–1797.

Efstratiadis, A., Posakony, J. W., Maniatis, T. and Lawn, R. M. (1980). The structure and evolution of the human β-globin gene family. *Cell* **21**, 653–668.

Fago A., Wells R.M., Weber R.E. (1997). Temperature-dependent enthalpy of oxygenation in Antarctic fish hemoglobins. *Comp. Biochem. Physiol. B.* **118**, 319–26.

Fairall, N. (1980) Growth and age determination in the hyrax Procavia capensis, *S. Afri. J. Zool.*, **15**, 16–21.

Fantoni, A., Farace, M. G. and Gambari, R. (1981). Embryonic hemoglobins in man and other mammals. *Blood* **57**, 623–633.

Farmer, M., Weber, R. E., Bonaventura, J., Best, R. C. and Domning, D. (1973). Functional properties of hemoglobin and whole blood in an aquatic mammal, the Amazonian manatee (Trichechus inunguis). *Comp. Biochem. Physiol. A.* **62**, 231–238.

Forget, B.G. (2001). Molecular mechanisms of beta thalassemia. In: Steinberg M, Forget B, Higgs D, Nagel R, editors. Disorders of hemoglobin: genetics, pathophysiology, and clinical management. Cambridge, UK. Cambridge University Press, 252–276.

Fronticelli, C., Pechik, I., Brinigar, W. S., Kowalczyk, J. and Gilliland, G. L. (1994).

Chloride ion independence of the Bohr effect in a mutant human hemoglobin beta (V1M+H2deleted). *J. Biol. Chem.* **269**, 23965–23969.

Fronticelli, C., Sanna, M. T., Perez-Alvarado, G. C., Karavitis, M., Lu, A. L. and Brinigar, W. S. (1995). Allosteric modulation by tertiary structure in mammalian hemoglobins. Introduction of the functional characteristics of bovine hemoglobin into human hemoglobin by five amino acid substitutions. *J. Biol. Chem.* **270**, 30588–30592.

Gallivan, G. J., Best, R. C. and Kanwisher, J. W. (1983). Temperature regulation in the Amazonian manatee Trichechus inunguis. *Physiol. Zool*. **1983**, 255-262.

Gallivan, G. J., Kanwisher, J. W. and Best, R. C. (1986). Heart rates and gas exchange in the Amazonian manatee (Trichechus inunguis) in relation to diving. *J. Comp. Physiol. B.* **156**, 415–423.

Garner, M. H., Bogardt, R. A. and Gurd, F. R. (1975). Determination of the pK values for the alpha-amino groups of human hemoglobin. *J. Biol. Chem*. **250**, 4398-4404.

Gatesy, J., Geisler, J. H., Chang, J., Buell, C., Berta, A., Meredith, R. W., Springer, M. S. and McGowen, M. R. (2012). A phylogenetic blueprint for a modern whale. *Mol. Phylogenet. Evol.* **66**, 479-506.

Gaudry, M. J., Storz, J. F., Butts, G. T., Campbell, K. L. and Hoffmann, F. G. (2014). Repeated evolution of chimeric fusion genes in the β-globin gene family of laurasiatherian mammals. *Gen. Biol. Evol.* **6**, 1219–1234.

Hardison, R. (1999). The Evolution of Hemoglobin Studies of a very ancient protein suggest

that changes in gene regulation are an important part of the evolutionary story. *Am. Scient.* **87**, 126.

Hardison, R. (2012). Evolution of hemoglobin and its genes. *Cold Spring. Harb. Perspect. Med.* **2**, a011627-a011627.

Hashimoto, M., Ishimori, K., Imai, K., Miyazaki, G., Morimoto, H., Wada, Y. and Morishima, I. (1993). Site-directed mutagenesis in hemoglobin: functional and structural study of the intersubunit hydrogen bond of threonine-38(C3)alpha at the alpha 1-beta 2 interface in human hemoglobin. *Biochemistry* **32**, 13688–13695.

He, Z. and Russell, J. E. (2001). Expression, purification, and characterization of human hemoglobins Gower-1 (zeta(2)epsilon(2)), Gower-2 (alpha(2)epsilon(2)), and Portland-2 (zeta(2)beta(2)) assembled in complex transgenic-knockout mice. *Blood* **97**, 1099–1105.

Hecht, F., Motulsky, A. G., Lemire, R. J. and Shepard, T. E. (1966). Predominance of hemoglobin Gower 1 in early human embryonic development. *Science* **152**, 91-92.

Higuchi, R., Bowman, B., Freiberger, M., Ryder, O. A. and Wilson, A. C. (1984). DNA sequences from the quagga, an extinct member of the horse family. *Nature* **312**, 282-284.

Hill, A. V. (1910). The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. *J. Physiol.* **40**, 4-7.

Hill, A. V. (1913). The Combinations of Haemoglobin with Oxygen and with Carbon Monoxide. *I. Biochem. J.* **7**, 471–480.

Hodges, E., Rooks, M., Xuan, Z., Bhattacharjee, A., Gordon, D. B., Brizuela, L., et al. (2009). Hybrid selection of discrete genomic intervals on custom-designed microarrays for massively parallel sequencing. *Nature protocols* **4**, 960–974.

Hoffmann, F. G., Opazo, J. C. and Storz, J. F. (2008). Rapid rates of lineage-specific gene duplication and deletion in the alpha-globin gene family. *Mol. Biol. Evol.* **25**, 591–602.

Huggett, A.S. (1927). Foetal blood-gas tensions and gas transfusion through the placenta of the goat. *J. Physiol.* **62**, 373–384.

Ikkala, E., Koskela, J., Pikkarainen, P., Rahiala, E.L., El-Hazmi, M.A., Nagai, K., Lang, A. and Lehmann, H. (1976). Hb Helsinki: A variant with a high oxygen affinity and a substitution at a 2, 3-DPG binding site (β82 [EF6] Lys→ Met). *Acta. haematol*. **56**, 257–275.

Ikeda-Saito, M., Yonetani, T. and Gibson, Q. H. (1983). Oxygen equilibrium studies on hemoglobin from the bluefin tuna (Thunnus thynnus). *J. Mol. Biol.* **168**, 673–686.

Imai, K. (1982). Allosteric effects in haemoglobin. Cambridge, UK. Cambridge University Press.

Imaizumi, K., Imai, K. and Tyuma, I. (1979). The linkage between the four-step binding of oxygen and the binding of heterotropic anionic ligands in hemoglobin. *J. Biochem.* **86**, 1829–1840.

Ingermann, R. L. (1997). Vertebrate hemoglobins. *Compr. Physiol*. **1997**, 357–408.

Irving, L. and Krog, J. (1955). Skin temperature as a heat regulator. *J. Ap.*

*Physio*l. **7,** 355–364

Jelkmann, W. and Bauer, C. (1978). Embryonic hemoglobins: dependency of functional characteristics on tetramer composition. *Pflugers. Arch.* **377**, 75–80.

Jensen, B., Storz, J. F. and Fago, A. (2016). Bohr effect and temperature sensitivity of hemoglobins from highland and lowland deer mice. *Comp. Biochem. Physiol. A.* **195**, 10–14.

Johnson, R. M., Buck, S., Chiu, C. H., Gage, D. A., Shen, T. L., Hendrickx, A. G., Gumucio, D. L. and Goodman, M. (2000). Humans and old world monkeys have similar patterns of fetal globin expression. *J. Exp. Zool.* **288**, 318–326.

Johnson, R. M., Buck, S., Chiu, C., Schneider, H., Sampaio, I., Gage, D. A., Shen, T. L., Schneider, M. P., Muniz, J. A., Gumucio, D. L., et al. (1996). Fetal globin expression in New World monkeys. *J. Biol. Chem.* **271**, 14684–14691.

Johnson, R. M., Gumucio, D. and Goodman, M. (2002). Globin gene switching in primates. *Comparative Biochem. Physiol. A.* **133**, 877–883.

Kendrew, J. C., Bodo, G., Dintzis, H. M., Parrish, R. G., Wyckoff, H. and Phillips, D. C. (1958). A three-dimensional model of the myoglobin molecule obtained by x-ray analysis. *Nature* **181**, 662–666.

Lahiri, S. (1975). Blood oxygen affinity and alveolar ventilation in relation in body weight in mammals. *Am. J. Physiol.* **229**, 529–536.

Lilly, L. E., Blinebry, S. K., Viscardi, C. M., Perez, L., Bonaventura, J. and McMahon, T. J. (2013). Parallel assay of oxygen equilibria of hemoglobin. *Anal. Biochem.* **441**, 63–68.

Lukin, J. A. and Ho, C. (2004). The Structure−Function Relationship of Hemoglobin in Solution at Atomic Resolution. *Chem. Rev.* **104**, 1219–1230.

Mairbäurl, H. and Weber, R. E. (2012). Oxygen transport by hemoglobin. *Compr. Physiol.* **2**, 1463–1489.

Manning, L. R., Russell, J. E., Padovan, J. C., Chait, B. T., Popowicz, A., Manning, R. S. and Manning, J. M. (2007). Human embryonic, fetal, and adult hemoglobins have different subunit interface strengths. Correlation with lifespan in the red cell. *Protein Sci.* **16**, 1641–1658.

Marsh, H., O'Shea, T.J. and Reynolds, J.E. (2012). Ecology and conservation of the Sirenia. Cambridge, UK. Cambridge University Press.

McCabe, M., Hamilton, R. and Marsh, H. (1978). Some studies on the oxygen affinity of haemoglobin from the dugong. *Comp. Biochem. Physiol. A.* **61**, 19-22.

McGowen, M. R., Gatesy, J. and Wildman, D. E. (2014). Molecular evolution tracks macroevolutionary transitions in Cetacea. *Trend. Ecol. Evol.* **29**, 336–346.

Meir, J. U. and Ponganis, P. J. (2009). High-affinity hemoglobin and blood oxygen saturation in diving emperor penguins. *J. Exp. Biol.* **212**, 3330–3338.

Meredith, R. W., Janecka, J. E., Gatesy, J., Ryder, O. A., Fisher, C. A., Teeling, E. C., Goodbla, A., Eizirik, E., Simao, T. L. L., Stadler, T., et al. (2011). Impacts of the Cretaceous Terrestrial Revolution and KPg Extinction on Mammal Diversification. *Science* **334**, 521–524.

Metzenberg, A. B., Wurzer, G., Huisman, T. H. and Smithies, O. (1991). Homology requirements for unequal crossing over in humans. *Genetics* **128**, 143–161.

Meyer, M., & Kircher, M. (2010). Illumina sequencing library preparation for highly multiplexed target capture and sequencing. *Cold. Spring. Harb. Protoc.* **2010**, t5448.

Mirceta, S., Signore, A. V., Burns, J. M., Cossins, A. R., Campbell, K. L. and Berenbrink, M. (2013). Evolution of mammalian diving capacity traced by myoglobin net surface charge. *Science* **340**, 1234192.

Moo-Penn, W. F., Jue, D. L., Bechtel, K. C., Johnson, M. H. and Schmidt, R. M. (1976). Hemoglobin Providence. A human hemoglobin variant occurring in two forms in vivo. *J. Biol. Chem.* **251**, 7557–7562.

Moraga, F., Monge, C., Riquelme, R. and Llanos, A. J. (1996). Fetal and maternal blood oxygen affinity: a comparative study in llamas and sheep. *Comp. Biochem. Physiol. A*. **115**, 111–115.

Nagylaki, T. (1984). The evolution of multigene families under intrachromosomal gene conversion. *Genetics* **106**, 529–548.

Natarajan, C., Hoffmann, F. G., Lanier, H. C., Wolf, C. J., Cheviron, Z. A., Spangler, M. L., Weber, R. E., Fago, A. and Storz, J. F. (2015). Intraspecific Polymorphism, Interspecific Divergence, and the Origins of Function-Altering Mutations in Deer Mouse Hemoglobin. *Mol. Biol. Evol.* **32**, 978–997.

Natarajan, C., Jiang, X., Fago, A., Weber, R. E., Moriyama, H. and Storz, J. F. (2011).

Expression and Purification of Recombinant Hemoglobin in Escherichia coli. *PLoS ONE* **6**, e20176.

Nery, M. F., Arroyo, J. I. and Opazo, J. C. (2013). Accelerated Evolutionary Rate of the Myoglobin Gene in Long-Diving Whales. *J. Mol. Evol.* **76**, 380–387.

Nigen, A. M. and Manning, J. M. (1975). The interaction of anions with hemoglobin carbamylated on specific NH2-terminal residues. *J. Biol. Chem.* **250**, 8248-8250.

Nigen, A. M., Manning, J. M. and Alben, J. O. (1980). Oxygen-linked binding sites for inorganic anions to hemoglobin. *J. Biol. Chem.* **255**, 5525–5529.

Noguchi, H., Campbell, K. L., Ho, C., Unzai, S., Park, S.-Y. and Tame, J. R. H. (2012). Structures of haemoglobin from woolly mammoth in liganded and unliganded states. *Acta. Crystallogr. D. Biol. Crystallogr.* **68**, 1441–1449.

Novy, M. J. and Parer, J. T. (1969). Absence of high blood oxygen affinity in the fetal cat. *Respir. Physiol.* **6**, 144–150.

O'Donnell, S., Mandaro, R. and Schuster, T. M. (1979). X-ray diffraction and solution studies of specifically carbamylated human hemoglobin A. Evidence for the location of a proton-and oxygen-linked chloride binding site at valine 1 alpha. *J. Biol. Chem.* **254**, 12204-12208.

Ohta, T. (1980). Evolution and variation of multigene families. Springer Science and Business Media.

Opazo, J. C., Hoffmann, F. G. and Storz, J. F. (2008). Differential loss of embryonic globin genes during the radiation of placental mammals. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 12950–

12955.

Opazo, J. C., Sloan, A. M., Campbell, K. L. and Storz, J. F. (2009). Origin and ascendancy of a chimeric fusion gene: the beta/delta-globin gene of paenungulate mammals. *Mol. Biol. Evol.* **26**, 1469–1478.

Orlando, L., Ginolhac, A., Zhang, G., Froese, D., Albrechtsen, A., Stiller, M., Schubert, M., Cappellini, E., Petersen, B., Moltke, I., et al. (2013). Recalibrating Equus evolution using the genome sequence of an early Middle Pleistocene horse. *Nature* **498**, 74–78.

Pääbo, S., Poinar, H., Serre, D., Jaenicke-Després, V., Hebler, J., Rohland, N., et al. (2004). Genetic analyses from ancient DNA. *Annu. Rev. Genet.*, **38**, 645–679.

Pataryas, H. A. and Stamatoyannopoulos, G. (1972). Hemoglobins in human fetuses: evidence for adult hemoglobin production after the 11th gestational week. *Blood* **39**, 688-696.

Perez-Stable, C. and Costantini, F. (1990). Roles of fetal G gamma-globin promoter elements and the adult beta-globin 3'enhancer in the stage-specific expression of globin genes. *Mol. Cell. Biol*. **10**, 1116–1125.

Perutz, M. F. (1970). Stereochemistry of cooperative effects in haemoglobin. *Nature* **228**, 726–739.

Perutz, M. F. (1983). Species adaptation in a protein molecule. *Mol. Biol. Evol.* **1**, 1–28.

Perutz, M. F., Fermi, G., Poyart, C., Pagnier, J. and Kister, J. (1993). A novel allosteric mechanism in haemoglobin. Structure of bovine deoxyhaemoglobin, absence of specific

chloride-binding sites and origin of the chloride-linked Bohr effect in bovine and human haemoglobin. *J. Mol. Biol.* **233**, 536–545.

Perutz, M. F., Fogg, J. H. and Fox, J. A. (1980). Mechanism of deamidation of haemoglobin providence Asn. *J. Mol. Biol.* **138**, 669–670.

Perutz, M. F., Shih, D. T. and Williamson, D. (1994). The chloride effect in human haemoglobin. A new kind of allosteric mechanism. *J. Mol. Biol.* **239**, 555–560.

Peterson, K. R. and Stamatoyannopoulos, G. (1993). Role of gene order in developmental control of human gamma-and beta-globin gene expression. *Mol. Cell. Biol.* **13**, 4836–4843.

Ponganis, P. J. (2011). Diving mammals. *Compr Physiol* **1**, 447–465.

Posada, D., Crandall, K.A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14,** 817–818.

Posthumus, K. (1933). The application of the van't Hoff-le Chatelier-Braun principle to chemical equilibria. *Recueil des Travaux Chimiques des Pays-Bas*. **52(1)**, 25–35.

Prinz, H. (2010). Hill coefficients, dose–response curves and allosteric mechanisms. *J. Chem. Biol. **3***, 37–44.

Purdie, A., Wells, R. and Brittain, T. (1983). Molecular aspects of embryonic mouse haemoglobin ontogeny. *Biochem. J*. **215**, 377–383.

Qvist, J., Weber, R. E. and Zapol, W. M. (1981). Oxygen equilibrium properties of blood and hemoglobin of fetal and adult Weddell seals. *J. Appl. Physiol. Respir. Environ. Exerc. Physiol.* **50**, 999–1005.

Rapoport, S. and Luebering, J. (1950). The formation of 2, 3-diphosphoglycerate in rabbit erythrocytes: the existence of a diphosphoglycerate mutase. *J. biol. Chem*. **183**, 507-516.

Raup, D. M. (1992). Extinction: bad genes or bad luck?. WW Norton & Company.

Richard, V., Dodson, G. G. and Mauguen, Y. (1993). Human deoxyhaemoglobin-2, 3-diphosphoglycerate complex low-salt structure at 2· 5 Å resolution. *J. Mol. Biol.* **233**, 270–274.

Richardson, R. S. and Newcomer, S. C. (2001). Skeletal muscle intracellular PO_2 assessed by myoglobin desaturation: response to graded exercise. *J. App. Physiol.* **91**, 2679-2685.

Riegel, K., Bartels, H., Buss, I. O. and Wright, P. G. (1967). Comparative studies of the respiratory functions of mammalian blood IV. Fetal and adult african elephant blood. *Resp. Physiol.* **2**, 182-195.

Riggs, A. (1971). Mechanism of the enhancement of the Bohr effect in mammalian hemoglobins by diphosphoglycerate. *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2062–2065.

Ropero, P., Fernández-Lago, C., Villegas, A., Polo, M., Mateo, M., Mora, A. and González, F. A. (2006). Hb LA Coruña [β38(C4)Thr→Ile]: A New Hemoglobin Variant Leading to Familial Polycythemia. *LHEM* **30**, 379–383.

Rossi-Fanelli, A. and Antonini, E. (1958). Studies on the oxygen and carbon monoxide equilibria of human myoglobin. *Arch. Biochem. Biophys.* **77**, 478–492.

Samuel, P. P., Smith, L. P., Phillips, G. N., Jr. and Olson, J. S. (2015). Apoglobin Stability Is the Major Factor Governing both Cell-free and in Vivo Expression of Holomyoglobin. *J.*

Biol. Chem. **290**, 23479–23495.

Schimenti, J. C. and Duncan, C. H. (1985). Structure and organization of the bovine beta-globin genes. *Mol. Biol. Evol.* **2**, 514–525.

Scholander, P. F. and Irving, L. (1941). Experimental investigations on the respiration and diving of the florida manatee. *J. Cell. Comp. Physiol.* **17**, 169–191.

Shih, D. T., Jones, R. T., Imai, K. and Tyuma, I. (1985). Involvement of Glu G3(101)beta in the function of hemoglobin. Comparative O2 equilibrium studies of human mutant hemoglobins. *J. Biol. Chem.* **260**, 5919–5924.

Signore, A. V., Stetefeld, J., Weber, R. E. and Campbell, K. L. (2012). Origin and mechanism of thermal insensitivity in mole hemoglobins: a test of the "additional" chloride binding site hypothesis. *J. Exp. Biol.* **215**, 518–525.

Sloan, A.M. (2005). Atypical molecular evolution of afrotherian and xenarthran b-globin cluster genes with insights into the b-globin cluter gene organization of stem eutherians. MSc. Thesis, University of Manitoba.

Snyder, G. K. (1983). Respiratory adaptations in diving mammals. *Respir. Physiol.* **54**, 269–294.

So, H.Y. (2010). Therapeutic hypothermia. *Korean J. Anesthes.* ***59*(5)**, 299–304.

Springer, M. S., Signore, A. V., Paijmans, J. L. A., Velez-Juarbe, J., Domning, D. P., Bauer, C. E., He, K., Crerar, L., Campos, P. F., Murphy, W. J., et al. (2015). Interordinal gene capture, the phylogenetic position of Steller's sea cow based on molecular and morphological

data, and the macroevolutionary history of Sirenia. *Mol. Phylogenet. Evol.* **91**, 178–193.

Stejneger, L. (1887). How the great northern sea-cow (Rytina) became exterminated. *Am Nat.* **21**, 1047–1054.

Steller, G.W. (1751). De Bestiis Marinis [On Beasts of the Sea]. *Novi. Commentarii. Aca. Sci. Imp. Petropoli.* **2**, 289–398.

Storz, J. F. (2016). Causes of molecular convergence and parallelism in protein evolution. *Nat. Rev. Genet.* **17**, 239–250.

Storz, J. F. and Moriyama, H. (2008). Mechanisms of Hemoglobin Adaptation to High Altitude Hypoxia. *High Alt. Med. Biol.* **9**, 148–157.

Storz, J. F., Runck, A. M., Sabatino, S. J., Kelly, J. K., Ferrand, N., Moriyama, H., Weber, R. E. and Fago, A. (2009). Evolutionary and functional insights into the mechanism underlying high-altitude adaptation of deer mouse hemoglobin. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 14450–14455.

Sugihara, J., Imamura, T., Nagafuchi, S., Bonaventura, J., Bonaventura, C. and Cashon, R. (1985). Hemoglobin Rahere, a human hemoglobin variant with amino acid substitution at the 2,3-diphosphoglycerate binding site. Functional consequences of the alteration and effects of bezafibrate on the oxygen bindings. *J. Clin. Invest.* **76**, 1169–1173.

Takenaka, O. and Morimoto, H. (1976). Oxygen equilibrium characteristics of adult and fetal hemoglobin of japanese monkey Macaca fuscata. *Biochim. Biophys. Acta (BBA)-Prot. Struct.* **446**, 457-462.

Tomita, S. (1981). Modulation of the oxygen equilibria of human fetal and adult hemoglobins by 2,3-diphosphoglyceric acid. *J. Biol. Chem.* **256**, 9495–9500.

Tufts, D. M., Natarajan, C., Revsbech, I. G., Projecto-Garcia, J., Hoffmann, F. G., Weber, R. E., Fago, A., Moriyama, H. and Storz, J. F. (2015). Epistasis Constrains Mutational Pathways of Hemoglobin Adaptation in High-Altitude Pikas. *Mol. Biol. Evol.* **32**, 287–298.

Tweeddale, P. M. (1973). DPG and the oxygen affinity of maternal and foetal pig blood and haemoglobins. *Respir. Physiol*. **19**, 12-18.

Uhen, M. D. (2007). Evolution of marine mammals: Back to the sea after 300 million years. *Anat. Rec.* **290**, 514–522.

van Beek, G. G., Zuiderweg, E. R. and de Bruin, S. H. (1979). The binding of chloride ions to ligated and unligated human hemoglobin and its influence on the Bohr effect. *Eur. J. Biochem.* **99**, 379–383.

Weber, R. E. (2007). High-altitude adaptations in vertebrate hemoglobins. *Resp. Phys. Neurobiol.* **158**, 132–142.

Weber, R. E. and Campbell, K. L. (2011). Temperature dependence of haemoglobin-oxygen affinity in heterothermic vertebrates: mechanisms and biological significance. *Acta Physiol.* **202**, 549–562.

Weber, R.E. (1992). Use of ionic and zwitterionic (Tris/BisTris and HEPES) buffers in studies on hemoglobin function. *J. Appl. Physiol*. **72**, 1611-1615.

Weber, R.E., Heath, M.E. and White, F.N. (1986). Oxygen binding functions of blood and hemoglobin from the Chinese pangolin, Manis pentadactyla: possible implications of burrowing and low body temperature. *Respir. Physiol.* **64,** 103–112.

Weber, R. E. and Fago, A. (2004). Functional adaptation and its molecular basis in vertebrate hemoglobins, neuroglobins and cytoglobins. *Resp. Physiol. Neurobiol.* **144**, 141–159.

Weber, R. E., Campbell, K. L., Fago, A., Malte, H. and Jensen, F. B. (2010). ATP-induced temperature independence of hemoglobin-O2 affinity in heterothermic billfish. *J. Exp. Biol.* **213**, 1579–1585.

Weber, R. E., Fago, A. and Campbell, K. L. (2014). Enthalpic partitioning of the reduced temperature sensitivity of O2 binding in bovine hemoglobin. *Comp. Biochem. Physiol. A. Mol. Int. Physiol.* **176**, 20–25.

Weber, R. E. (1994). Hemoglobin-based O2 transfer in viviparous animals. *Israel. J. Zool.* **40**, 541-550.

Weber, R. E., Kleinschmidt, T. and Braunitzer, G. (1987). Embryonic pig hemoglobins Gower I (ζ2ϵ2), Gower II (α2ϵ2), Heide I () and Heide II (): oxygen-binding functions related to structure and embryonic oxygen supply. *Respir. Physiol.* **69**, 347–357.

Weber, R. E., Lalthantluanga, R. and Braunitzer, G. (1988). Functional characterization of fetal and adult yak hemoglobins: An oxygen binding cascade and its molecular basis. *Arch. Biochem. Biophys.* **263**, 199–203.

Wertheim J.O., Murrell B., Smith M.D., Pond S.L., Scheffler K. (2014). RELAX: detecting

relaxed selection in a phylogenetic framework. *Mol. Biol. Evol*. **32**, 820–832.

Weiss, J. N. (1997). The Hill equation revisited: uses and misuses. *FASEB*. **11**, 835–841.

Wells, R. (1981). Transition to cooperative oxygen-binding by embryonic haemoglobin in mice. *J. Exp. Biol.***90**, 351–355.

White, J. R., Harkness, D. R., Isaacks, R. E. and Duffield, D. A. (1976). Some studies on blood of the Florida manatee, Trichechus manatus latirostris. *Comp. Biochem. Physiol. A. Comp. Physiol.* **55**, 413–417.

Wood, W. G. (1976). Haemoglobin synthesis during human fetal development. *Br. Med. Bull.* **32**, 282–287.

Wyman J., Gill S.J., Gaud H.T., Colosimo A., Giardina B.R., Kuiper H.A., Brunori M. (1978). Thermodynamics of ligand binding and allosteric transition in hemoglobins. Reaction of Hb trout IV with CO. *J. Mol. Biol.* **124**, 161–175.

Yang, Z. (2007). PAML 4: Phylogenetic Analysis by Maximum Likelihood. *Mol. Biol. Evol.* **24**, 1586–1591.

Yuan, Y., Byrd, C., Shen, T.-J., Simplaceanu, V., Tam, T. C. S. and Ho, C. (2013). Role of β/δ101Gln in Regulating the Effect of Temperature and Allosteric Effectors on Oxygen Affinity in Woolly Mammoth Hemoglobin. *Biochemistry* **52**, 8888–8897.

Yuan, Y., Shen, T.-J., Gupta, P., Ho, N. T., Simplaceanu, V., Tam, T. C. S., Hofreiter, M., Cooper, A., Campbell, K. L. and Ho, C. (2011). A biochemical--biophysical study of hemoglobins from woolly mammoth, Asian elephant, and humans. *Biochemistry* **50**, 7350–7360.

Zheng, T., Brittain, T., Watmough, N. J. and Weber, R. E. (1999). The role of amino acid

alpha38 in the control of oxygen binding to human adult and embryonic haemoglobin Portland. *Biochem. J.* **343**, 681–685.

APPENDICES

Appendix 1. GenBank and European Nucleotide Archive accession numbers for globin gene sequences used in this study. Loci marked "N/A" had no available sequence data.

Gene	*E. maximus*	*M. primigenius*	*L. africana*	*D. dugon*	*H. gigas*	*T. manatus*	*P. capensis*
HBB/HBD	gb|FJ716086	gb|FJ716094	gb| FJ716092			gb|DQ091203	gb|AC242819
HBA	gb|FJ716082	gb| FJ716093	gb|FJ716089			gb|AHIN01032287	gb|AC206382
HBE	SRA|ERR361995 SRA|ERR361996	ena|PRJEB7929	gb|AAGU03086368			gb|AHIN01136518	gb|AC242819
HBG	gb|DQ091216	ena|PRJEB7929	gb|DQ091215			gb|DQ091217	N/A
HBZ-T1	SRA|PRJNA281811	ena|PRJEB7929	gb|AAGU03068855			gb|AHIN01032286	gb|AC206382
Mb	gb|KC524737	gb|KC524742 SRA|SRR006703 SRA|SRR006706 SRA|SRR006684 SRA|SRR006668 SRA|SRR006720 SRA|SRR006731 SRA|SRR006711	gb|AAGU03077599	gb|KC524735	gb|KC524738	gb|XM_004373813	gb|ABRQ01421420 gb|ABRQ01816509 gb|ABRQ01421419
Ngb	SRA|ERR180975 SRA|ERR361995 SRA|ERR361996	ena|PRJEB7929	gb|AAGU03025367			gb|AHIN01016700	SRA|SRR1580937 SRA|SRR1580943 SRA|SRR1580948 SRA|SRR1580938 SRA|SRR1580940
Cygb	SRA|ERR180972 SRA|ERR361995 SRA|ERR361996	ena|PRJEB7929	SRA|SRR1041765			gb|AHIN01037489	ti|1202135783 ti|1244931539 ti|1292254337 ti|1202135783 ti|1263956455

Appendix 2. Alignment of paenungulate HBA protein coding sequences.

```
P.capensis          GTGCTGTCTGCTGCTGACAAGACCAACGTGAAGGGGGCCTGGGAAAAGGTTGGCAGCCAT
L.africana          GTGCTGTCTGATAACGACAAGACCAACGTCAAGGCCACCTGGAGCAAGGTTGGCGACCAC
E.maximus           GTGCTGTCTGATAAGGACAAGACCAACGTCAAGGCCACCTGGAGCAAGGTTGGCGACCAC
M.primigenius       GTGCTGTCTGATAACGACAAGACCAACGTCAAGGCCACCTGGAGCAAGGTTGGCGACCAC
T.manatus           GTGCTGTCTGATGAGGACAAGACCAACGTGAAGACCTTCTGGGGCAAGATTGGCACCCAC
D.dugon             GTGCTGTCTGCTGAGGACAAGACCAACGTGAAGACCTTCTGGGGCAAGCTGGGCGCCCAC
H.gigas             GTGCTGTCTGCTGAGGACAAGACCAACGTGAAGACCTTCTGGGGCAAGCTGGGCGCCCAC
                    ********** *   ************** ***     ****   *** * ***  ***

P.capensis          GCAGGAGAATATGGTGCTGAAGCCCTGGAGAGGATGTTCCTCTCCTTCCCCACTACCAAG
L.africana          GCTTCGGATTATGTCGCCGAGGCCCTGGAGAGGATGTTCTTCTCCTTTCCCACCACCAAG
E.maximus           GCTTCGGATTATGTCGCCGAGGCCCTGGAGAGGATGTTCTTCTCCTTTCCCACCACCAAG
M.primigenius       GCTTCGGATTATGTCGCCGAGGCCCTGGAGAGGATGTTCTTCTCCTTTCCCACCACCAAG
T.manatus           ACCGGAGAGTATGGCGGCGAGGCCCTGGAGAGGATGTTCCTCTCCTTCCCCACCACCAAG
D.dugon             ACTGCAGAGTATGGCAGCGAGGCCCTGGAGAGGATGTTCAACGCCTTCCCCGCCACCAAG
H.gigas             ACTGCAGAGTATGGCGGCGAGGCCCTGGAGAGGATGTTCCTCTCCTTCCCCACCACCAAG
                     *    ** ****     ** ******************  * **** *** * ******

P.capensis          ACCTACTTTCCTCACTTTGACCTGACGCACGGCTCCGCCCAGGTCAAAGGCCATGGCCAG
L.africana          ACCTACTTTCCTCACTTCGACCTGGGCCATGGCTCTGGCCAGGTCAAGGCACATGGCAAG
E.maximus           ACCTACTTTCCTCACTTCGACCTGAGCCATGGCTCTGGCCAGGTCAAGGGACATGGCAAG
M.primigenius       ACCTACTTTCCTCACTTCGACCTGAGCCATGGCTCTGGCCAGGTCAAGGGACATGGCAAG
T.manatus           ACCTACTTTCCTCACTTCGACCTGAGCCACGGCTCCGGCCAGATCAAGGCCCACGGCAAG
D.dugon             ACCTACTTTCCTCACTTCGACATGAGCCACGGCTCCGACCAGATCAAGGCCCACGGCAAG
H.gigas             ACCTACTTTCCTCACTTCGACATGAAGCACGACTCCGACCAGATCAAGGCCCACGGCAAG
                    ***************** *** **   ** * *** * **** **** *  ** *** **

P.capensis          AAGGTGGGGGCAGCACTGACCAAAGCTGTCGGCCACTTGGATGACCTGCCCAACGCCCTG
L.africana          AAGGTGGGGGAAGCACTGACCCAAGCTGTTGGCCACCTGGATGACCTGCCTAGCGCCCTG
E.maximus           AAGGTGGGGGAAGCACTGACCCAAGCTGTTGGCCACCTGGATGACCTGCCTAGCGCCCTG
M.primigenius       AAGGTGGGGGAAGCACTGACCCAAGCTGTTGGCCACCTGGATGACCTGCCTAGCGCCCTG
T.manatus           AAGGTGGCGGACGCGCTGACCCGCGCTGTCGGCCACCTGGAGGACCTGCCTGGCACTCTG
D.dugon             AAGGTGGCGGAGGCGCTGACCCGCGCTGTCGGCCACCTGGACGACCTCCCTGGCACCCTG
H.gigas             AAGGTGGCGGACGCGCTGACCCGCGCTGTCGGCCACCTGGACGACCTCCCTGGCACCCTG
                    ******* **  ** ******   ***** ****** **** ***** **   * * ***

P.capensis          TCTGACCTGAGTGACCTACATGCTCACAAGCTGAGGGTTGACCCCGTCAACTTCAAGCTC
L.africana          TCTGCACTCAGCGACCTGCACGCTCACAAGCTGAGGGTGGACCCTGTCAACTTCAAGCTC
E.maximus           TCTGCACTCAGCGACCTGCACGCTCACAAGCTGAGGGTGGACCCTGTCAACTTCAAGCTC
M.primigenius       TCTGCACTCAGCGACCTGCACGCTCACAAGCTGAGGGTGGACCCTGTCAACTTCAAGCTC
T.manatus           TCTGAGCTGAGCGACCTGCATGCTCACAGGTTGAGGGTGGACCCCGTCAACTTCAAGCTC
D.dugon             TCTGACCTGAGCGACCTGCATGCTCACAGATTGAGGGTGGACCCCATCAACTTCAAGCTC
H.gigas             TCTGAGCTGAGCGACCTGCATGCTCACAAACTGAGGGTGGACCCCATCAACTTCAAGCTC
                    ****  ** ** ***** ** *******   ******* *****  **************

P.capensis          CTGAGCCACTGCCTGCTGGTGACTCTAAGCCGCCATCTCCCGGAAGAGTTCACCCCTGCC
L.africana          CTGAGCCACTGCCTGCTGGTGACTCTGAGCAGCCACCARCCCACGGAGTTCACCCCTGAG
E.maximus           CTGAGCCACTGCCTGCTGGTGACTCTAAGCAGCCACCAACCCACGGAGTTCACCCCTGAG
M.primigenius       CTGAGCCACTGCCTGCTGGTGACTCTGAGCAGCCACCAACCCACGGAGTTCACCCCTGAG
T.manatus           CTGAGCCACTGCCTGCTGGTGACTCTGAGCAGCCACCTCCGTGAAGATTTCACCCCTTCC
D.dugon             CTGAGCCACTGCCTGCTGGTGACTCTGAGCAACCACCTCCCTGACGATTTCACCCCTCCC
H.gigas             CTGAGCCACTGCCTGCTGGTGACTCTGAGCGGCCACCTCCCTGAAGACTTCACCCCTCCC
                    ************************** ***  *** *  *     ** *********

P.capensis          GTCCACGCCTCTCTGGACAAGTTCTTCAGCAACGTGAGCACCGTGCTGACCTCCAAGTAT
L.africana          GTCCATGCCTCCCTGGACAAGTTCCTCAGCAACGTGAGCACCGTGCTGACCTCCAAATAT
E.maximus           GTCCATGCCTCCCTGGACAAGTTCCTTAGCAACGTGAGCACCGTGCTGACCTCCAAATAT
M.primigenius       GTCCATGCCTCCCTGGACAAGTTCCTCAGCAACGTGAGCACCGTGCTGACCTCCAAATAT
T.manatus           GTCCACGCTTCCCTGGACAAGTTCCTCAGCAGCGTTAGCACCGTGCTGACCTCCAAATAT
D.dugon             GTCCACGCTTCCCTGGACAAGTTCCTCAGCAACGTTAGCACCGTGCTGACCTCCAAATAT
H.gigas             GTCCACGCTTCCCTGGACAAGTTCCTCAGCAACGTTAGCACCGTGCTGACCTCCAAATAT
                    ***** ** ** ************ * **** *** ******************** ***

P.capensis          CGT
L.africana          CGT
E.maximus           CGT
M.primigenius       CGT
T.manatus           CGT
D.dugon             CGT
H.gigas             CGT
                    ***
```

Appendix 3. Alignment of paenungulate HBB/HBD protein coding sequences.

```
P.capensis            GTGCATCTGACTGACGCCGAGAAGGCTGCGGTCACCGGCCTGTGGGGCAAAGTGAAAGTG
M.primigenius         GTGAATCTGACTGCTGCTGAGAAGACACTAGTCGCCAACCTGTGGGACAAGGTGAATGTG
L.africana            GTGAATCTGACTGCTGCTGAGAAGACACAAGTCACCAACCTGTGGGGCAAGGTGAATGTG
E.maximus             GTGAATCTGACTGCTGCTGAGAAGACACAAGTCACCAACCTGTGGGGCAAGGTGAATGTG
T.manatus             GTGCATCTGACTCCTGAAGAGAAGGCTTTGGTCATCGGCCTGTGGGCCAAGGTGAACGTG
D.dugon               GTGCATCTGACTGCTGATGAGACGGCTTTGGTCACCGGCCTGTGGGCCAAGGTGAACGTG
Stellers_sea_cow      GTGCATCTGACTGCTGATGAGAAGGCTTTGGTCACCGGCCTGTGGTCCAAGGTGAACGTG
                      *** ********   *  **** * *    ***  *  *******  *** ***** ***

P.capensis            GATGAATACGGTGGGGAGGCCTTGGGCAGGTTGCTGGTCGTCTACCCATGGACCCAGAGG
M.primigenius         AAAGAGCTTGGTGGTGAGGCCCTGAGCAGGCTGCTGGTGGTCTACCCATGGACCCGGAGG
L.africana            AAAGAGCTTGGTGGTGAGGCCCTGAGCAGGCTGCTGGTGGTCTACCCATGGACCCGGAGG
E.maximus             AAAGAGCTTGGTGGTGAGGCCCTGAGCAGGCTGCTGGTGGTCTACCCATGGACCCGGAGG
T.manatus             AAAGAATATGGTGGTGAGGCCCTGGGCAGGCTGTTGGTTGTCTACCCATGGACCCAGAGG
D.dugon               AAAGAATATGGTGGTGAGGCCCTGGGCAGGCTGTTGGTTGTCTACCCATGGACCCAGAGG
Stellers_sea_cow      AAAGAATATGGGGGTGAGGCCCTGGGCAGGCTGTTGGTTGTCTACCCATGGACCCAGAGG
                       * **    ** ** ****** ** ***** ** **** ***************** ****

P.capensis            TTCTTTGAACACTTTGGGGACCTGTCCAGTGCTGACGCTATCATGCACAACCCTAAAGTG
M.primigenius         TTCTTTGAACACTTTGGGGACCTGTCCACTGCTGACGCTGTCCTGCACAACGCTAAAGTG
L.africana            TTCTTTGAACACTTTGGGGACCTGTCCACTGCTGAAGCTGTCCTGCACAACGCTAAAGTG
E.maximus             TTCTTTGAACACTTTGGGGACCTGTCCACTGCTGACGCTGTCCTGCACAACGCTAAAGTG
T.manatus             TTCTTTGAGCACTTTGGGGACCTGTCCTCTGCTTCGGCTATCATGAACAACCCTAAAGTG
D.dugon               TTCTTTGAGCACTTTGGGGACCTGTCCTCTGCTTCGGCTGTCATGCACAACCCTAAAGTG
Stellers_sea_cow      TTCTTTGAGCACTTTGGGGACCTGTCCTCTGCTTCGGCTGTCATGCACAACTCTAAAGTG
                      ******** ******************  ****   *** ** ** ***** ********

P.capensis            CTGGCCCATGGCAAGAAAGTGCTGTCCTCCTTTGGGGAGGGCCTGAATCACCTGGACAAC
M.primigenius         CTGGCCCATGGCGAGAAAGTGTTGACCTCCTTTGGTGAGGGCCTGAAGCACCTGGACAAC
L.africana            CTGGCCCATGGCGAGAAAGTGTTGACCTCCTTTGGTGAGGGCTTGAAGCACCTGGACAAC
E.maximus             CTGGCCCATGGCGAGAAAGTGTTGACCTCCTTTGGTGAGGGCCTGAAGCACCTGGACAAC
T.manatus             AAGGCCCATGGCGAGAAAGTGTTCACCTCCTTTGGTGACGGCCTGAAGCACCTGGAAGAC
D.dugon               AAGGCCCATGGCGAGAAAGTGTTGGCCTCCTTTGGTGACGGCCTGAAGCACCTGGACGAC
Stellers_sea_cow      CAGACCCATGGCGAGAAAGTGTTGGCCTCCTTTGGTGATGGCCTGAAGCACCTGGACAAC
                        * ******** ******** *  ********** ** *** **** ********  **

P.capensis            CTCAAGGGCACCTTTGCCCAGCTGAGCGAGCTGCACTGTGACAAGCTGCATGTGGACCCT
M.primigenius         CTCAAGGGCACCTTTGCCGATCTGAGCGAGCTGCACTGTGACAAGCTGCACGTGGATCCT
L.africana            CTCAAGGGCACCTTTGCCGATCTGAGCGAGCTGCACTGTGACAAGCTGCACGTGGATCCT
E.maximus             CTCAAGGGCACCTTTGCCGATCTGAGCGAGCTGCACTGTGACAAGCTGCACGTGGATCCT
T.manatus             CTCAAGGGTGCTTTTGCTGAGCTGAGTGAGCTGCACTGTGACAAGTTGCACGTGGATCCT
D.dugon               CTCAAGGGCGCCTTTGCTGAGCTGAGTGCGCTGCACTGTGAGAAGTTGCACGTGGATCCT
Stellers_sea_cow      CTCAACAGCGCCTTTGCTGAGCTGAGTGAGCTGCACTGTGACAAGTTGCACGTGGATCCT
                      *****  *  * *****  * ***** * ************ *** **** ***** ***

P.capensis            GAGAACTTCAGGCTCCTGGGCAATGTCTTGGTGGTTGTCCTGGCCCGGCACTTCCGTGAG
M.primigenius         CAGAATTTCAGGCTCCTGGGCAATGTGCTGGTGATTGTCCTGGCCCGCCACTTTGGCAAG
L.africana            GAGAATTTCAGGCTCCTGGGCAATGTGCTGGTGATTGTCCTGGCCCGCCACTTTGGCAAG
E.maximus             GAGAATTTCAGGCTCCTGGGCAATGTGCTGGTGATTGTCCTGGCCCGCCACTTTGGCAAG
T.manatus             GAGAACTTCAGGCTCCTGGGCAATGTGCTGGTGTGTGTGCTGGCCCGCCACTTTGGCAAG
D.dugon               CAGAACTTCAAGCTCCTGGGCAATATGCTGGTGTGTGTGCTGTCCCGCCACTTGGGCAAG
Stellers_sea_cow      GAGAACTTCAGGCTCCTGGGCAATGTGCTGGTGTGTGTGCTGGCCCGCCACTTGGGCAAG
                       **** **** ************* *  *****  *** *** **** *****  *  **

P.capensis            GAGTTCACCCCAGATGTTCAGGCTGCCTTTCAGAAGGTTGTGACTGGTGTGGCAAATGCT
M.primigenius         GCATTCACCCCAGATGTTCAGGCTGCCTATGAGAAGGTTGTGGCTGGTGTGGCGAATGCC
L.africana            GAATTCACCCCAGATGTTCAGGCTGCCTATGAGAAGGTTGTGGCTGGTGTGGCGAATGCC
E.maximus             GAATTCACCCCAGATGTTCAGGCTGCCTATGAGAAGGTTGTGGCTGGTGTGGCGAATGCC
T.manatus             GAATTCTCTCCAGAGGCACAGGCTGCCTATCAGAAGGTTGTGGCTGGTGTGGCGAACGCC
D.dugon               GAATTCTCTCCACAGGCACAGGCTGCCTATGAGAAGGTTGTGGCTGGGGTGGCGAACGCC
Stellers_sea_cow      GAATTCTCTCCACAGGCACAGGCTGCCTATCAGAAGGTTGTGGCTGGGGTGGCGAACGCC
                      *  *** * *** * *  ********** * ************ **** ***** ** **

P.capensis            TTGGCTCACAAATACCAC
M.primigenius         CTGGCTCACAAATACCAC
L.africana            CTGGCTCACAAATACCAC
E.maximus             CTGGCTCACAAATACCAC
T.manatus             TTGGCTCACAAATACCAC
D.dugon               TTGGCTCACAAATACCAC
Stellers_sea_cow      TTGGCTCACAAATACCAC
                       ******************
```

Appendix 4. Alignment of paenungulate HBE protein coding sequences.

```
P.capensis          GTGCATTTTACTGCTGAAGAGAAGGCTGCTATCACAGGCCTGTGGGGCAAGGTCAATGTG
L.africana          GTGCATTTTACTGCTGAGGAGAAGTCTGCTGTCACAAGTCTGTGGGGCAAGGTGAATGTG
E.maximus           GTGCATTTTACTGCTGAGGAGAAGTCTGCTGTCACAAGTCTGTGGGGCAAGGTGAATGTG
M.primigenius       GTGCATTTTACTGCTGAGGAGAAGTCTGCTGTCACAAGTCTGTGGGGCAAGGTGAATGTG
T.manatus           GTGCATTTTACTGCTGAGGAGAAGTCTGCTGTCACAAGTCTGTGGGGCAAGGTGAATGTG
D.dugon             GTGCATTTTACTGCTGAGGAGAAGTCTGCTGTCACAAGTCTGTGGGGCAAGGTGAATGTG
H.gigas             GTGCATTTTACTGCTGAGGAGAAGTCTGCTGTCACAAGTCTGTGGGGCAAGGTGAATGTG
                    ***************** ****** ***** ***** * ************** ******

P.capensis          GAAGAGGCTGGAGGAGAAGCCTTGGGAAGGCTCCTGGTTGTCTATCCCTGGACCCAGAGG
L.africana          GAAGAGGCTGGAGGTGAAGCCTTGGGAAGGCTCCTGGTTGTCTACCCCTGGACCCAGAGG
E.maximus           GAAGAGGCTGGAGGTGAAGCCTTGGGAAGGCTCCTGGTTGTCTACCCCTGGACCCAGAGG
M.primigenius       GAAGAGGCTGGAGGTGAAGCCTTGGGAAGGCTCCTGGTTGTCTACCCCTGGACCCAGAGG
T.manatus           GAAGAGGCTGGAGGCGAAGCCTTGGGAAGGCTCCTGGTTGTCTACCCTTGGACCCAGAGG
D.dugon             GAAGAGGCTGGAGGCGAAGCCTTGGGAAGGCTCCTGGTTGTCTACCCTTGGACCCAGAGG
H.gigas             GAAGAGGCTGGAGGTGAAGCCTTGGGAAGGCTCCTGGTTGTCTACCCTTGGACCCAGAGG
                    ************** ***************************** ** ************

P.capensis          TTTTTTGACAGCTTTGGCAACCTGTCCTCTGCCTCTGCCATCATGGGCAACCCCAAGGTC
L.africana          TTTTTTGACAGCTTTGGTAACCTGTCCTCGGCCTCTGCCATCATGGGCAACCCCAAGGTC
E.maximus           TTTTTTGACAGCTTTGGTAACCTGTCCTCGGCCTCTGCCATCATGGGCAACCCCAAGGTC
M.primigenius       TTTTTTGACAGCTTTGGTAACCTGTCCTCGGCCTCTGCCATCATGGGCAACCCCAAGGTC
T.manatus           TTTTTTGACACATTTGGCAACCTGTCCTCTGCCTCTGCCATCATGGGCAACCCCAAGGTC
D.dugon             TTTTTTGACACATTTGGCAACCTGTCCTCTGCCTCTGCCATCATGGGCAACCCCAAGGTC
H.gigas             TTTTTTGACACATTTGGCAACCTGTCCTCTGCCTCTGCCATCATGGGCAACCCCAAGGTC
                    **********  ***** *********** ******************************

P.capensis          AAGGCTCATGGCAAGAAGGTGCTGACCTCCTTTGGAGAAGCTATCAAGAACATGGACAAC
L.africana          AAGGCTCATGGAAAGAAGGTGCTGACCTCCTTTGGAGATGCTGTTAAGAACATGGACAAC
E.maximus           AAGGCTCATGGAAAGAAGGTGCTGACCTCCTTTGGAGATGCTGTTAAGAACATGGACAAC
M.primigenius       AAGGCTCATGGAAAGAAGGTGCTGACCTCCTTTGGAGATGCTGTTAAGAACATGGACAAC
T.manatus           AAGGCTCATGGCAAGAAGGTGTTGACCTCCTTTGGAGATGCTGTTAAGAACATGGACAAC
D.dugon             AAGGCTCATGGCAAGAAGGTGTTGACCTCCTTTGGAGATGCTGTTAAGAACATGGACAAC
H.gigas             AAGGCTCATGGCAAGAAGGTGTTGACCTCCTTTGGAGATGCTGTTAAGAACATGGACAAC
                    *********** ********* ***************** *** * ***************

P.capensis          CTCAAGGGCACCTTTGCTAAGCTGAGCGAGCTGCACTGTGACAAGCTGCACGTGGATCCT
L.africana          CTCAAAGGCGCCTTTGCTAAGCTGAGCGAACTGCATTGTGACAAGCTGCACGTGGATCCT
E.maximus           CTCAAAGGCGCCTTTGCTAAGCTGAGCGAACTGCATTGTGACAAGCTGCACGTGGATCCT
M.primigenius       CTCAAAGGCGCCTTTGCTAAGCTGAGCGAACTGCATTGTGACAAGCTGCACGTGGATCCT
T.manatus           CTCAAGAATGCCTTTGCGAAGCTGAGTGAGCTGCATTGTGACAAGCTGCATGTGGATCCT
D.dugon             CTCAAGAATGCCTTTGCTAAGCTGAGTGAGCTGCATTGTGACAAGCTGCATGTGGATCCT
H.gigas             CTCAAGAATGCCTTTGCTAAGCTGAGTGAGCTGCATTGTGACAAGCTGCATGTGGATCCT
                    *****     ******* ******** ** ***** ************** *********

P.capensis          GAGAACTTCAGGCTCCTGGGCAATGTGCTGGTGATTATTCTGGCTTCTCATTTTGGCAGA
L.africana          GAGAACTTCCGGCTCCTGGGCAACGTGCTGGTAATTATTCTGGCTTCTCATTTTGGCAGA
E.maximus           GAGAACTTCCGGCTCCTGGGCAACGTGCTGGTAATTATTCTGGCTTCTCATTTTGGCAGA
M.primigenius       GAGAACTTCCGGCTCCTGGGCAACGTGCTGGTAATTATTCTGGCTTCTCATTTTGGCAGA
T.manatus           GAGAACTTCCGGCTCCTGGGCAACGTTCTGGTGATTATTCTGGCTTCTCATTTTGGCAAG
D.dugon             GAGAACTTCCGGCTCCTGGGCAACGTTCTGGTGATTATTCTGGCTTCTCATTTTGGCAAG
H.gigas             GAGAACTTCCGGCTCCTGGGCAACGTTCTGGTGATTATTCTGGCTTCTCATTTTGGCAAG
                    ********* ************* ** ***** ************************

P.capensis          GAATTCACCCCTGATGTGCAGGCTGCTTGGCAGAAACTCGTGGCTGGTGTTGCCACTGCT
L.africana          GAATTCACCCCCGATATGCAGGCTGCTTGGCAGAAACTGGTGTCTGGTGTTGCCAATGCT
E.maximus           GAATTCACCCCCGATATGCAGGCTGCTTGGCAGAAACTGGTGTCTGGTGTTGCCAATGCT
M.primigenius       GAATTCACCCCCGATATGCAGGCTGCTTGGCAGAAACTGGTGTCTGGTGTTGCCAATGCT
T.manatus           GAATTCACCCCTGATATGCAGGCTGCTTGGCAGAAACTGGTGTCTGGTGTTGCCAATGCT
D.dugon             GAATTCACCCCTGATACGCAGGCTGCTTGGCAGAAACTGGTGTCTGGTGTTGCCAATGCT
H.gigas             GAATTCACCCCTGATACGCAGGCTGCTTGGCAGAAACTGGTGTCTGGTGTTGCCAATGCT
                    *********** ***  ********************** *** ************ ****

P.capensis          CTGTCCCACAAGTACCAC
L.africana          CTGTCCCACAAGTACCAC
E.maximus           CTGTCCCACAAGTACCAC
M.primigenius       CTGTCCCACAAGTACCAC
T.manatus           CTGTCCCACAAGTACCAC
D.dugon             CTGTCCCACAAGTACCAC
H.gigas             CTGTCCCACAAGTACCAC
                    ******************
```

Appendix 5. Alignment of paenungulate HBG protein coding sequences.

```
L.africana          GTGCATTTTACTGCCGAAGAGAAGGCTGCTATCACAAGCCTATGGGGCCAGGTGAATGTG
E.maximus           GTGCATTTTACTGCCGAAGAGAAGGCTGCTATCACAAGCCTATGGGGCCAGGTGAATGTG
M.primigenius       GTGCATTTTACTGCCGAAGAGAAGGCTGCTATCACAAGCCTATGGGGCCAGGTGAATGTG
T.manatus           GTGGATTTTACTGCTGAAGAGAAGGCTGCTATCACAAGGCTGTGGGGCAAGATGAATGTG
D.dugon             GTGTATTTTACTGCTGAAGAGAAGGCTGCTATCACAAGCCTGTGGGGCAAGGTGAATGTG
H.gigas             GTGTATTTTACTGCTGAAGAGAAGGCTGCTATCACAAGCCTGTGGGGCAAGGTGAACGTG
                    *** ********** ********************** ** ****** ** **** ***

L.africana          GAAGAGACCGGAGGCGAGGCCCTGGGCAGGCTTCTGGTTGTCTACCCTTGGACCCAGAGG
E.maximus           GAAGAGACCGGAGGCGAGGCCCTGGGCAGGCTTCTGGTTGTCTACCCTTGGACCCAGAGG
M.primigenius       GAAGAGACCGGAGGCGAGGCCCTGGGCAGGCTTCTGGTTGTCTACCCTTGGACCCAGAGG
T.manatus           GAAGAGGCTGGAGGCAAGGCCCTGGGCAGGCTCCTGATTGTCTACCCTTGGACCCAGAGG
D.dugon             GAAGAGGCTGGAGGCAAGGCCCTAGGCAGGCTCCTGATTGTCTACCCTTGGACCCAGAGG
H.gigas             GAAGAGGCTGGAGGCACGGCCCTAGGCAGGCTCCTGATTGTCTACCCTTGGATCCAGAGG
                    ****** * ******  ****** ******** *** *************** *******

L.africana          TTTTTTGACACCTTTGGCAACCTATCCTCTGCCTCTGCCATCATGGGCAACCCCAGGGTC
E.maximus           TTTTTTGACACCTTTGGCAACCTATCCTCTGCCTCTGCCATCATGGGCAACCCCAGGGTC
M.primigenius       TTTTTTGACACCTTTGGCAACCTATCCTCTGCCTCTGCCATCATGGGCAACCCCAGGGTC
T.manatus           TTTTTTGACAACTTTGGCAACCTATCCTCTGCCTCTGCCATCATGGGCAACCCCAAGGTC
D.dugon             TTTTTTGACAAATTTGGCAACCTATCCTCTGCCTCTGCCATCATGGGCAACCCCAAGATC
H.gigas             TTTTTTGACAAATTTGGCAACCTATCCTCTGCCTCTGCCATCATGGGCAACCCCAAGGTC
                    **********  ****************************************** * **

L.africana          AAGGCCCATGGCAAGAAGGTGCTGACCTCCTTTGGAGATGCTGTTAAGAACCTGGACAAC
E.maximus           AAGGCCCATGGCAAGAAGGTGCTGACCTCCTTTGGAGATGCTGTTAAGAACCTGGACAAC
M.primigenius       AAGGCCCATGGCAAGAAGGTGCTGACCTCCTTTGGAGATGCTGTTAAGAACCTGGACAAC
T.manatus           AAGGCCCATGGCAAGAAGGTGCTGAACTCCTTTGGAGATGCCGTTAAGAACCCGGACAAC
D.dugon             AAGGCCCATGGCAAGAAGGTGCTGAACTCCTTTGGCGATGCCGTTGAGAACCCGGACAAC
H.gigas             AAGGCCCATGGCAAGAAGGTGCTGAACTCCTTTGGCGATGCCGTTAAGAACCCGGACAAC
                    ************************* ********* ***** *** ****** *******

L.africana          CTCAAGGGCACCTTTGCTAAGCTGAGCGAGCTGCACTGTGACAAGCTGCACGTGGATCCT
E.maximus           CTCAAGGGCACCTTTGCTAAGCTGAGCGAGCTGCACTGTGACAAGCTGCACGTGGATCCT
M.primigenius       CTCAAGGGCACCTTTGCTAAGCTGAGCGAGCTGCACTGTGACAAGCTGCACGTGGATCCT
T.manatus           CTCAAGGGTACCTTTGCTAAGCTGAGTGAGCTGCACTGTGACAAGCTGCTTGTGGATTCT
D.dugon             CTCAAGGGTACCTTTGCTAAGCTGAGTGAGCTGCACTGTGACAAGCTGCTTGTGGATCCT
H.gigas             CTCAAGGGTACCTTTGCTAAGCTGAGTGAGCTGCACTGTGACAAGCTGCTTGTGGATCCT
                    ******** ***************** **********************  ****** **

L.africana          GAGAACTTCAGGCTCCTGGGAAATGTGCTAGTGATTGTCTTGGCAAACCACTTTGGCAAA
E.maximus           GAGAACTTCAGGCTCCTGGGAAATGTGCTAGTGATTGTCTTGGCAAACCACTTTGGCAAA
M.primigenius       GAGAACTTCAGGCTCCTGGGAAATGTGCTAGTGATTGTCTTGGCAAACCACTTTGGCAAA
T.manatus           GAGAACTTCAGGCTCCTGGGAAACGTGCTAGTGATTGTCTTGGCAAACCACTTTGGCAAA
D.dugon             GAGRACTTCAGGCTCCTGGGAAACGTGATAGTGATTGTCTTGGCAAACCACTTTGGCAAA
H.gigas             GACAACTTCAGGCTCCTGGGAAACATGATAGTGATTGTCTTGGCAAACCACTTTGGCAAA
                    **  ******************  ** ********************************

L.africana          GAATTCACCCCCCAGGTGCAGGCTGCCTGGCAGAAGATGGTGACGGGCGTGGCCAATGCC
E.maximus           GAATTCACCCCCCAGGTGCAGGCTGCCTGGCAGAAGATGGTGACGGGCGTGGCCAATGCC
M.primigenius       GAATTCACCCCCCAGGTGCAGGCTGCCTGGCAGAAGATGGTGACGGGCGTGGCCAACGCC
T.manatus           GAATTTACCCCCCAGGTGCAGGCTGCCTGGCAGAAGATGGCGACTGGTGTGGCCAGTGCC
D.dugon             GAATTTACCCCCCAGGTGCAGGCTGCCTGGCAGAAGATGGTGACTGGTGTGGCCAGTGCC
H.gigas             GAATTTACCCCCCAGGTGCAGGCTGCCTGGCAGAAGATGGTGACTGGTGTGGCCAGTGCC
                    ***** ********************************** *** ** *******  ***

L.africana          CTGGCCTACAAGTATCAC
E.maximus           CTGGCCTACAAGTATCAC
M.primigenius       CTGGCCTACAAGTATCAC
T.manatus           GTGGCCCGCAAGTATCAC
D.dugon             CTGGCCCGCAAGTATCAC
H.gigas             CTGGCCCGCAAGTATCAC
                     *****  **********
```

Appendix 6. Alignment of paenungulate HBZ-T1 protein coding sequences.

```
P.capensis          TCTCTGACCAAAGCCGAGAGGGCCGTCATCGCATCGGTGTGGGGCAAGGTCTCCACCCAG
L.africana          TCTCTGACCAAGGCCGAGAGGACCATCATCGTGTCCATGTGGAACAAGGTGTCCACACAG
M.primigenius       TCTCTGACCAAGGCCGAGAGGACCATCATCGTGTCCATGTGGAACAAGGTGTCCACACAG
T.manatus           TCTCTGACCAAGGCCGAGAGGACCATCATCATAACCATGTGGAGTAAGGTCTCCACACAG
D.dugon             TCTCTGACCAAGGCCGAGAGGACCATCATCATAACCATGTGGGGTAAGATCTCCACACAG
H.gigas             TCTCTGACCAAGGCCGAGAGGACCATCATCATAACCATGTGGGGTAAGGTCTCCACACAG
                    *********** ********* ** *****    *  *****   *** * ***** ***

P.capensis          GCCGAGAGCATCGGCACCGAGGCCCTGGAGAGGCTCTTCGCCAGCTACCCCCAGACCAAG
L.africana          GCTGACAGTATTGGCACCGAAGCCCTGGAGAGGCTCTTCGCCAGCTACCCCCAGACCAAG
M.primigenius       GCTGACAGTATTGGCACCGAAGCCCTGGAGAGGCTCTTCGCCAGCTACCCCCAGACCAAG
T.manatus           GCCGACAGCATTGGCACCGAGGCCCTGGAGAGGCTCTTCGCCAGCTACCCCCAGACCAAG
D.dugon             GCCGACAGCATTGGCACCGAGGCCCTGGAGAGGCTCTTCGCCAGCTACCCCCAGACCAAG
H.gigas             GCCGACAGCATTGGCACTGAGGCCCTGGAGAGGCTCTTCGCCAGCTACCCCCAGACCAAG
                    ** ** ** ** ***** ** ****************************************

P.capensis          ACCTACTTCCCGCACTTCGACCTGCACCCGGGCTCGGCGCAGCTGCGCGCGCACGGCTCC
L.africana          ACTTACTTCCCGCACTTCGACCTGCACCCTGGCTCGGCGCAGCTGCGCGCGCACGGCTCC
M.primigenius       ACTTACTTCCCGCACTTCGACCTGCACCCTGGCTCGGCGCAGCTGCGCGCGCACGGCTCC
T.manatus           ACCTACTTCCCGCACTTCGACCTGCACCCGGGCTCGGCGCAGCTGCGCACGCACGGCTCC
D.dugon             ACCTACTTCCCGCACTTCGACCTGCACCCGGGCTCGGCGCAGCTGCGCACGCACGGCTCC
H.gigas             ACCTACTTCCCGCACTTCGACCTGCACCCGGGCTCGGCGCAGCTGCGCACGCACGGCTCC
                    ** ************************** ****************** ***********

P.capensis          AAGGTGGTGGCCGCCCTGGGCGACGCGGTCAGGAGCCTGGACAACGTCTCGAGCGCGCTG
L.africana          AAGGTGGTGGCCGCTGTGGGTGACGCGGTCAAGAGCATCGACAATATCTCGGGCGCGCTG
M.primigenius       AAGGTGGTGGCCGCTGTGGGTGACGCGGTCAAGAGCATCGACAATATCTCGGGCGCGCTG
T.manatus           AAGGTGGTGGCCGCCGTGGGCGACGCGGTCAAGAGCGTCGACAACATCTCGGGCGCGCTG
D.dugon             AAGGTGGTGGCCGCCGTGGGCGACGCGGTCAAGAGCGTCGACAACATCGCGGGCGCGCTG
H.gigas             AAGGTGGTGGCCGCCGTGGGCGACGCGGTCAAGAGCGTCGACAACATCGCGGGCGCGCTG
                    **************  **** ********** **** * *****  ** ** ********

P.capensis          TCCAAGCCCAGCGAGCTGCACGCCTACGTCCTGCGCGTGGACCCGGTGAACTTCAAGTTC
L.africana          TCCAAGCTGAGCGAGCTGCACGCCTACATCCTGCGCGTGGACCCGGTCAACTTCAAGCTC
M.primigenius       TCCAAGCTGAGCGAGCTGCACGCCTACATCCTGCGCGTGGACCCGGTCAACTTCAAGCTC
T.manatus           TCCAAGCTGAGCGAGCTGCACGCCTACATCCTGCGCGTGGACCCGGTCAACTTCAAGCTC
D.dugon             TCCAAGCTGAGCGAGCTGCACGCCTACATCCTGCGCGTGGACCCGGTCAACTTCAAGCTC
H.gigas             TCCAAGCTGAGCGAGCTGCACGCCTACATCCTGCGCGTGGACCCGGTCAACTTCAAGCTT
                    *******  ****************** ****************** ********* *

P.capensis          CTGTCGCACTGCCTGCTGGTAACCCTGGCCACGCGCTTCCCCGCCGACTTCACGGCCGAG
L.africana          CTGTCACACTGTCTGCTGGTGACCTTGGGCACGCGCTTCCCCGCTGACTTCACGGCCGAG
M.primigenius       CTGTCACACTGTCTGCTGGTGACCTTGGGCACGCGCTTCCCCGCTGACTTCACGGCCGAG
T.manatus           CTGTCGCACTGCCTGCTGGTGACCGTGGCCACGCGCTTCCCCGCCGACTTCACGGCCGAG
D.dugon             CTGTCGCACTGCCTGCTGGTGACCGTGGCCACGCGCTTCCCCGCCGACTTCACGGCCGAG
H.gigas             CTGTCGCACTGCCTGCTGGTGACCGTGGCCACGCGCTTCCCCGCGGACTTCACGGCCGAG
                    ***** ***** ******** *** *** *************** ***************

P.capensis          GCCCACGCCGCCTGGGACAAGTTCCTGTCCGTCGTGTCTTCCGTCCTGACCGAGAAGTAC
L.africana          GCCCACGCCGCCTGGGACAAATTCCTGTCCGTTGTGTCTTCCGTCCTGACCGAGAAGTAC
M.primigenius       GCCCACGCCGCCTGGGACAAATTCCTGTCCGTTGTGTCTTCCGTCCTGACCGAGAAGTAC
T.manatus           GCCCACGCCGCCTGGGACAAGTTCCTGTCCATCGTGTCTTCCGTCCTGACCGAGAAGTAC
D.dugon             GCCCACGCCGCCTGGGACAAGTTCCTGTCCGTCGTGTCTTCCGTCCTGACCGAGAAGTAC
H.gigas             GCCCACGCCGCCTGGGACAAGTTCCTGTCCATCGTGTCTTCCGTCCTGACCGAGAAGTAC
                    ******************** ********* * ****************************

P.capensis          CGC
L.africana          CGC
M.primigenius       CGC
T.manatus           CGC
D.dugon             CGC
H.gigas             CGC
                    ***
```

Appendix 7. Alignment of paenungulate MB protein coding sequences.

```
L.africana          GGACTCAGCGACGGGGAATGGGAGTTGGTGTTGAAAACCTGGGGGAAAGTGGAGGCTGAC
E.maximus           GGACTCAGCGACGGGGAATGGGAGTTGGTGTTGAAAACCTGGGGGAAAGTGGAGGCTGAC
M.primigenius       GGACTCAGCGACGGGGAATGGGAGTTGGTGTTGAAAACCTGGGGGAAAGTGGAGGCTGAC
P.capensis          GGGCTCAGCGACGGGGAATGGCAGTTGGTGTTGAACGCCTGGGGGAAGGTGGAGGCTGAC
T.manatus           GCGCTGAGCGACGGGGAATGGCAGTTGGTGTTGAACGTCTGGGGGAAGGTGGAGGCTGAT
D.dugon             GTGCTGAGCGACGGGGAATGGCAGTTGGTGTTGAACGTCTGGGGGAAGGTAGAGGCTGAT
H.gigas             GTGCTGAGCGACGGGGAATGGCAGTTGGTGTTGAACGTCTGGGGGAAGGTGGAGGCTGAT
                    *  ** ***************** *************   ********* ** ********

L.africana          ATCCCGGGCCATGGGCTGGAAGTCTTCGTCAGGCTCTTCACAGGTCATCCTGAGACCCTG
E.maximus           ATCCCGGGCCATGGGCTGGAAGTCTTCGTCAGGCTCTTCACAGGTCATCCCGAGACCCTG
M.primigenius       ATCCCGGGCCATGGGCTGGAAGTCTTCGTCAGGCTCTTCACAGGTCATCCCGAGACCCTG
P.capensis          ATCCCAGGCCATGGGCAGGAAGTCCTCATCAGACTCTTCCAGGGTCATCCTGAGACCCTG
T.manatus           ATCGCAGGCCATGGGCTGGAAGTCCTCATCAGCCTCTTCAAGGGTCATCCTGAGACCCTG
D.dugon             ATCGCGGGCCATGGGCTGGAAGTCCTCATCAGCCTCTTCAAGGGTCATCCTGAGACCCTG
H.gigas             ATCGCGGGCCATGGGCTGGAAGTCCTCATCAGCCTCTTCAAGGGTCATCCTGAGACCCTG
                    *** * ********** ******* ** **** ******   ******** *********

L.africana          GAGAAGTTCGACAAGTTTAAGCACCTGAAGACAGAGGGCGAGATGAAGGCCTCCGAGGAC
E.maximus           GAGAAGTTTGACAAGTTTAAGCACCTGAAGACAGAGGGCGAGATGAAGGCCTCCGAGGAC
M.primigenius       GAGAAGTTCGACAAGTTTAAGCACCTGAAGACAGAGGGCGAGATGAAGGCCTCCGAGGAC
P.capensis          GAGAAGTTTGACAAGTTCAAGCACCTGAAGACAGCAGATGAGATGAAGGCCTCTGAGGAC
T.manatus           GAGAAGTTCGACAAGTTCAAGCACCTGAAGTCAGAGGAAGAAATGAAGGCCTGTGAAGAC
D.dugon             GAGAAGTTCGACAAGTTCAAGCACCTGAAGACAGAGGAAGAAATGAAGGCCTGTGAGGAC
H.gigas             GAGAAGTTCGACAAGTTCAAGCACCTGAAGACAGAGGAAGAAATGAAGGCCTGTGAGGAC
                    ******** ******** ************ ***  *  ** **********  ** ***

L.africana          CTGAAGAAGCAAGGTGTTACTGTGCTCACTGCCCTGGGGGGCATCCTCAAGAAGAAAGGG
E.maximus           CTGAAGAAGCAAGGTGTTACTGTGCTCACTGCCCTGGGGGGCATCCTCAAGAAGAAAGGG
M.primigenius       CTGAAGAAGCAAGGTGTTACTGTGCTCACTGCCCTGGGGGGCATCCTCAAGAAGAAAGGG
P.capensis          CTGAAGAAGCATGGCGGTGTCGTGCTCACTGCCCTGGGTGGCATCCTCAAGAAGAAGGGG
T.manatus           CTGAAGAAGCACGGCGTCACCGTGCTTACTGCCTTGGGGGGCATCCTGAAGAAGAAGGGG
D.dugon             CTGAAGAAGCACGGCACCACTGTGCTTACTGCCTTGGGGGGCATCCTGAAGAAGAAGGGG
H.gigas             CTGAAGAAGCACGGCACCACCGTGCTTACTGCCTTGGGGGGCATCCTGAAGAAGAAGGGG
                    *********** **       ***** ****** **** ******** ******** ***

L.africana          CATCACCAGGCGGAGATTCAGCCCCTGGCCCAGTCTCATGCCACCAAGCACAAGATCCCC
E.maximus           CATCACCAGGCGGAGATTCAGCCCCTGGCCCAGTCTCATGCCACCAAGCACAAGATCCCC
M.primigenius       CATCACCAGGCGGAGATTCAGCCCCTGGCCCAGTCTCATGCCACCAAGCACAAGATCCCC
P.capensis          GATCACCAGGCAGAGATTCAGCCCCTGGCCCAGTCCCATGCTACCAAACACAAGATTCCC
T.manatus           CATCACCAGGCGGAGATTCAGCCCTTGGCCCAGTCCCATGCCACCAAGCACAAGATCCCT
D.dugon             CATCACCAGGCGGAGATTCAGCCCTTGGCCCAGTCCCATGCCACCAAGCACAAGATCCCT
H.gigas             CATCACCAGGTGGAGATTCAGCCCTTGGCCCAGTCCCACGCCACCAAGCACAAGATCCCC
                     *********  ************ ********** ** ** ***** ******** **

L.africana          ATCAAGTATCTGGAGTTCATCTCGGACGCCATCATCCACGTTCTGCAAAGCAAGCATCCC
E.maximus           ATCAAGTATCTGGAGTTCATCTCGGACGCCATCATCCACGTTCTGCAAAGCAAGCATCCT
M.primigenius       ATCAAGTATCTGGAGTTCATCTCGGACGCCATCATCCACGTTCTGCAAAGCAAGCATCCT
P.capensis          ATCAAGTACTTGGAGTTCATCTCAGAAGCCATCATCCATGTTCTGCAAAGCAAGCATGCT
T.manatus           GTCAAGTACCTGGAGTTCATCTCGGAAGCCATCATCCACGTTCTGCAGAGCAAGCACCCT
D.dugon             GTCAAGTACCTGGAGTTCATCTCGGAAGCCATCATCCACGTTCTGCAGAGCAAGCACCCT
H.gigas             GTCAAGTACCTGGAGTTCATCTCGGAAGCCATCATCCACGTTCTGCAGAGCAAGCACCCT
                     *******  ************* ** *********** ******** ********  *

L.africana          GCGGAATTTGGCGCTGATGCCCAGGCAGCCATGAAAAAGGCCTTGGAGCTGTTCCGGAAT
E.maximus           GCGGAATTTGGCGCTGATGCCCAGGGAGCCATGAAAAAGGCCTTGGAGCTGTTCCGGAAT
M.primigenius       GCGGAATTTGGCGCTGATGCCCAGGGAGCCATGAAAAAGGCCTTGGAGCTGTTCCGGAAT
P.capensis          GCAGACTTCGGTGCAGATGCCCAGGGAGCCATGCGCAAGGCCCTGGAGCTCTTCCGGAAT
T.manatus           GGAGACTTTGGCGCTGATGCCCAGGGAGCCATGAGCAAGGCCTTGGAGCTGTTCCGGAAT
D.dugon             GGAGACTTTGGTGCCGATGCCCAGGGAGCCATGAGGAAGGCCTTGGAGCTGTTCCGGAAT
H.gigas             GGAGACTTTGGTGCCGATGCCCAGGGAGCCATGAGGAAGGCCTTGGAGCTGTTCCGGAAT
                    *  ** ** ** ** ********** *******   ****** ******* *********

L.africana          GACATTGCGGCCAAGTACAAGGAGCTGGGGTTCCAGGGC
E.maximus           GACATTGCGGCCAAGTATAAGGAGCTGGGGTTCCAGGGC
M.primigenius       GACATTGCGGCCAAGTATAAGGAGCTGGGGTTCCAGGGC
P.capensis          GACATCGCAGCCAAGTACAAGGAGCTGGGCTTCCAGGGC
T.manatus           GCCATGGCTGCCAACTACAAGGAGCTGGGATTCCAGGGC
D.dugon             GACATGGCTGCCAAGTACAAGGAGCTGGGATTCCAGGGC
H.gigas             GACATGGCTGCCAAGTACAAGGAGCTGGGATTCCAGGGC
                    * *** ** ***** ** *********** *********
```

Appendix 8. Alignment of paenungulate NGB protein coding sequences.

```
L.africana          CAGCGCCCCGAGCACGAGCTGATCCGGCAGAGCTGGCGCGTGGTGAGCCGCAGCCCGCTG
E.maximus           CAGCGCCCCGAGCACGAGCTGATCCGGCAGAGCTGGCGCGTGGTGAGCCGCAGCCCGCTG
M.primigenius       CAGCGCCCCGAGCACGAGCTGATCCGGCAGAGCTGGCGCGTGGTGAGCCGCAGCCCGCTG
P.capensis          CAGCGCCCGGAGCACGAGCTGATCCGACAGAGCTGGCGCGTGGTGAGCCGCAGCCCGCTG
T.manatus           CAGCGCCCGGAGCACGAGCTGATCCGGCAGAGCTGGCGCGTGGTGAGCCGCAGCCCCCTA
D.dugon             CAGCGCCCGGAGCACGAGCTGATCCGGCAGAGCTGGCGCGTGGTGAGCCGCAGCCCCCTG
H.gigas             CAGCGCCCGGAGCACGAGCTGATCCGGCAGAGCTGGCGCGTGGTGAGCCGCAGCCCCCTG
                    ******** ***************** ***************************** **

L.africana          GAGCACGGTACCGTCCTGTTCGCCAGGCTGTTTGACCTGGAGCCAGACCTGCTGCCCCTC
E.maximus           GAGCACGGTACCGTCCTGTTCGCCGGGCTGTTTGACCTGGAGCCAGACCTGCTGCCCCTC
M.primigenius       GAGCACGGTACCGTCCTGTTCGCCAGGCTGTTTGACCTGGAGCCAGACCTGCTGCCCCTC
P.capensis          GAACATGGCACGGTGCTGTTCGCCAGGCTGTTTGACCTGGAACCTGACCTACTGCCGCTC
T.manatus           GAGCACGGCACCGTCCTGTTCGCCAGGCTGTTTGACCTGGAACCCGACCTGCTGCCCCTC
D.dugon             GAGCACGGCACCGTCCTGTTCGCCAGGCTGTTTGACCTGGAACCCGACCTGCTGCCCCTC
H.gigas             GAGCACGGCACCGTCCTGTTCGCCAGGCTGTTTGACCTGGAACCCGACCTGCTGCCCCTC
                    ** ** ** ** ** ********* *************** ** ***** ***** ***

L.africana          TTCCAATACAACTGCCGCCAGTTCTCCAGCGTGAATGACTGCCTCTCCTCCCCAGAGTTC
E.maximus           TTCCAATACAACTGCCGCCAGTTCTCCAGCGTGGATGACTGCCTCTCCTCCCCAGAGTTC
M.primigenius       TTCCAATACAACTGCCGCCAGTTCTCCAGCGTGGATGACTGCCTCTCCTCCCCAGAGTTC
P.capensis          TTCCAGTACAACTGCCGCCAGTTCTCCAGCGTGGAGGACTGCCTATCCTCCCCAGAGTTC
T.manatus           TTCCAGTACAACTGCCGCCAGTTCTCCAGCGTGGAGGACTGCCTCTCCTCCCCAGAGTTC
D.dugon             TTCCAGTACAACTGCCGCCAGTTCTCCAACGTGGAGGACTGCCTCTCCTCCCCAGAGTTC
H.gigas             TTCCAGTACAACTGCCGCCAGTTCTCCAACGTGGAGGACTGCCTCTCTTCCCCAGAGTTC
                    ***** ********************** **** * ******** ** ************

L.africana          CTGGACCACATCCGGAAGGTGATGGTCGTGATTGATGCTGCAGTGACCAATGTGGAGGAT
E.maximus           CTGGACCACATCCGGAAGGTGATGGTCGTGATTGATGCTGCAGTGACCAATGTGGAGGAT
M.primigenius       CTGGACCACATCCGGAAGGTGATGGTCGTGATTGATGCTGCAGTGACCAATGTGGAGGAT
P.capensis          CTGGACCACATCCGAAAGGTGATGCTAGTGATTGACGCTGCAGTGACCAATGTGGAGGAC
T.manatus           CTGGACCACATCCGAAAGGTGATGCTCGTGATTGACGCTGCAGTGACGAACGTGGAGGAC
D.dugon             CTGGACCACATCCGAAAGGTGATGCTCGTGATTGACGCTGCAGTGACGAACGTGGAGGAC
H.gigas             CTGGACCACATCCGAAAGGTGATGCTCGTGATTGACGCTGCAGTGACGAACGTGGAGGAC
                    ************** ********* * ******** ************ ** ********

L.africana          CTGTCCTCACTGGAGGAGTACCTTGCCGGCCTGGGCAAGAAGCACCGGTTGGTGGGTGTG
E.maximus           CTGTCCACACTGGAGGAGTACCTTGCCGGCCTGGGCAAGAAGCACCGGTTGGTGGGTGTG
M.primigenius       CTGTCCACACTGGAGGAGTACCTTGCCGGCCTGGGCAAGAAGCACCGGTTGGTGGGTGTG
P.capensis          TTGTCATCGCTGGAGGAGTACCTTGCTGGCCTGGGCAGGAAGCACCGGGTGGTGGGTGTG
T.manatus           CTGTCCTCACTGGAGGAGTACCTTGCCGGCCTGGGCAAGAAGCATCGGGTGGTGGGTGTG
D.dugon             CTGTCCTCGCTGGAGGAGTACCTTGCCGGCCTGGGCAAGAAGCATCGGGTGGTGGGTGTG
H.gigas             CTGTCCTCGCTGGAGGAGTACCTTGCCAGCCTGGGCAAGAAGCATCGGGTGGTGGGTGTG
                     ****  * *****************  ********* ****** *** ************

L.africana          AAGCTCAGCTCATTCTCGACGGTGGGTGAGTCCCTGCTCTACATGCTGGAGAAGTGCCTG
E.maximus           AAGCTCAGCTCATTCTCGACGGTGGGTGAGTCCCTGCTCTACATGCTGGAGAAGTGCCTG
M.primigenius       AAGCTCAGCTCATTCTCGACGGTGGGTGAGTCCCTGCTCTACATGCTGGAGAAGTGCCTG
P.capensis          AAGCTCAGCTCCTTCTCGACGGTGGGTGAGTCCCTGCTCTACATGCTGGAGAAGTGCCTG
T.manatus           AAGCTCAGCTCCTTCTCGACAGTGGGCGAGTCTCTGCTCTACATGCTGGAGAAGTGCCTG
D.dugon             AAGCTCAGCTCCTTCTCGACAGTGGGCGAGTCTCTGCTCTACATGCTGGAGAAGTGCCTG
H.gigas             AAGCTCAGCTCCTTCTCGACAGTGGGCGAGTCTCTGCTCTACATGCTGGAGAAGTGCCTG
                    *********** ******** ***** ***** ***************************

L.africana          GGTCCCGCCTTCACGCCAGCCACGAAGGCCGCCTGGAGTCAGCTCTACGGGGCTGTGGTG
E.maximus           GGTCCCGCCTTCACGCCAGCCACGAAGGCCGCCTGGAGTCAGCTCTACGGGGCTGTGGTG
M.primigenius       GGTCCCGCCTTCACGCCAGCCACGAAGGCCGCCTGGAGTCAGCTCTACGGGGCTGTGGTG
P.capensis          GGTCCAGCCTTCACGCCAGCCATGCGGGCTGCCTGGAGCCAGCTCTATGGGGCTGTGGTG
T.manatus           GGCCCTGCCTTCACGCCAGCCATGCGGGCTGCCTGGAGCCAGCTCTATGGGGCCGTGGTG
D.dugon             GGCCCTGCCTTCACGCCAGCCATGCGGGCTGCCTGGAGCCAGCTCTATGGGGCTGTGGTG
H.gigas             GGCCCTGCCTTCACGCCAGCCATGCGGGCTGCCTGGAGCCAGCTCTATGGGGCTGTGGTG
                    ** ** ***************** *  *** ******** ******** ***** ******

L.africana          CAGGCCATGAGTCGAGGCTGGGACGGCGAG
E.maximus           CAGGCCATGAGTCGAGGCTGGGACGGCGAG
M.primigenius       CAGGCCATGAGTCGAGGCTGGGACGGCGAG
P.capensis          CAGGCCATGAGTCGTGGCTGGGACGGCGAG
T.manatus           CAGGCCATGAGTCGGGGCTGGGACAACGAG
D.dugon             CAGGCCATGAGTCGGGGCTGGGACAACGAG
H.gigas             CAGGCCATGAGTCGGGGCTGGGACAACGAG
                    ************** *********  ****
```

Appendix 9. Alignment of paenungulate CYGB protein coding sequences.

```
P.capensis      GAGAAAGTACCGGGCGAGATGGAGACCGAGCGCAGGGAGCGGAGCGAGGAGATGTCCGAG
T.manatus       GAGAAAGTGCTGGGCGAGATGGAGATTGAGCGCAGGGAGCGGAGCGAGGAGCTGTCCGAG
H.gigas         GAGAAAGTGCTGGGCGAGATGGAGATTGAGCGCAGGGAGCGGAGCGAGGAGCTGTCCGAG
D.dugon         GAGAAAGTGCTGGGCGAGATGGAGATTGAGCGCAGGGAGCGGAGCGAGGAGCTGTCCGAG
L.africana      GAGAAAGTGCCGGGCGAGATGGAGATCGAGCGCAGGGAGCGGAGCGAGGAGCTGTCCGAG
E.maximus       GAGAAAGTGCCGGGCGAGATGGAGATCGAGCGCAGGGAGCGGAGCGAGGAGCTGTCCGAG
                ******** * **************  ************************ ********

P.capensis      GCGGAGAGGAAGGCGGTGCAGGAGACGTGGGCCCGGCTCTATGCCAACTACGAGGACGTG
T.manatus       GCGGAGAGGAAGACCGTGCAGGCGACGTGGGCCCGGCTGTATGCCAACTGCGAGGACGTG
H.gigas         GCGGAGAGGAAGACCGTGCAGGCGACGTGGGCCCGGCTGTATGCCAACTGCGAGGACGTG
D.dugon         GCGGAGAGGAAGACCGTGCAGGCGACGTGGGCCCGGCTGTATGCCAACTGCGAGGACGTG
L.africana      GCAGAGAGGAAGGCGGTGCAGGCGACGTGGGCCCGGCTGTATGCCAACTGCGAGGACGTG
E.maximus       GCAGAGAGGAAGGCGGTGCAGGCGACGTGGGCCCGGCTGTATGCCAACTGCGAGGACGTG
                ** ********* * ******* *************** ********** **********

P.capensis      GGGGTGGCCATCCTGATGAGGTTCTTCGTGAACTTCCCGTCGGGCAAGCAGTACTTCAGC
T.manatus       GGGGTGGCCATCCTGGTGAGGTTCTTTGTGAACTTCCCATCGGGCAAGCAGTACTTCAGC
H.gigas         GGGGTGGCCATCCTGGTGAGGTTCTTTGTGAACTTCCCATCGGGCAAGCAGTACTTCAGC
D.dugon         GGGGTGGCCATCCTGGTGAGGTTCTTTGTGAACTTCCCATCGGGCAAGCAGTACTTCAGC
L.africana      GGGGTGGCCATCCTGGTGAGGTTCTTTGTGAACTTCCCGTCCAGCAAGCAGTACTTCAGC
E.maximus       GGGGTGGCCATCCTGGTGAGGTTCTTTGTGAACTTCCCGTCCAGCAAGCAGTACTTCAGC
                *************** ********** *********** **  ******************

P.capensis      CAGTTCAAACACATGGTGGAGCCCCTGGAGATGGAGCGCAGCCCCCAGCTGCGGAAGCAC
T.manatus       CAGTTCAAGCACATGGTGGATCCCCTGGAAATGGAGCGGAGTCCCCAGCTACGGAAGCAT
H.gigas         CAGTTCAAGCACATGGTGGATCCCCTGGAAATGGAGCGGAGTCCCCAGCTACGGAAGCAT
D.dugon         CAATTCAAGCACATGGTGGATCCCCTGGAAATGGAGCGGAGTCCCCAGCTACGGAAGCAT
L.africana      CAGTTCAAGCACATGGTGGAGCCCCTGGAAATGGAGCGGAGCCCCCAGCTGCGGAAGCAC
E.maximus       CAGTTCAAGCACATGGTGGAGCCCCTGGAAATGGAGCGGAGCCCCCAGCTGCGGAAGCAC
                ** ***** ************ ******** ******** ** ******** ********

P.capensis      GCCTGCCGGGTCATGGGGGCCCTCAACACCGTTGTGGAGAACCTGCACGACCCTGACAAG
T.manatus       GCCTGCCGGGTCATGGGGGCCCTCAACACGGTTGTGGAGAACCTGCATGACCCCGACAAG
H.gigas         GCCTGCCGGGTCATGGGGGCCCTCAACACAGTTGTGGAGAACCTGCATGACCCCGACAAG
D.dugon         GCCTGCCGGGTCATGGGGGCCCTCAACACGGTTGTGGAGAACCTGCATGACCCCGACAAG
L.africana      GCCTGCCGGATCATGGGGGCCCTCAACACAGTCGTGGAGAACCTACATGACCCTGACAAG
E.maximus       GCCTGCCGGATCATGGGGGCCCTCAACACAGTCGTGGAGAACCTACATGACCCTGACAAG
                ********* ******************* ** ************ ** ***** ******

P.capensis      GTGTCCTCTGTGCTAGGCCTCTTGGGCAAAGCTCACGCCCTCAAGCACAAGGTGGAGCCT
T.manatus       GTGTCCTCCGTGCTGGCCCTCGTGGGCAAAGCTCACGCCCTCAAGCACAAGGTGGAGCCT
H.gigas         GTGTCCTCCGTGCTGGCCCTCGTGGGCAAAGCTCACGCCCTCAAGCACAAGGTGGAGCCT
D.dugon         GTGTCCTCCGTGCTGGCCCTCGTGGGCAAAGCTCACGCCCTCAAGCACAAGGTGGAGCCT
L.africana      GTGTCCTCCGTGCTGGCCCTCCTGGGCAAAGCTCACGCCCTCAAGCACAAGGTGGAGCCT
E.maximus       GTGTCCTCCGTGCTGGCCCTCCTGGGCAAAGCTCACGCCCTCAAGCACAAAGTGGAGCCT
                ******** ***** * **** ************************** *********

P.capensis      GTGTATTTCAAGATCCTCTCTGGGATTATTCTGGAAGTGGTCGCTGAGGAGTTTGCCAAC
T.manatus       GTGTACTTCAAGATCCTCTCTGGGGTCATCCTGGAGGTGGTTGCCGAGGAGTTTGCCAAT
H.gigas         GTGTACTTCAAGATCCTCTCTGGGGTCATCCCGGAGGTGGTTGCCGAGGAGTTTGCCAAT
D.dugon         GTGTACTTCAAGATCCTCTCTGGGGTCATCCTGGAGGTGGTTGCCGAGGAGTTTGCCAAT
L.africana      GTGTACTTCAAGATCCTCTCTGGAATCATTCTGGAAGTGGTGGCTGAGGAGTTTGCCGAT
E.maximus       GTGTACTTCAAGATCCTCTCTGGAATCATTCTGGAAGTGGTGGCTGAGGAGTTTGCCGAC
                ***** *****************  * ** * *** ***** ** ************ *

P.capensis      GATTTCCCGCCTGAGACACAGAGAGCCTGGACCAAGCTGCGTGGCCTCATCTACAGCCAT
T.manatus       GACTTCCCACCCGAGACGCAGAGAGCCTGGGCCAAGCTGCGTGCCCTCATCTACAGCCAT
H.gigas         GACTTCCCACCCGAGACGCAGAGAGCCTGGGCCAAGCTGCGTGGCCTCATCTACAGCCAT
D.dugon         GACTTCCCACCCGAGACGCAGAGAGCCTGGGCCAAGCTGCGTGGCCTCATCTACAGCCAT
L.africana      GACTTCCCGCCCGAGACGCAGAGAGCCTGGGCCAAGCTGCGTGGCCTCATCTACAGCCAT
E.maximus       GACTTCCCGCCCGAGACGCAGAGAGCCTGGGCCAAGCTGCGTGGCCTCATCTACAGCCAT
                ** ***** ** ***** ************ ************ ****************

P.capensis      GTGACCGCTGCCTACAAGGAAGTAGGCTGGGTACAGCAGGTCCCCAATGCCACCATCCCG
T.manatus       GTGACTGCTGCCTACAAGGAAGTGGGCTGGGTACAGCAGGTCCCCAACACCACCATCCCA
H.gigas         GTGACTGCTGCCTACAAGGAAGTGGGCTGGGTACAGCAGGTCCCCAACGCCACCATCCCA
D.dugon         GTGACTGCTGCCTACAAGGAAGTGGGCTGGGTACAGCAGGTCCCCAACGCCACCATCCCA
L.africana      GTGATCGCTGCCTACAAGGAAGTGGGCTGGGTACAGCAGGTCCCCAACGTCACCATCCCA
E.maximus       GTGACCGCTGCCTACAAGGAAGTGGTCTGTGTACAGCAGGTCCCCAACGTCACCATCCCA
                ****  ***************** * *** *****************   *********

P.capensis      CCGGCCACTCTGCCCTCTTCGGGGACA
T.manatus       CCGGCCACAGTACCCTCCTCGGGGCCG
H.gigas         CCGGCCACAGTACCCTCCTCGGGGCCG
D.dugon         CCGGCCACAGTACCCTCCTCGGGGCCG
L.africana      CCGGCCACAGTGCCCTCTTCCGGACCA
E.maximus       CCGGCCACAGTGCCCTCTTCCGGACCA
                ********  * ***** ** **  *
```

Appendix 10. Nucleotide alignment highlighting specific non-synonymous substitutions in fetal (HBG) and adult (HBA and HBB/HBD) hemoglobin genes of three *Hydrodamalis gigas* isolates. Question marks denote missing data. Sequence coverage depth following removal of sequence duplicates for each *H. gigas* isolate are indicated. The embryonic expressed hemoglobin genes of *H. gigas* (HBE and HBZ-T1) are identical to that of the sirenian ancestor (data not shown).

HBA

Nucleotide position	142	156	265	273	327	336
T.manatus translation	Leu**Ser**His	**Gly**Ser	His	**Arg**Leu	Ser	**Ser**His
T. manatus	...**C**TGAGCCACGGCTCC...		CACAGGTTG...		AGCAGCCAC...	
D. dugon	...ATGAGCCACGGCTCC...		CACAGATTG...		AGCA**A**CCAC...	
H. gigas 6852 (n=13,24,20)	...ATGA**AG**CACG**A**CTCC...		CACA**A**ACTG...		AGC**GG**CCAC...	
H. gigas 17170 (n=1,3,2)	...ATGA**AG**CACG?????...		CACA**A**ACTG...		AGC**GG**CCAC...	
H. gigas 6853 (n=1,2,1)	...ATGA**AG**CACG**A**CTCC...		CACA**A**ATTG...		AGC**GG**CCAC...	
H.gigas translation	Met**Lys**His	**Asp**Ser	His	**Lys**Leu	Ser	**Gly**His

HBB/HBD

Nucleotide position	43	51	172	186	235	249
T.manatus translation	Trp**Ala**Lys		**Pro**LysVal	**LysAla**	Glu**Asp**Leu	**LysGly**
T. manatus	...TGGGCCAAG...		CCTAAAGTGAAGGCC...		GA**A**GACCTCAAGGGT...	
D. dugon	...TGGGCCAAG...		CCTAAAGTGAAGGCC...		GACGACCTCAAGGGC...	
H. gigas 6852 (n=27,56,29)	...TGG**T**CCAAG...		**T**CTAAAGTG**C**AG**A**CC...		GAC**A**ACCTCAA**CA**GC...	
H. gigas 17170 (n=4,3,6)	...TGG**T**CCAAG...		**T**CTAAAGTG**C**AG**A**CC...		GAC**A**ACCTCAA**CA**GC...	
H. gigas 6853 (n=1,0,1)	...TGG**T**CCAAG...		????????????????...		GAC????????**CA**GC...	
H.gigas translation	Trp**Ser**Lys		**Ser**LysVal	**GlnThr**	Asp**Asn**Leu	**AsnSer**

HBG

Nucleotide position	73	81	109	117	298	306	322	330
T.manatus translation	Gly**Lys**Ala		Trp**Thr**Gln		Ser**Glu**Asn		Asn**Val**Leu	
T. manatus	...GGCAAGGCC...		TGGACCCAG...		**T**CTGAGAAC...		AACGTG**C**TA...	
D. dugon	...GGCAAGGCC...		TGGACCCAG...		CCTGAGAAC...		AACGTGATA...	
H. gigas 6852 (n=16,8,33,5)	...GGCA**C**GGCC...		TGGA**T**CCAG...		CCTGA**C**AAC...		AAC**A**TGATA...	
H. gigas 17170 (n=3,4,1,0)	...GGCA**C**GGCC...		TGGA**T**CCAG...		CCTGA**C**AAC...		????????...	
H. gigas 6853 (n=0,0,3,0)	...????????...		????????...		CCTGA**C**AAC...		????????...	
H.gigas translation	Gly**Thr**Ala		Trp**Ile**Gln		Pro**Asp**Asn		Asn**Met**Ile	

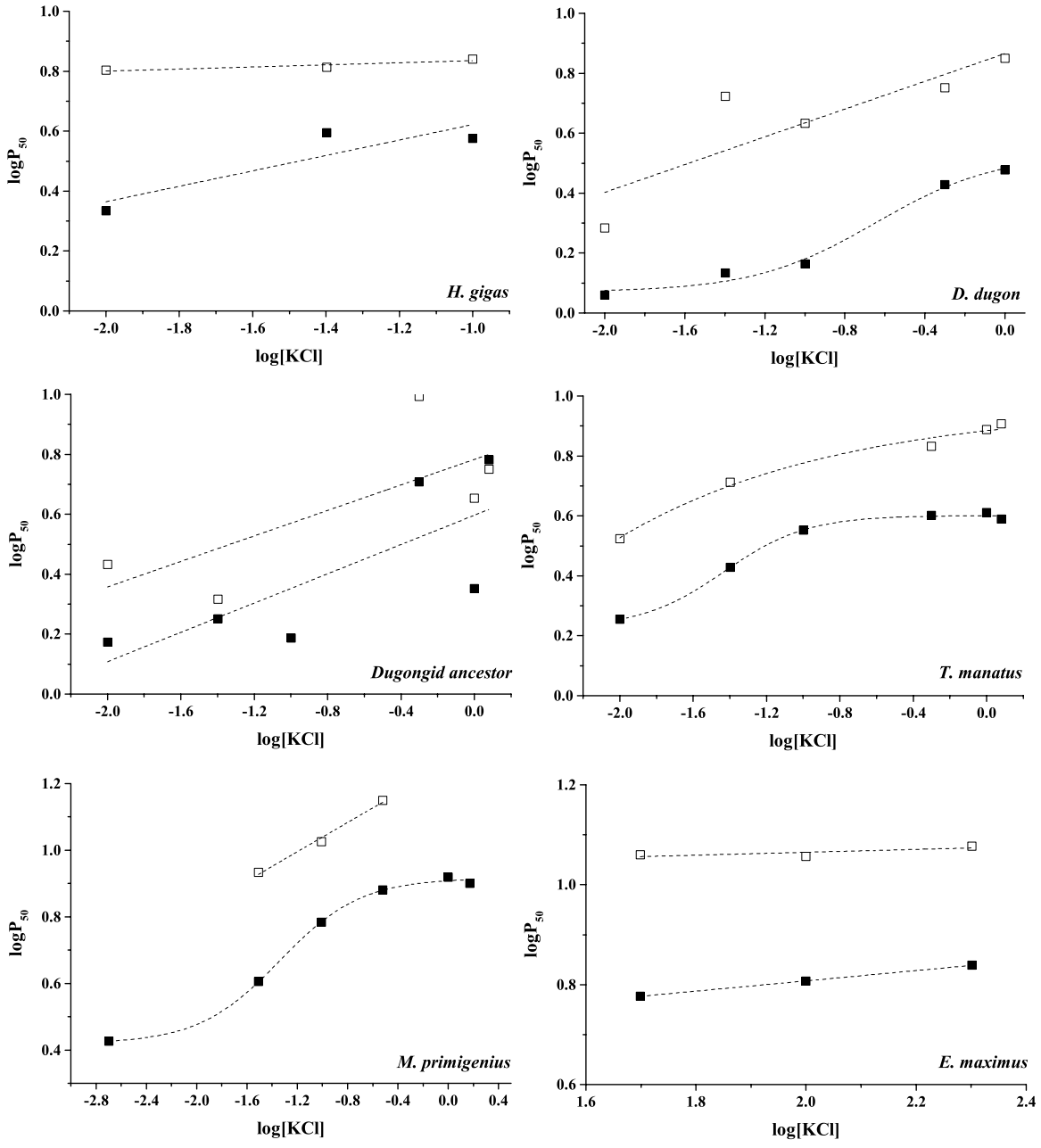
Appendix 11. Estimates of ω at specified foreground branches for each globin gene and the resulting likelihood ratio tests between H_a (estimate of ω) H_0 (ω fixed to 1). Asterisks denote branches with an ω value significantly ($p < 0.05$) different than 1.

Gene	Foreground Branch	Branch ω	lnL H_a	lnL H_0	2Δℓ	p-value
HBB/D	Stem Sirenia	0.3532	-1175.64	-1178.11	4.94	0.026*
	Stem Dugongid	1.0972	-1175.95	-1175.96	0.01	0.940
	T. manatus	1.1726	-1175.61	-1175.63	0.04	0.848
	D. dugon	>10	-1171.94	-1174.31	4.75	0.029*
	H. gigas	1.0716	-1175.75	-1175.75	0.01	0.936
HBA	Stem Sirenia	0.5420	-1170.65	-1171.04	0.79	0.375
	Stem Dugongid	0.4824	-1171.07	-1171.41	0.68	0.408
	T. manatus	0.9777	-1170.69	-1170.69	0.00	0.987
	D. dugon	>10	-1166.34	-1168.03	3.39	0.066
	H. gigas	0.6069	-1170.94	-1171.06	0.23	0.628
HBE	Stem Sirenia	0.1324	-792.75	-798.11	10.71	0.001*
	Stem Dugongid	>10	-791.64	-791.93	0.56	0.453
	T. manatus	0.0001	-792.43	-793.73	2.60	0.107
	D. dugon	0.0001	-792.80	-792.80	0.00	0.995
	H. gigas	0.0001	-792.42	-793.78	2.73	0.098
HBG	Stem Sirenia	0.0001	-799.45	-801.52	4.13	0.042*
	Stem Dugongid	0.3929	-801.46	-801.85	0.79	0.375
	T. manatus	>10	-798.63	-800.23	3.19	0.074
	D. dugon	>10	-800.57	-801.13	1.12	0.290
	H. gigas	1.3533	-801.10	-801.14	0.08	0.783
HBZ-T1	Stem Sirenia	0.5220	-775.33	-775.37	0.08	0.784
	Stem Dugongid	>10	-774.80	-775.00	0.39	0.532
	T. manatus	>10	-776.25	-776.33	0.15	0.703
	D. dugon	>10	-774.82	-775.01	0.38	0.535
	H. gigas	0.0001	-774.60	-781.24	13.28	0.0003*
Mb	Stem Sirenia	0.1461	-1063.03	-1066.62	7.18	0.007*
	Stem Dugongid	0.9778	-1062.21	-1062.21	0.00	0.993
	T. manatus	1.0308	-1061.18	-1061.18	0.00	0.988
	D. dugon	0.0001	-1062.32	-1065.09	5.54	0.019*
	H. gigas	0.1065	-1063.12	-1064.72	3.18	0.074

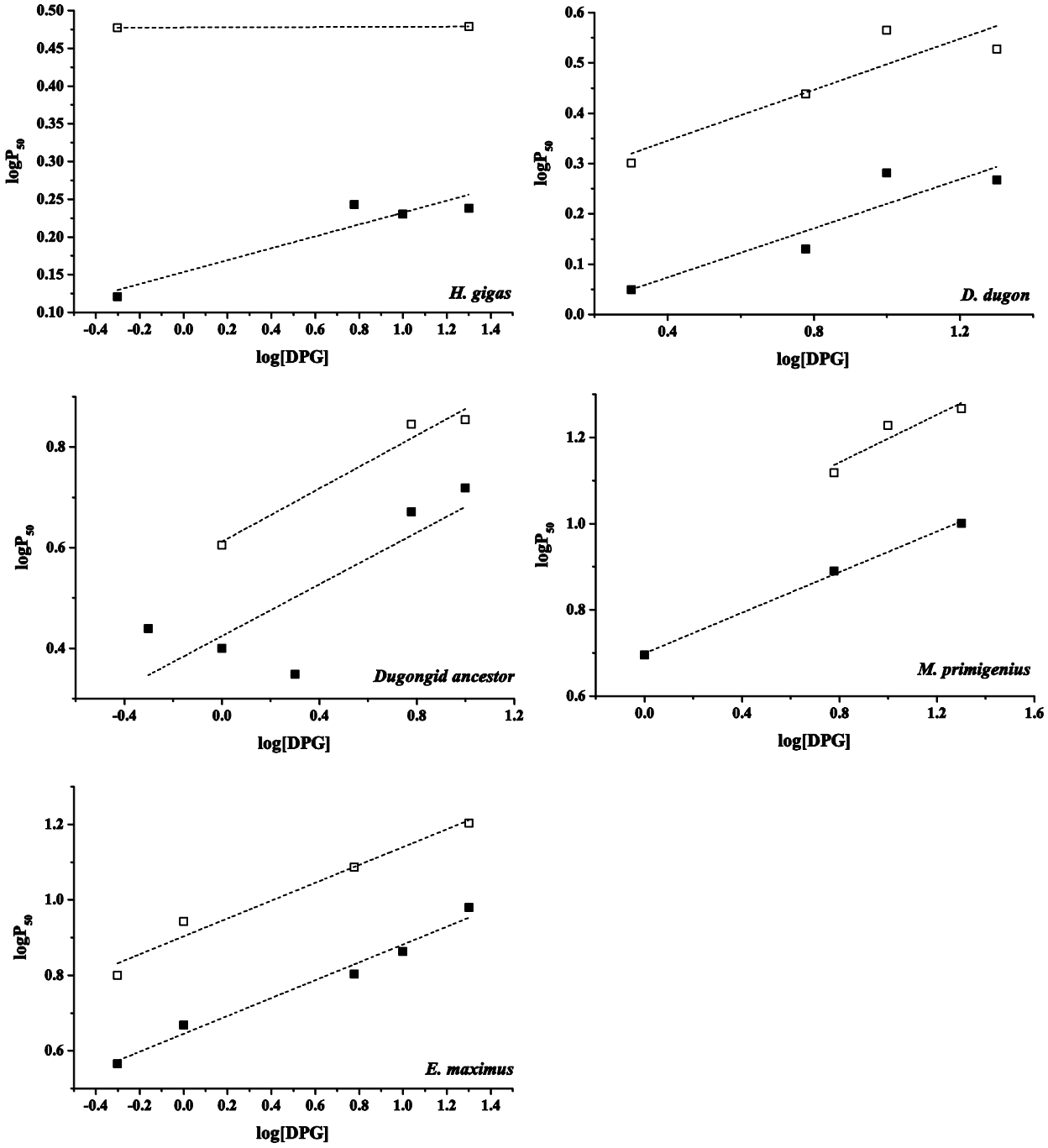
	Stem Sirenia	0.0324	-852.20	-859.79	15.17	0.0001*
	Stem Dugongid	0.2317	-852.06	-852.25	0.38	0.538
Ngb	*T. manatus*	0.0001	-852.02	-854.37	4.71	0.030
	D. dugon	0.7965	-852.46	-852.46	0.00	0.998
	H. gigas	0.2103	-852.02	-852.58	1.10	0.293
	Stem Sirenia	0.0488	-1067.57	-1080.82	26.51	0.0000002*
	Stem Dugongid	0.4982	-1068.27	-1068.27	0.00	0.998
Cygb	*T. manatus*	>10	-1065.79	-1066.15	0.72	0.397
	D. dugon	0.0001	-1067.89	-1069.75	3.72	0.054
	H. gigas	0.3124	-1067.96	-1068.06	0.20	0.653

Appendix 12. The probability of single amino acids within sirenian globin genes evolving under positive selection. Asterisks denote sites with a posterior probability >0.95 under the Bayes empirical Bayes method.

Gene	Stem Sirenia	Stem Dugongid	*T. manatus*	*D. dugon*	*H. gigas*
HBB/HBD	52 (0.782) 112 (0.582)	None	56 (0.592) 68 (0.555) 79 (0.605)	8 (0.937) 90 (0.910) 94 (0.913) 101 (0.823) 104 (0.933) 109 (0.858) 115 (0.928) 131 (0.812)	62 (0.614) 82 (0.623) 83 (0.614)
HBA	None	48 (0.515) 96 (0.542)	74 (0.795) 114 (0.874) 131 (0.876)	34 (0.826) 35 (0.836) 38 (0.819)	49 (0.525) 51 (0.723)
HBE	44 (0.778) 83 (0.922) 120 (0.629)	126 (0.519)	126 (0.519)	None	None
HBG	None	None	2 (0.705) 13 (0.901) 18 (0.760) 100 (0.845) 134 (0.760) 141 (0.884)	60 (0.669) 76 (0.698)	26 (0.556) 38 (0.642) 101 (0.644) 109 (0.640)
HBZ-T1	12 (0.892) 57 (0.755) 109 (0.863)	15 (0.602) 77 (0.935)	None	None	None
Mb	121 (0.527)	None	1 (0.542) 51 (0.937) 141 (0.957*) 145 (0.961*)	None	84 (0.720)
Ngb	149 (0.876)	50 (0.807)	None	None	70 (0.808)
Cygb	None	None	155 (0.982*) 177 (0.885)	None	131 (0.824)



Appendix 13. Plots of log[KCl] vs. log[P_{50}] for paenungulate adult Hbs (see Chapter 4 for details). Curved and straight dashed lines represent four parameter logistic and linear fits, respectively.



Appendix 14. Plots of log[DPG] vs. log[P_{50}] for paenungulate adult Hbs (see Chapter 4 for details). Straight dashed lines represent linear fits.