Molecular evolution and structure-function relationships of myoglobin in diving mammals

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Abstract

Remarkable feats of breath-hold endurance are observed in diving mammals, with some species routinely diving for an hour. During most mammalian dives metabolism remains aerobic in nature, which is accomplished by restricting the blood flow to parts of the body through peripheral vaso-constriction and bradycardia. This mechanism preserves essential oxygen (O_2) for heart and brain function, but also means some parts of the body, including locomotory muscles, become isolated and have to rely on O_2 stored within the tissues. Due to the isolation of skeletal muscles, mammalian divers must be able to buffer large quantities of H^+ ions due to the production of CO_2 during aerobic metabolism and acidic end products of anaerobic metabolism once muscle O_2 stores have been consumed. The protein responsible for storing molecular O_2 is myoglobin (Mb), a small 17 kDa monomeric globular haemoprotein with the primary function of reversible O_2 binding and facilitated diffusion of O_2 to the mitochondria. A hallmark of mammalian divers is increased Mb concentrations ([Mb]), with divers exhibiting concentrations up to thirty times those seen in non-diving species. Previous research has found that proteins at high concentrations are prone to form aggregations leading to non-functioning protein. This raises the question of Mb solubility at such high concentrations as observed in mammalian divers.

The central hypothesis of this thesis is that mammalian myoglobin has undergone previously unrecognised, parallel and adaptive evolution in several lineages of mammalian divers that has profoundly increased their maximal physiological diving capacity.

To test the hypothesis, Mb amino acid sequences of 124 mammals, including 24 newly determined sequences, are analysed for the content and individual buffering properties of their ionisable amino acids. This is used to calculate the specific Mb buffer value (β_{Mb}) for each species, which is experimentally verified by acid-base titration of purified Mb. Together with known [Mb], the contribution of Mb to whole muscle buffer capacity ($\beta_{muscleMb}$) is then quantified. Amino acid sequences are assessed for substitutions that increase modelled net Mb charge in mammalian divers compared to terrestrial species and predictions are confirmed by measuring electrophoretic mobility of purified Mb. Observed changes in Mb buffer properties and net surface charge are mapped on a composite mammalian phylogeny to test whether they are significantly linked to the evolution of diving behaviour. Observed molecular changes in Mb amino acid sequence are integrated with diving capacity, producing a model that allows prediction of maximal dive duration from Mb amino acid sequence and body mass. Using ancestral Mb sequence reconstructions and body mass estimates, the model is applied to infer the evolution of maximal diving capacity in the cetacean lineage.

Results suggest a general trend towards increasing β_{Mb} due to increased Mb histidine content in mammalian divers. $\beta_{muscleMb}$ is significantly higher in divers compared to terrestrial species, and can account for up to 45%, of the increase in whole muscle buffering observed in diving mammals. This study shows a remarkable trend in all diving species to significantly increase the net charge of the Mb protein, which would convey increased Mb solubility. This is supported by a significant correlation between Mb net charge and maximal [Mb]. Evolutionary analysis shows that high Mb net charge is significantly linked to the occurrence of diving. Contrary to previous findings, the model developed here finds that increases in [Mb] convey greater increases in dive duration than similar increases in body mass.

This study provides novel insights into how cumulative substitutions on the molecular surface of Mb can have profound adaptive effects on the physiological properties conveyed to the whole animal. It suggests that adaptation to diving in multiple lineages of mammals involved not only evolution of increased expression levels of Mb, but also substantial qualitative changes to the protein to avoid aggregation and increase solubility and buffering.

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List of abbreviations used in this study

Amino acids commonly referred to in this study:

Ala	Alanine	Gly	Glycine
Arg	Arginine	His	Histidine
Asn	Asparagine	Pro	Proline
Asp	Aspartic acid	Val	Valine
Cys	Cysteine	Ser	Serine
Gln	Glutamine	Thr	Threonine
Glu	Glutamic acid	Tyr	Tyrosine

ANCOVA	Analysis of covariance
apoMb	Myoglobin protein without its associated haem group
β_{Mb}	Specific myoglobin buffer value
$\beta_{muscleNB}$	Whole muscle non-bicarbonate buffering capacity
$\beta_{muscleMb}$	The contribution that myoglobin conveys towards whole muscle non-bicarbonate buffering capacity
BMR	Basal metabolic rate
bpm	Beats per minute
cADL	Calculated aerobic dive limit
cDNA	Complementary DNA strand synthesised from messenger RNA
CDS	Coding sequence
C-term	Carboxyl terminus at the end of a protein
deoxyMb	Myoglobin protein with no oxygen ligand present
DLT	Diving lactate threshold, defined as the duration of a dive after which post-dive blood lactate concentrations increased above pre-dive levels
DMR	Diving metabolic rate
FMR	Field metabolic rate
H^+	Hydrogen ion (proton) responsible for decreasing pH
Hb	Haemoglobin
HbCO	Carbonmonoxy haemoglobin, CO ligand present in the Hb
holoMb	Myoglobin containing its associated haem group
log t_{max}	Calculated average maximum dive duration
Mb	Myoglobin
[Mb]	Myoglobin content
[Mb_{max}]	Maximum myoglobin content
MbCO	Carbonmonoxy myoglobin, CO ligand present in the Mb
MetMb	Myoglobin in the oxidised met form
MetMbCN	Metmyoglobincyanide, a stable form of myoglobin commonly used in experimentation
MYA	Million years ago
NCBI	National Centre for Biotechnology Information
NMR	Nuclear magnetic resonance
NO	Nitric oxide
N-term	Amino terminal residue at the beginning of a protein
O_{2capMb}	Oxygen binding capacity of myoglobin
OD	optical density
OLR	Ordinary least squares regression
oxyMb	Oxygenated form of myoglobin
P_{50}	Partial pressure of oxygen at which 50% of the Hb/Mb is saturated
PAGE	Polyacrylamide gel electrophoresis

PCR	Polymerase chain reaction
p*I*	Isoelectric point, the point at which the is no overall charge on a protein
PIC	Phylogenetic independent contrasts
p*K*a	The pH at which a given ionisable group is half-maximal occupied by a proton
RACE	Rapid amplification of cDNA ends
RMR	Resting metabolic rate
Slyke	Defined as μmoles of titrant per gram wet weight of muscle tissue required to change the pH of the homogenate by one pH unit
SMART	Switching Mechanism At 5' end of RNA Transcript, a method to create cDNA with a built in primer ready for PCR
STPD	Standard temperature and pressure in dry condition
Taq	A polymerase enzyme used in PCR
TIGR	The Institute of Genomic Research also known as TGI
UTR	Un-translated region
V_{Blood}	Total blood volume
V_{Lung}	Total lung volume
VO_{2Blood}	Total blood oxygen store
VO_{2Lung}	Total lung oxygen store
$VO_{2Muscle}$	Total muscle oxygen stores,
5' end	Five prime end refers to the beginning of a gene sequence
3' end	Three prime end refers to the end of a gene sequence

Chapter 1 - Introduction

This study aims to look at the molecular evolution and structure-function relationships of myoglobin in diving mammals. For the purpose of this study diving is defined as a bout of breath-hold underwater submergence that relies upon oxygen (O_2) taken on at the surface. A dive begins as soon as an animal's head is submerged and terminates as soon as the animal returns to the surface to take another breath. A mammalian diver is a species that regularly undergoes breath-hold diving for all or part of its life's needs, no matter whether the purpose of the dive is for foraging, reduction of metabolic costs of locomotion or predator evasion (Berta et al., 2006).

Mammalian divers can be either fully aquatic or semi aquatic. Only two groups of mammalian divers, the cetaceans and sirenians, are fully aquatic, spending all of their lives in water, even to give birth. The sea otter although being able to move on land is also capable of giving birth in water and spending all of their lives in water (VanBlaricom, 2001). All other mammalian divers are semi-aquatic, spending some proportion of their lives on land, to rest, moult or to breed and give birth. In the species observed in this study diving behaviour has independently evolved nine times (**Fig 4.1** shown below, see **Chapter 4** for in depth methods and discussion). Each time diving behaviour has evolved, the problems associated with diving for air breathing mammals have been overcome through a series of physiological adaptations. The degree of adaptation tends to reflect the amount of time spent in water, with the fully aquatic cetaceans showing the biggest number of adaptations (Berta et al., 2006).

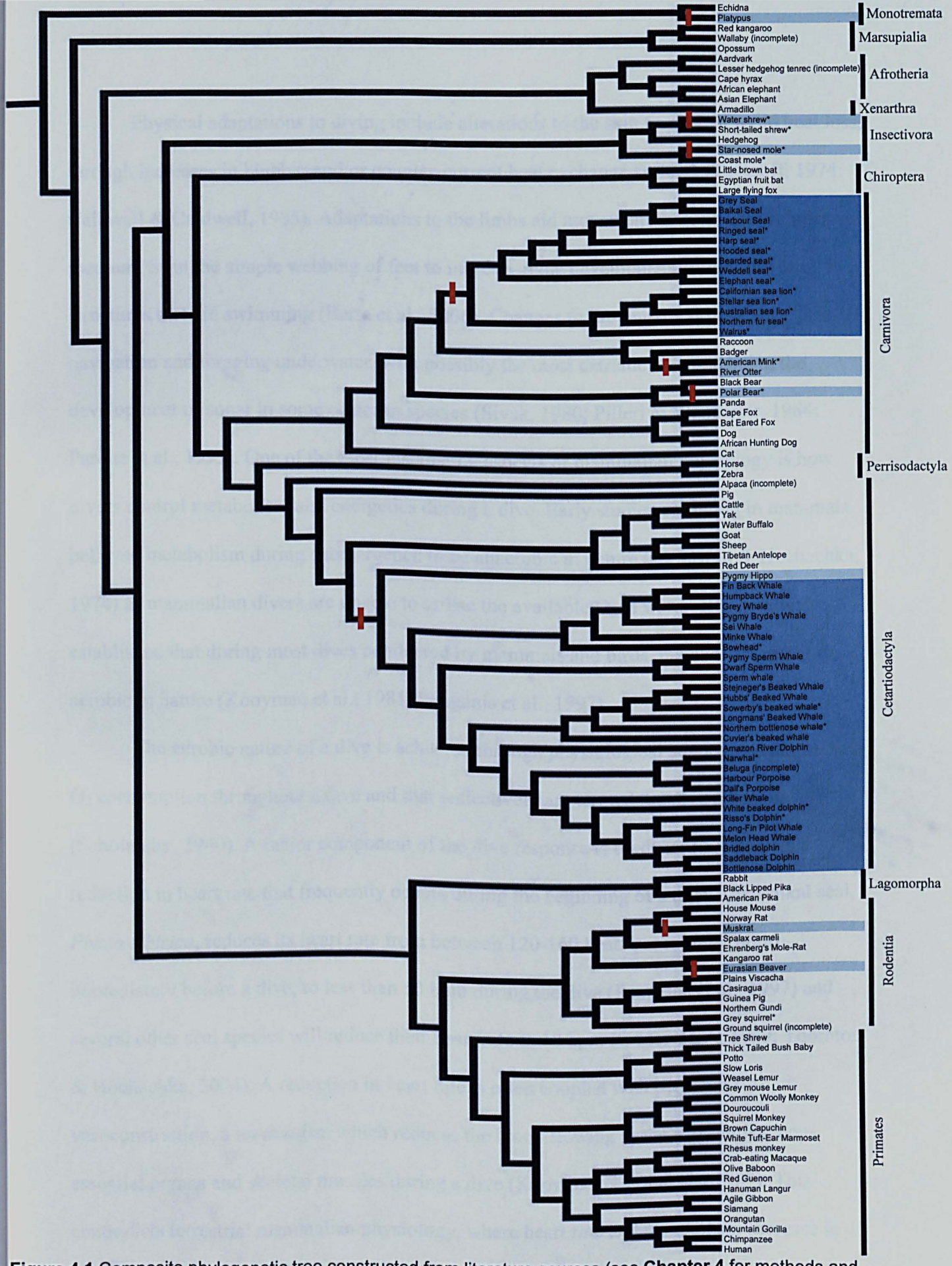


Figure 4.1 Composite phylogenetic tree constructed from literature sources (see **Chapter 4** for methods and discussion). Species in dark blue boxes are proficient divers, those in light blue are semi-aquatic divers with shorter maximum dive durations. Red bars indicate where diving behaviour evolved in a parsimony reconstruction. Black bars indicate phylogenetic orders. Species highlighted with an asterisk were sequenced for the first time in this study

Physical adaptations to diving include alterations to the skin to prevent rapid heat loss, through increases in blubber and/or counter-current heat exchange systems (Tarasoff, 1974; Caldwell & Caldwell, 1985). Adaptations to the limbs aid movement through a more viscous medium, from the simple webbing of feet to more extreme development of fins and tail structures that aid swimming (Berta et al., 2006). Changes to the sensory organs aid navigation and foraging underwater, with possibly the most extreme example being the development of sonar in some cetacean species (Sivak, 1980; Pilleri & Wanderler, 1964; Pardue et al., 1993). One of the most fascinating aspects of mammalian physiology is how divers control metabolism and energetics during a dive. Early studies on diving in mammals believed metabolism during submergence to be anaerobic in nature (see Owen & Hochachka, 1974) as mammalian divers are unable to utilise the available O_2 in water. It is now well established that during most dives conducted by mammals and birds, metabolism remains aerobic in nature (Kooyman et al., 1981; Ponganis et al., 1997).

The aerobic nature of a dive is achieved through physiological adaptations that reduce O_2 consumption throughout a dive and that collectively are termed the dive response (Scholander, 1940). A major component of the dive response is bradycardia, which is a reduction in heart rate that frequently occurs during the beginning of a dive. The Baikal seal, *Phoca sibirica*, reduces its heart rate from between 120-160 beats per minute (bpm) immediately before a dive, to less than 50 bpm during the dive (Ponganis et al., 1997) and several other seal species will reduce their heart rate to 10 bpm (Odden et al., 1999; Thornton & Hochachka, 2004). A reduction in heart rate is often coupled with peripheral vasoconstriction, a mechanism which reduces the blood flowing to the extremities, non-essential organs and skeletal muscles during a dive (Kooyman & Ponganis, 1989). This contradicts terrestrial mammalian physiology, where heart rate and metabolism increase in response to exercise (Borg et al., 1985). One of the earliest encounters of peripheral

vasoconstriction, although it was not known at the time, is found in the first description of the now extinct Steller's sea cow (*Hydrodamalis gigas*) in which it is noted that blood spurting from a back wound 'like a fountain' stopped as the animal submerged its head and blood erupted once again when the head came out of the water (Steller, 1751). The dive response can be enhanced by behavioural changes to further reduce the consumption of O_2, including modification of swimming to incorporate less energetically costly styles of locomotion such as gliding and sinking (Williams et al., 2000).

Table 1.1 Average maximum dive duration and depth data for, carnivoran, cetacean and rodent divers (highlighted in blue boxes). Maximum myoglobin (Mb) content is given for divers and close terrestrial relatives for comparison. See appendix **Table A2** for more detailed information on diving characteristics.

Species	Body mass (kg)	Dive duration (s)	Ref	Dive depth (m)	Ref	Max Mb content (g 100 g^{-1} w.wt)	Ref
Grey Seal	217	1265	1	235	1	5.4	7
Harp seal	77	1200	2	261.4	1	9.7	8
Elephant seal	655	2518	1	1034.6	1	7.9	9
California sea lion	111	462	1	224.1	1	4.9	10
Australian sea lion	125	360	1	84	1	2.7	11
Dog						0.6	12
Cat						0.3	13
Bowhead	79400	3300	1	208	1	3.5	14
Northern bottlenose whale	4750	3299	1	1152.5	1	6.3	15
Cuvier's beaked whale	2112	5220	1	1888	5	4.3	14
Bridled dolphin	80	242	1	104.3	1	2.5	12
Bottlenose Dolphin	200	480	1	213	6	2.7	14
Pig						0.5	16
Cow						0.6	17
Muskrat	1	120	3		1	1.4	18
Eurasian Beaver	18	900	4		1	1.3	19
House Mouse						0.6	20
Norway Rat						0.6	20

1 Halsey et al., 2006
2 Irving & Orr, 1935
3 Dyck & Romberg, 2007
4 MacArthur, 1992
5 Tyack et al., 2006
6 Hastie et al., 2006
7 Reed et al., 1994
8 Lestyk et al., 2009
9 Hassrick et al., 2010
10 Weise & Costa, 2007
11 Fowler et al., 2007
12 Castellini & Somero, 1981
13 Schuder et al., 1979
14 Noren & Williams, 2000
15 Scholander, 1940
16 Lawrie, 1950
17 Lawrie, 1953
18 MacArthur et al., 2001
19 McKean & Carlton, 1977
20 O'Brien et al., 1992

Mammalian divers vary in their diving abilities, which can be judged by dive duration and dive depth. The diving prowess of a species can reflect the amount of time spent in water, with the most proficient divers being the cetaceans and pinnipeds. However there is variation in diving ability within all groups of divers. Within pinnipeds phocid seals are generally able to dive deeper and longer than otariid seals (**Table 1.1**) and the most proficient divers of all are the phocid elephant seals and the cetacean beaked whales (**Table 1.1**). The duration of a dive depends upon the amount of O_2 within the body and the rate at which is consumed (Scholander 1940; Butler & Jones, 1997). There are three main reservoirs of O_2 within the body: O_2 stored in the lung, bound to haemoglobin (Hb) in the blood and bound to myoglobin (Mb) in skeletal and cardiac muscle tissue (discussed further in **Chapter 5** of this thesis). High Mb content ([Mb]) is seen as one of the key adaptations in diving mammals (Dolar et al., 1999) with divers exhibiting [Mb] up to thirty times that of close terrestrial relatives (Castellini et al., 1981; Noren and Williams, 2000) (Table 1.1).

Mb is a small 17 kDa monomeric globular haemoprotein. It was one of the first proteins to have its three dimensional structure determined in the ground-breaking work of Kendrew et al. (1958), that identified the crystal structure of sperm whale, *Physeter catodon*, Mb. The amino acid sequence for sperm whale Mb was later established by Edmundson (1965). Each mammalian Mb protein consists of 153 amino acids and one haem complex in the centre of the molecule that contains a single iron atom, which serves to reversibly bind one O_2 molecule (Kendrew, 1962) (**Fig 1.1**). Mb has been used as a model protein for understanding the general structure and functional properties of proteins and as a result many Mb amino acid sequences exist for a variety of species with over ninety protein sequence entries for mammals alone in the National Centre for Biotechnology Information database.

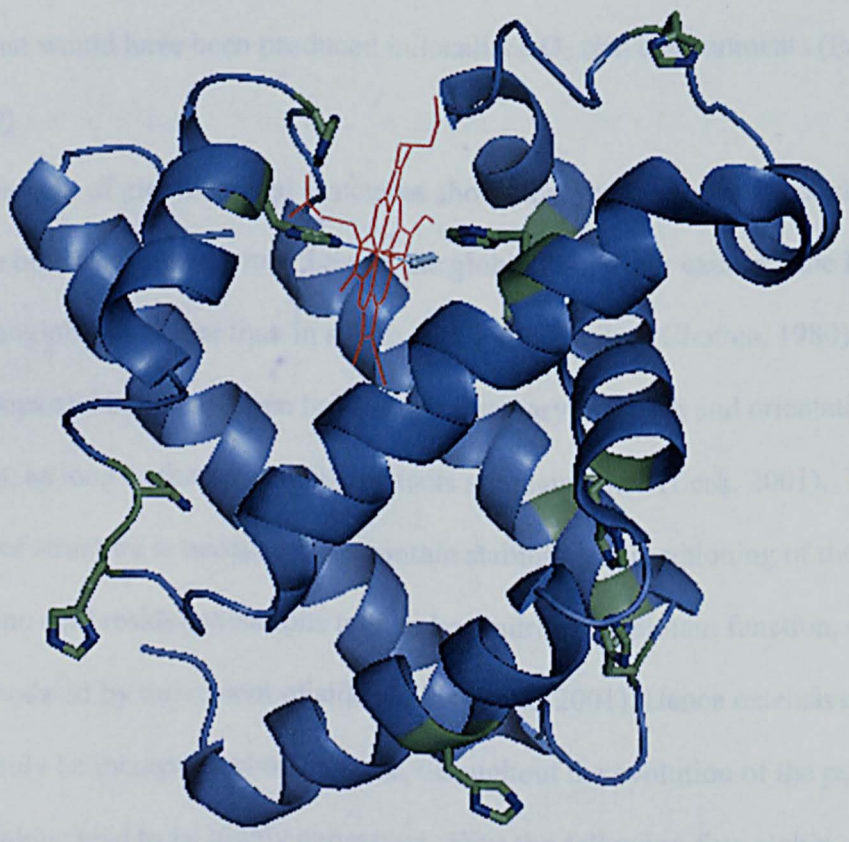


Figure 1.1 Three-dimensional structure of sperm whale oxymyoglobin. Image was created using PDB file 1MBO (Phillips, 1980) and The PyMOL Molecular Graphics System (Schrodinger, 2010). Helix backbone structure is shown in blue with the haem oxygen complex shown in red and cyan respectively. Green sticks represent histidine (His) residues, highlighting that His can occur internally, interacting with the haem pocket and externally on the surface of the protein.

Mb is a member of the globin family of proteins which are present in a large number of prokaryotes, plants and animals. Globin-like sequences are reported in archaeal, eukaryotic and bacterial genomes and are all believed to have evolved from one common ancestor (Vinogradov et al., 2007). The secondary structure of globins consists of 8 helices notated A-H. However, in mammalian globins the F helix is often divided into two segments giving 9 helices. This general trend of 8 or 9 helices has been conserved throughout globin evolution (Lesk & Chothia, 1980), which reflects the similarity in the roles that globins generally have in binding or sensing of oxygen. The earliest ancestral globin is alleged to have been used to buffer excess O_2 that would have been toxic to early anaerobic bacteria and/or sense localised increases in O_2 in a predominantly anoxic environment (Vinogradov et al., 2007). Another role believed to have been exhibited by the ancestral globin is preventing the toxicity of nitric

oxide (NO) that would have been produced in localised O_2 rich environments (Poole & Hughes, 2000).

Comparison of globin crystal structures shows that the same above noted helices can vary in length between different members of the globin family, for example the E helix in plant leghaemoglobin is longer than in sperm whale Mb (Lesk & Chothia, 1980). The connecting loops and bends between helices can also vary in length and orientation between globin species, as long as the helix-helix contacts are maintained (Lesk, 2001). This conservation of structure is necessary to maintain stability and functioning of the protein. Thus any amino acid residue mutations tend to be neutral, to maintain function, or they have to be accommodated by movement of side chains (Lesk, 2001). Hence extensive sequence changes can only be incorporated one by one, throughout the evolution of the protein. For this reason globins tend to be highly conserved. Thus the following five globin proteins, which are all primarily involved in O_2 handling, are approximately 35% identical: sperm whale and horse, *Equus caballus*, Hb alpha and beta chains, and sperm whale Mb (Lesk, 2001). In contrast, complete Mb sequences from 118 mammalian species in this study have a range of identity of 72-100%, between species. This shows that the structure and therefore presumably the functional properties of Mb are highly conserved between different animals, something that is not shared with other proteins. For example Mb's larger, tetrameric 'cousin' Hb is a protein that shows structural complexity and functional variability that is famously adapted to different animals' needs (Rossi-Fanelli et al., 1960; Perutz, 1983; Berenbrink et al., 2005; Weber & Campbell, 2011).

The primary function of Mb is the storage of O_2 in skeletal and cardiac muscle tissue of vertebrates where it can be released in response to increased O_2 requirements during bursts of exercise or breath-hold diving . Another major function is the facilitated diffusion of O_2 from the sarcolemma to the mitochondria within oxidative muscle cells, (Wittenberg &

Wittenberg, 2003). The exact mechanisms of the facilitated diffusion of O_2 have yet to be established but it is known that molecular O_2 will be bound and released by many Mb molecules before the mitochondria are reached (Gros et al., 2010).

Mb has a high O_2 affinity, allowing it to remove a large proportion of oxygen from the blood. The partial pressure where 50% of the protein is saturated with O_2 (P_{50}) under physiological conditions in muscle and blood is 0.43 mmHg for horse Mb (Wittenberg & Wittenberg, 2007) and 0.73 mmHg for human Mb (Rossi-Fanelli et al., 1960) compared to the P_{50} of human Hb of 26.6 mmHg (Dejours, 1981). This may account for blood flow to the muscles being restricted during dives, ensuring that the vital organs, such as the heart and brain, receive a constant supply of oxygen (Davis et al., 2004). Under the same experimental conditions the P_{50} of Mb is very similar between species with a P_{50} of 0.9 mmHg and 0.73 mmHg reported for tuna and human Mb respectively (Rossi-Fanelli et al., 1960). It has also been noted that Mb O_2 affinity is not affected by pH, and that both mammalian Mb and tuna Mb show complete absence of the Bohr effect between pH 7 and 8 (Rossi-Fanelli et al., 1960).

In recent years Mb has been the subject of numerous investigations, due to the exciting discovery of novel functions of the Mb protein. Previously believed to exist only in cardiac and oxidative skeletal muscle tissues, Mb has recently been found to be expressed in non-muscle tissues, including liver, kidney, gill and brain of carp, *Cyprinus carpio*, and zebrafish, *Danio rerio*, (Cossins et al., 2009), suggesting that Mb has additional roles that are not yet completely understood. One of the most astonishing findings has been the creation of a Mb knockout mouse that is able to perform normally under hypoxia and exercise (Garry et al., 1998). The knockout mouse shows increase in capillary concentration both within cardiac (Gödecke et al., 1999) and skeletal muscle tissue (Grange et al., 2001; Meeson et al., 2001), but also shows an increase in the expression of hypoxia induced genes which may serve to

regulate the physical alterations observed in order to maintain O_2 delivery (Grange et al., 2001).

A novel function concerning the binding of NO has been the focus of recent studies due to medical implications. The properties of NO mean it can be both advantageous and harmful. NO can act as a vaso-dilator but can also interfere with the functioning of mitochondrial respiration by inhibiting cytochrome *c* oxidase (Brunori, 2001). As Mb is able to bind NO it has been shown to play a role in scavenging NO in a normoxic environment (Brunori, 2001; Hendgen-Cotta et al., 2010). On the other hand, in hypoxic environments deoxygenated Mb has been shown to produce NO from nitrite (Hendgen-Cotta et al., 2010), aiding in the preservation of mitochondrial function. This is supported by observations that hypoxia induced cardiac injury in Mb knockout mice can be alleviated by NO synthases (Mammen et al., 2003). NO binding has also been shown to aid in hypoxia tolerance in goldfish, *Carassius auratus*, by increasing myocardial efficiency and maintaining mitochondrial function in extreme hypoxia (Pedersen et al., 2010).

Mb has been extensively studied for fifty years and been used as a model to discover functional aspects of other proteins. Some studies have looked at mutating amino acid residues within the Mb protein of a few species and attempted to discover functional properties of amino acid changes based on these mutations (DiMarchi et al., 1978a; Quillin et al., 1993; Scott et al., 2000). Mb amino acid sequences are known for many species and authors usually compare a new Mb amino acid sequence to the first Mb to be sequenced, that of the sperm whale, or to other closely related species (Bradshaw & Gurd, 1969; Dwulet et al., 1975; Wang et al., 1977; DiMarchi et al., 1978b). It seems strange that no study has systematically looked at the naturally occurring differences of Mb between species and attempted to explain the functional differences conveyed by amino acid substitutions to whole organism physiology in what is a highly conserved protein. This is the more so as

recent evidence has shown that Mb is more than just an O_2 store in cardiac and skeletal muscle tissue as, previously believed.

This thesis aims to explore specific amino acid substitutions in the Mb sequence of the widest possible range of mammalian species in an attempt to explain any functional properties that those substitutions may convey towards increased diving ability. It will then look at how and when these substitutions may have originated throughout the evolution of multiple lineages of diving mammals

Tissue proton buffers are generally divided into bicarbonate and non-bicarbonate buffers. The magnitude of the former depends on the tissue bicarbonate concentration, which is similar in all mammals (Fernandez et al., 1989). Diving mammals have been shown to have increased whole muscle non-bicarbonate buffering ($\beta_{muscleNB}$) compared to terrestrial species (Castellini & Somero, 1981). Although most dives remain aerobic in nature (Kooyman et al., 1981) all mammalian divers regularly exceed their diving lactate threshold (DLT), defined as an increase in post dive lactate concentration above pre-dive levels, and when this happens muscle tissues have to rely on anaerobic metabolism for locomotion (see **Chapter 5** for discussion). Dives that exceed DLT tend to be predator evading or foraging dives and have been observed in Weddell seals, *Leptonychotes weddellii*, and beaked whales, *Ziphius* spp. and *Mesoplodon* spp, that perform very long, deep foraging dives followed by shorter, shallower non-foraging dives, believed to be necessary to repay the oxygen debt built up during the previous deep dive (Castellini et al., 1992; Tyack et al., 2006). This capacity for anaerobic exercise allows diving mammals to remain submerged for the maximum durations observed (Ponganis et al., 1990). During these times of anaerobic metabolism, acidic end products need to be buffered in order for the muscle to continue functioning properly. Proton buffering by non-bicarbonate buffers is primarily achieved through free histidine (His), His in proteins and His related dipeptides (Abe, 2000). Most protein side chains are either

hydrophobic or hydrophilic meaning they occupy internal or external positions within the tertiary structure of a protein (Lesk, 2001). His in Mb is a rare, polar, exception that has internal residues in contact with the haem group, as well as external residues (Kendrew, 1962). It has been shown that maximum Mb concentrations correlate with $\beta_{muscleNB}$ (Castellini & Somero, 1981). Although not completely responsible for elevated $\beta_{muscleNB}$ in divers, Mb may play an important role in buffering acidic end products of anaerobic dives. This would be shown by increases in His residues in the Mb protein of mammalian divers compared to terrestrial species.

As previously mentioned, increased Mb concentrations are an important adaptation in diving mammals for the provision of O_2 during a dive. However some studies have shown that at high concentrations proteins will cause aggregations (Rumen & Appella, 1962; Fink, 1998). In the most severe cases this can cause the protein to become non-functional and protein aggregations are linked to a number of human medical conditions including; Alzheimer's disease (Joachim and Selkoe, 1992), Huntingdon's disease (Scherzinger et al., 1997) and Parkinson's disease (Lansbury, 1999). The elevated concentrations of Mb in mammalian divers may be high enough to cause issues with aggregation, unless alterations are made to the protein to increase solubility. The structure, solubility, stability and functioning of a protein depends on the net charge of that protein and on the ionization state of the individual residues within the amino acid sequence. A protein is least soluble at its iso-electric point (p*I*), where the overall net charge of the protein is zero (Pace et al., 2009). Research has shown that the addition of strongly positively charged residues by mutating the primary sequence of ribonuclease Sa, an enzyme involved in RNA degradation, the solubility of the protein can be dramatically increased (Shaw et al., 2001). Therefore alterations in the primary sequence that increase the overall net charge of the Mb protein and any less stable precursors, may provide an advantage to mammalian divers as it may facilitate high Mb

expression rates without the negative consequences of increased aggregation and loss of function.

The central hypothesis of this thesis is that mammalian Mb protein has undergone previously unrecognised, parallel and adaptive evolution in several lineages of mammalian divers that has profoundly increased their maximal physiological diving capacity.

To test the hypothesis, a number of interrelated approaches are taken. Firstly, the Mb amino acid sequences of 124 mammals, including from 9 diving lineages and 24 here newly determined sequences, are analysed for the content and individual buffering properties of their ionisable amino acids (**Chapter 2**). This information is used to calculate the total specific Mb buffer value for every species, which is then experimentally verified by acid-base titration of purified Mb protein from selected mammalian divers and their close terrestrial relatives. Together with known Mb muscle contents, this allows to quantify the contribution of Mb to the increased whole muscle buffer capacity described in mammalian divers (**Chapter 2**).

Second, amino acid sequences are assessed for substitutions that increase the modelled overall net charge of Mb in mammalian divers compared to terrestrial species. These predictions are experimentally confirmed by determining the electrophoretic mobility of purified Mb from selected diving and terrestrial mammals (**Chapter 3**).

Third, the observed changes in Mb buffer properties and net surface charge are mapped on a composite mammalian phylogeny to test whether they are significantly linked to the evolution of diving behaviour (**Chapter 4**).

Fourth, the observed molecular changes in Mb amino acid sequence are integrated with whole animal diving capacity. A model is developed that allows prediction of maximal

dive duration in living mammals, from water shrew to sperm whale, based on their body mass and Mb amino acid sequence alone. Using ancestral Mb sequence reconstructions and body mass estimates, the model is finally applied to infer the evolution of maximal diving capacity in the cetacean lineage (**Chapter 5**)

Support and weaknesses of the hypothesis are generally discussed in a concluding final chapter (**Chapter 6**).

Chapter 2 – Myoglobin (Mb) specific buffer capacity and the contribution of Mb to whole muscle non-bicarbonate buffering capacity

Introduction

Metabolism during most mammalian dives has been shown to be aerobic in nature (Kooyman et al., 1980) and in order to achieve this; mammalian divers have evolved many specialised adaptations. One of these is localised vaso-constriction upon initiation of a dive (Kooyman & Ponganis, 1998). This enables blood oxygen (O_2) to be reserved for essential organs. However, as a result the skeletal muscle beds become isolated and have to rely on their own O_2 stores in order to maintain aerobic respiration. This is achieved through elevated muscle myoglobin (Mb) content which is seen as a hallmark of mammalian divers. Mb content can increase over 15-fold in locomotory muscles of divers compared to those of their close terrestrial relatives (Castellini et al., 1981). The sperm whale, *Physeter catodon*, locomotory muscle (longissimus dorsi) has a Mb content of 7.0 g 100 g^{-1} wet weight (Sharp & Marsh, 1953) whereas the pig, *Sus scrofa*, locomotory muscle (psoas) has a Mb content of 0.43 g 100 g^{-1} wet weight (Lawrie, 1952). Assuming a typical mammalian muscle water content of 75% (Sharp & Marsh, 1953) then Mb would constitute almost 30% of muscle dry matter in the sperm whale.

There is increasing support suggesting that O_2 reservoirs (lung, blood and muscle) in diving mammals are consumed separately during a dive and that a dive is terminated when one or more stores are exhausted. Models of calculating aerobic dive limits (cADL) have indicated that cADL is reached as muscle O_2 stores become fully desaturated, yet at this point arterial and venous blood may still contain 45 and 23% O_2 respectively (Davis & Kanatous, 1999). This has been most clearly shown recently in Emperor penguins, *Aptenodytes forsteri*, demonstrating that lung and blood O_2 stores are reserved for essential organs and tissues and are never fully desaturated during a dive (Ponganis et al., 2010). Muscle stores however,

being isolated from circulating blood, are reliant on Mb bound O_2 to power locomotion. It has been shown in the Emperor penguin that muscle O_2 stores can be completely desaturated during some dives. Thus, when the penguins reach their aerobic dive limit (ADL) at 5.6 min, any continuation in dive duration means that the skeletal muscles are solely reliant on anaerobic metabolism (Williams et al., 2011). For further discussion on diving capacity see **Chapter 5**.

Even with high Mb concentrations providing a store of O_2, all mammalian divers exceed their aerobic dive limits on occasions (Kooyman et al., 1980; Ponganis et al., 1997; Butler, 2006), this tends to occur during predator evasion or foraging dives (Butler & Jones, 1997). When this happens mammalian divers need to be able to buffer large accumulations of H^+ ions due to lactic acid production (Scholander, 1940; Costa et al., 2004). Even during dives within their ADL divers have to be capable of buffering acidic conditions due to the production of CO_2 during aerobic metabolism, which cannot be washed out from the muscle due to localised vaso-constriction that redirects blood flow and isolates muscle beds. There is evidence to show that the increase in muscle H^+ during exercise is not the result of lactic acid production but rather the hydrolysis of non-mitochondrial ATP during aerobic cellular metabolism (Gevers, 1977; Hochachka & Mommsen, 1983; Busa & Nuccitelli, 1984; Reviewed in Robergs et al., 2004). This therefore suggests that diving species must be able to buffer increases in proton concentrations during all dives.

Tissue proton buffers are generally divided into bicarbonate and non-bicarbonate buffers and the magnitude of the former depends on the tissue bicarbonate concentration, which is similar in all mammals (Fernandez et al., 1989). It has been noted that mammalian divers have significantly increased whole muscle non-bicarbonate buffering capacity compared to their terrestrial counterparts (Castellini & Somero, 1981). Apart from organic and inorganic phosphates, intracellular non-bicarbonate buffering in muscle tissue occurs

predominately due to the uptake of protons by the imidazole group of histidine (His) residues in proteins, free L-histidine and His-related dipeptides (Abe, 2000). Studies have revealed that the concentrations of three His-related dipeptides, namely balenine, carnosine and anserine, vary considerably among mammalian species (Crush, 1970; Abe, 1995). Some cetaceans have large amounts of balenine in their muscles, the fin, *Balaenoptera physalus*, sei, *Balaenoptera borealis*, blue, *Balaenoptera musculus* and Northern bottlenose, *Hyperoodon ampullatus*, whales have approximately 4.27 mmoles 100 g^{-1} (Crush, 1970), which would considerably aid non-bicarbonate buffering capacity. This is not the case for all cetaceans; the bottlenose dolphin, *Tursiops truncatus* and Risso's dolphin, *Grampus griseus*, have approximately 2.01 mmoles 100 g^{-1} balenine in the muscles of adults (Crush, 1970). This is comparable to the carnosine content of 2.05 mmoles $100g^{-1}$ in the gluteus muscles of horse, *Equus caballus* and the anserine content of 2.00, 2.08, 1.87, and 1.33 mmoles $100g^{-1}$ in the muscles of the cat, *Felis catus*, rabbit, *Oryctolagus cuniculus*, viscacha, *Lagostomus maximus* and rat (unnamed species), respectively (Crush, 1970). The carnosine content in human, *Homo sapiens*, muscle for comparison is 0.8 mmoles 100 g^{-1} (Abe, 1995). Therefore His-related dipeptides will vary in their contributions towards whole muscle non-bicarbonate buffering among different mammalian species.

A cursory look at published amino acid sequences indicates that mammalian divers tend to have an increased number of His residues in their Mb sequence compared to their terrestrial relatives. For example, within the order Cetartiodactyla, the sperm whale has 12 His residues compared to 9 His in the pig. Within Carnivora the grey seal has 13 His while the dog has 10 His. Similarly, within Rodentia, the muskrat has 11 His while the rat only has 6 His residues (**Table 2.1**).

Table 2.1 Number of His residues in Mb of some mammalian species. Species highlighted in blue are diving species.

Species	Scientific name	Mb Accession No	His No
Sperm whale	*Physeter catodon*	P02185	12
Pig	*Sus scrofa*	NP_999401	9
Grey Seal	*Halichoerus grypus*	P68081	13
Dog	*Canis lupus familiaris*	P63113	10
Muskrat	*Ondatra zibethicus*	P32428	11
Norway Rat	*Rattus norvegicus*	NP_067599	6

Curiously these His changes seem not to have been noted before or at least have not been incorporated into a detailed analysis that may convey an understanding of their properties and what this potentially means for whole muscle non-bicarbonate buffering. This preliminary comparison suggests that Mb His residues could aid whole muscle proton buffering in diving species. Indeed Castellini and Somero (1981) found a strong positive correlation between Mb concentration and whole muscle non-bicarbonate buffering capacity, however these authors stressed that this relationship was likely not causal and that even though Mb existed in high concentrations in muscles of diving mammals, it was not the main component of muscle non-bicarbonate buffering capacity (Castellini and Somero, 1981).

This study sets out to quantify the extent to which Mb contributes to whole muscle buffering capacity in four orders of mammalian divers and their closest terrestrial relatives. To this end the translated amino acid sequence of 22 diving and 4 terrestrial mammals were newly determined from their cDNA. Using acid-base titration of purified Mb from 4 terrestrial and 6 diving mammals a method was then developed that allowed accurate modelling of the specific buffer value (β_{Mb}) for any Mb from its amino acid sequence. Using this model it is possible to calculate the contribution of Mb to whole muscle non-bicarbonate buffering capacity for any species where Mb concentration and whole muscle buffering capacity data is available.

Methods

Tissue samples

Tissue samples were obtained from various sources (**Table A4**). Whole hearts of cow, *Bos taurus*, sheep, *Ovis aries* and pig, *Sus scrofa*, were obtained from slaughter houses in the area of Liverpool, Merseyside, UK. Samples of the left ventricle were freed from fat and connective tissue and snap frozen in liquid nitrogen within two hours after the death of the animals.

Muscle biopsies from the majority of pinniped species were provided by Dr Jennifer Burns, University of Alaska, Anchorage. They were taken according to U.S legislation and frozen as soon as possible and imported on dry ice under Defra licence POAO/2009/58. These samples comprised ringed seal, *Phoca hispida*, harp seal, *Pagophilus groenlandicus*, hooded seal, *Cystophora cristata*, bearded seal, *Erignathus barbatus*, Weddell seal, *Leptonychotes weddellii*, Northern elephant seal, *Mirounga angustirostris*, Californian sea lion, *Zalophus californianus*, Steller sea lion, *Eumetopias jubatus*, Australian sea lion, *Neophoca cinerea*, Northern fur seal, *Callorhinus ursinus*, and walrus, *Odobenus rosmarus*.

Some tissue samples could be obtained from stranded animals in the UK. Thus samples from minke whale, *Balaenoptera acutorostrata*, humpback whale, *Megaptera novaeangliae*, Risso's dolphin, *Grampus griseus*, Northern-bottlenose whale, *Hyperoodon ampullatus*, white beaked dolphin, *Lagenorhynchus albirostris,* and Sowerby's beaked whale, *Mesoplodon bidens*, were obtained through collaboration with Dr Robert Deaville, project manager of the UK Cetacean Strandings Investigation Programme, Institute of Zoology, Zoological Society of London.

Tissue samples from stranded common dolphin, *Delphinus delphis*, and harbour porpoise, *Phocoena phocoena*, were donated by Dr James Barnett & Dr Nick Davison, Veterinary Laboratories agency, UK and from the grey seal, *Halichoerus grypus*, by Dr

Dominic McCafferty, University of Glasgow, UK. Samples were taken as soon as possible after death, however they were not always fresh and some species had been deceased for an unknown time before samples could be collected. Tissues were transported on ice to a facility where they were subsequently frozen.

Tissue samples received from Dr Kevin Campbell, University of Manitoba, Canada; water shrew, *Sorex palustris*, short-tailed shrew, *Blarina brevicauda*, star-nosed mole, *Condylura cristata*, coast mole, *Scapanus orarius*, muskrat, *Ondatra zibethicus*, narwhal, *Monodon monoceros* and bowhead, *Balaena mysticetus*. Narwhal and bowhead whale tissues were further processed (see below) in the laboratory of Dr Kevin Campbell.

Tissue samples received from Dr Julian Chantrey, University of Liverpool, UK; grey squirrel, *Sciurus carolinensis* and Dr Xavier Lambin, University of Aberdeen, UK; American mink, *Neovison vison*, were the result of trapping events and hence tissues were not taken immediately after death, but taken as and when traps were checked and then dissected and frozen.

Tissue samples from the raccoon, *Procyon lotor*, and the polar bear, *Ursus maritimus*, were obtained from animals killed by licenced hunters and kindly provided, respectively, by Heinz Pauleickhoff, Hövelriege, Germany, and Dr. Einar Arnason, Institute of Biology, University of Iceland.

A sample for the pygmy hippo, *Choeropsis liberiensis*, was provided from Dr Edmund Flach, Institute of Zoology, Zoological Society of London, from an individual that died while at Whipsnade zoo. All tissues, once received at The University of Liverpool, were stored at -80°C until further processed (**Table A4**).

Whole muscle homogenate titration

Whole muscle non-bicarbonate buffering capacity was determined according to the methods described by Castellini and Somero (1981). Briefly, half a gram of frozen tissue was thawed and homogenised in 10 ml of ice cold normal saline (0.9% NaCl), using an Ultra-Turrax T25 basic, setting 3 (13,500 rpm), and then equilibrated to 37 °C. The sample was then titrated between approximately pH 6 and 7 with 0.1M NaOH using a PHM82 standard pH meter, and G2040C pH glass electrode with a K4040 calomel reference electrode (all Radiometer, Copenhagen, Denmark). Buffering capacity was measured in slykes, defined as μmoles of titrant per gram wet weight of muscle tissue required to change the pH of the homogenate by one pH unit (between pH 6.0-7.0).

Myoglobin content determination

Mb content [Mb] was determined for white-beaked dolphin, Risso's dolphin and raccoon using a modification of Reynafarje's method (Reynafarje, 1963), which allows quantification of [Mb] in the presence of Hb by using differences in the extinction coefficients of the carbonmonoxy (CO) derivatives of these globins. Briefly, thawed muscle tissue (approximately 150 mg) was homogenised in ice cold 0.04 M phosphate buffer pH 6.6 using an Ultra-Turrax T25 basic, setting 3 (13,500 rpm) in a ratio of 24.25 ml per g of tissue for the cetaceans and 9.25 ml per g of tissue for the raccoon. Homogenisation periods were three times of 10 sec with 1 min standing on ice, ensuring that the sample was uniformly homogenised without undue warming. The homogenate was then centrifuged for 70 min at 15,600 *g* and 4°C. The supernatant was then transferred to an especially manufactured Eschweiler glass tonometer (Eschweiler, Kiel, Germny) to which a 1 cm path-length optical glass cuvette had been sealed. The sample was then equilibrated with humidified ~10% carbon monoxide (CO) for 10 min and the conversion of the mixture of oxy and deoxy Mb

and Hb to MbCO and HbCO, respectively, and was followed spectrometrically using a Unicam UV500 spectrophotometer over a wavelength range of 500-700 nm. Any metmyoglobin or methaemoglobin (MetMb or MetHb) present was reduced by addition of sodium dithionite crystals and the sample was equilibrated with CO for another 3 min. Again the completion of the reaction to the CO derivatives of the globins was followed spectrometrically. The optical density was then taken at 538 and 568 nm and [Mb] was determined from the difference between the optical densities at the two wavelengths, according to **Equation 2.1** (Reynafarje, 1963)

$$[Mb]\ (mg\ g^{-1}) = (OD_{538} - OD_{568}) \times a \qquad \textbf{Equation 2.1}$$

where OD is the optical density and *a* is a multiplication factor that corresponds to the volume of the sample used (see Reynafarje, 1963), in this case *a* equals 146.625 for cetaceans and 58.56 for the raccoon. [Mb] is given for these three species along with reported values for other mammalian species in **Table A3**.

RNA extraction and cDNA synthesis

Approximately 50 mg of tissue was blade homogenised using an Ultra-Turrax T25 basic, setting 6 (24,000 rpm). Total RNA was extracted following the Trizol method (Invitrogen). The concentration and quality of RNA was determined using a labtech NanoDrop ND-1000 spectrophotometer and by 1.5% agarose gel electrophoresis (for technique see Sambrook and Russell, 2001). 5 μg/μl of total RNA was then used in a standard SuperScript II (Invitrogen) reverse transcriptase reaction, to create first strand cDNA.

PCR

Myoglobin (Mb) cDNA coding sequences (CDS) of cetaceans, seals and polar bear were amplified by polymerase chain reaction (PCR) using forward and reverse primers (Sigma; **Table 2.2**) that were designed based on consensus alignments in the 5' and 3' un-translated regions (UTRs) of known Mb nucleotide sequences from closely related species . Primers for rodent Mb were taken from Bianchi et al. (2003). Insectivore PCRs were conducted using rodent primers to obtain a core fragment of the Mb sequence. 3' rapid amplification of cDNA ends (RACE) was then conducted following the SMART method (Invitrogen), using a SMART reverse primer and a consensus forward primer (3' Mb probe) based on a conserved region between position 306 and 326 in the mammalian Mb nucleotide sequence. Primers were diluted to 10 μM working stocks and PCR was carried out using Fast Start High Fidelity Taq DNA Polymerase (Roche) in a reaction mix containing: 5 μl 10X reaction buffer, 1 μl DMSO, 1 μl dNTP mix (10 mM each), 0.5 μl enzyme mix, 2 μl each primer, 2 μl of first stand cDNA and made up to 50 μl with nuclease-free water. Initial denaturation took place at 95°C for 2 min, followed by 30 cycles of (95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds) with a final extension step of 72°C for 7 min. RACE PCR was conducted according to the SMART manual (Invitrogen).

Table 2.2 Oligonucleotide primer sequences that were used during PCR. Rodent primers were taken from Bianchi et al. (2003). Mb = myoglobin, Fwd = forward primer, Rev = reverse primer, Cet = cetacean and Gen = a general forward primer based in the 5' UTR.

Oligonucleotide	Sequence
Cet.Mb.Fwd	5'-AGCTGTCGGAGCCAGGAYAC-3'
Cet.Mb.Rev	5'-GCCYCTCACAAACAAAGCAGG-3'
Seal.Mb.Fwd	5'-CCCAGCTGTCAGAGCCAGGACACC-3'
Seal.Mb.Rev	5'-CAAAGCAGACACTCAGAAGCAAAC-3'
Rodent.Mb.Fwd	5'-GGAATTCCATATGGGGCTCAGTGATGGGGAGTGGCAGC-3'
Rodent.Mb.Rev	5'-GTACAAGGAGCTAGGCTTCCAGGGCTAAGGATCCGGG-3'
Gen.Mb.Fwd 1	5'-CTTCAGACTGTGCCATGGGGCTCAG-3'
Gen.Mb.Fwd 2	5'-CTTCAGACTGTGCCATGGTGCTCAG-3'
3'.Mb.Probe	5'-CAAGTACCTGGAGTTCATCTC-3'

Sequencing

Unused primers and excess nucleotides were removed with ExoSap-IT (USB Corp) and sequencing products were generated using Big-Dye v3.1 terminators (Applied Biosystems), these sequencing products were then precipitated using ethanol and subjected to electrophoresis on an Applied Biosystems ABI3130XL capillary sequencer.

Alignments

A search of scientific databases including the National Centre for Biotechnology Information (NCBI), The Institute of Genomic Research (TIGR) and the Ensembl genome browser, for mammalian Mb amino acid and nucleotide sequences was conducted from Oct 2007 to Nov 2010. The extensive search was conducted on published sequence data and un-annotated genomes for sequences or partial sequences matching Mb.

Newly formed nucleotide sequences were aligned using Clustal W multiple alignment algorithm in BioEdit v7.0.5 (Hall, 2001), amino acid coding sequences were determined by comparisons to published sequences of closely related species. The nucleotide sequences were then translated to amino acids using the translate function within the BioEdit program and combined into an alignment with published mammalian protein sequences (**Table A1**).

Mb Purification

Muscle tissues from eight species; Sowerby's beaked whale, Northern bottlenosed whale, Risso's dolphin, humpback whale, minke whale, cow, sheep, and pig, were thawed and homogenised on ice using an Ultra-Turrax T25 basic, once at setting 2 (9,500 rpm), for 10 sec, followed by three times at setting 3 (13,500 rpm) in cold 20 mM phosphate buffer at a ratio of 5 ml g^{-1} of tissue (pH 6.0 for sheep, pig, humpback whale and minke whale and pH 6.5 for the rest). The samples were centrifuged at 10,500 *g* for 20 min at 4°C and the supernatant filtered through Whatman 'grade A' filter paper, then transferred to a clean beaker where the pH was adjusted with phosphoric acid back to the original pH of the buffer used for that sample.

This study used a two-step approach of cation exchange followed by gel filtration, removing the need for initial ammonium sulphate precipitation and therefore reducing loss of protein (O'Brien, 1992). Cation exchange chromatography was used to separate Mb from the majority of proteins within the raw muscle homogenate, including haemoglobin. Cation exchange was carried out at room temperature on a column (2 X 50 cm) containing SP Sepharose fast flow (Sigma) at a flow rate of 2 ml min^{-1} using the same buffer as above. Elution of Mb was achieved with the same buffer containing 40 mM NaCl at the same pH as above. Fractions containing Mb were identified using spectrophotometry (Unicam UV500) over a wavelength range of 500-700 nm. The collected Mb fractions were concentrated using Amicon Ultra-15 centrifugal filter unit (Millipore) with a 10 kDa membrane size, following manufacturer guidelines. Mb was separated from any remaining proteins by size exclusion gel filtration chromatography using a Hi-Load 16/60 Superdex 75 prep grade column (GE healthcare) equilibrated with 100 mM KCl using a flow rate of 0.5 ml min^{-1} at a temperature of 4°C. The Mb purity was analysed by SDS PAGE and fractions were pooled and

concentrated using Amicon Ultra-15 centrifugal filter units (Millipore) following manufactures guidelines, ready for subsequent experiments.

Purified Mb Titration

Deoxygenated and oxygenated Mb (deoxyMb, oxyMb) with Fe^{2+} are easily oxidised to MetMb with Fe^{3+}, which binds a water molecule at the Fe that can release a proton. The p*K*a of this reaction is between 8.0 and 9.0 (Sundberg & Martin, 1974), which could be potentially problematic during the acid-base titration of Mb. For this reason, three ml of purified Mb (approximately 0.13 mM, in 100 mM KCl) from each of the eight species above, was converted to cyanometmyoglobin (MetMbCN) by adding 200 µl of 10 X modified Drabkin's reagent, consisting of (in non-concentrated form): $NaHCO_3$ (11.9 mmol l^{-1}), $K_3[Fe(CN)_6]$ (0.61 mmol l^{-1}) and KCN (0.77 mmol l^{-1}) (Völkel & Berenbrink, 2000). After excess Drabkin's reagent was removed by gel filtration using a sephadex G-25M desalting PD-10 column (GE healthcare), this gave approximately 300 nmoles of Mb in total. The conversion to and concentration of MetMbCN was checked using a Unicam UV500 spectrophotometer over a wavelength range of 500-700 nm and by using a MetMbCN extinction coefficient of 11.0 l mol^{-1} cm^{-1} at 540 nm. The MetMbCN protein solution was incubated at 25°C for half an hour under water vapour saturated nitrogen gas to remove any CO_2, before the volume of sample was recorded gravimetrically and the isoelectric pH measured using a Radiometer PHM82 standard pH meter and a G2040C pH glass electrode with a K4040 calomel reference electrode (Radiometer, Copenhagen, Denmark). The sample was then titrated at 25°C in the absence of CO_2 using 0.1 M NaOH or HCl, starting from the isoelectric point, in appropriate volumes to give approximately 0.1 pH unit changes for each addition of titrant, over the range of pH 5.8 - 8.0.

Determining the Mb specific buffer value

Mb proton charge ΔzH^+ was calculated as the total nmoles of titrant added to the sample, divided by the total number of nmoles of Mb in the sample (Jensen, 1989; Berenbrink et al., 2005 supplemental data) and plotted against pH. The Mb specific buffer value (β_{Mb}) (mol H^+ mol Mb^{-1} pH $unit^{-1}$) was determined by first fitting a polynomial curve to the experimental titration data. Then the first derivative of each point in the polynomial curve was calculated, giving β_{Mb}, over the range of pH 5-9, in increments of 0.01. In order to create a model for the calculation of β_{Mb} for species that did not have experimentally measured data, a titration curve had to be estimated first from the amino acid sequence. This was achieved by calculating the Mb net surface charge as a function of pH

Mb net surface charge determination

Mb net surface charge was calculated from the amino acid composition of each sequence by totalling the estimated charge contribution of each ionisable residue. The charge of each residue at every pH value ranging from 4-11 with increments of 0.01 was calculated using the equation of a hyperbolic saturation curve according to the formula

$$S = [H^+]/([H^+]+a) \qquad \textbf{Equation 2.2}$$

where S is the fractional occupation of a given proton binding site, $[H^+]$ is the hydrogen ion activity [(-)log pH] and a is (-)log p*K*a. The p*K*a thus corresponds to the pH at which a given ionisable group is half-maximal occupied by a proton. Charges of each ionisable residue at each pH were based on experimentally determined p*K*a values obtained from the literature data where possible. p*K*a values for specific His residues in Mb were obtained from NMR studies of MetMb (Bothelo et al., 1978; Kao et al., 2000) or carbonmonoxymyoglobin

(MbCO) (Basford et al., 1993), or, when this information was not available, based on an average p*K*a for His in folded proteins (Pace et al., 2009). The p*K*a values used to determine the charge of other ionisable residues and the carboxyl terminus were also taken as the average p*K*a for that residue in folded proteins (Pace et al., 2009). The p*K*a value for the valine amino terminus was taken from sperm whale (Kao et al., 2000) and the amino terminal glycine p*K*a was taken as that of the horse (Wilbur & Allerhand, 1977). For each of the two haem proprionates a p*K*a value was obtained from sperm whale MetMb (Friend & Gurd, 1979). Wherever possible the p*K*a chosen from the literature was measured close to the ionic strength assumed in mammalian muscle tissues, i.e. 0.15 M. The standard temperature for p*K*a determination is 25°C and most p*K*a values are only available at this temperature. Since p*K*a values increase as ionic strength increases (Kao et al., 2000) and decrease as temperature increases (Bhattacharya & Lecomte, 1997), it was decided that experimental titrations in this study would be conducted at 25°C and at 0.1 M KCl to enable the use of published p*K*a values in modelling experimentally determined net charge and buffer values (see **Table 2.3** for more details).

The p*K*a value for His 88 was determined in the present study, using the titration curves of sheep and pig MetMbCN. Amino acid sequence analysis revealed that sheep and pig Mb differ by three His residues and one charged residue. In contrast to the pig, sheep Mb has histidines at position 88, which is occupied by proline in all other mammals apart from other ruminants, and at positions 113 and 116, which tend to be either also His or glutamine in other mammals. The pig further has a lysine at position 34, which is a threonine in the ruminants and old world monkeys. Known values of p*K*a for residues 113 and 116 and a non-linear iterative curve fit algorithm was used to estimate the p*K*a value for the unknown histidine residue that would allow the pig titration curve plus 3 extra His and minus one Lys to match the titration curve of the sheep (**Fig 2.1**). This technique was also applied to fit the

titration curve of sheep Mb, to that of cow Mb, two animals that differ in terms of ionisable Mb residues only by one His residue at position 152. This found a p*K*a value of 5.99 for the cow His at position 152, which falls within the experimental range of p*K*a values from 5.95 in the Amazon river dolphin, *Inia geoffrensis*, to 6.29 in the harbour seal, *Phoca vitulina*, measured in seven other species at this position (Bothelo et al., 1978). For further detailed discussions of calculating Mb net charge see **Chapter 3**.

Estimated titration curves were then compared to the measured titration data for the eight species involved in this study, which allowed minor adjustments to be made to the modelling procedure, until a good relationship was determined for each species. One of the adjustments was that of the p*K*a of His 88 discussed above. Another was the inclusion of His 82 as a titratable residue. This residue is highly conserved among mammalian Mbs and has been deemed un-titratable in several 1D and 2D NMR studies (Bothelo et al., 1978; Cocco et al., 1992; Bashford et al., 1993; Bhattacharya & Lecomte, 1997 and Kao et al., 2000). However neutron diffraction has shown that this residue is titratable and fully charged at pH 5.7 indicating a relatively high p*K*a in MbCO (Cheng & Schoeborn, 1991). Initial titration curve models consistently indicated an additional titratable group in the physiological pH range in all species for which experimental titrations were carried out. For this reason His 82 was included in the estimated titration curve model, using an average His pKa value of 6.6 for an unconstrained His residue (Pace et al., 2009)

Once the estimated titration curve model was established the β_{Mb} was determined for each point in the curve over a pH range from 5.0-8.0 in increments of 0.01. This was done by calculating the slope of the titration curve at the three points surrounding each pH value, the slope of this line is negative but for convenience absolute values will be used for the remainder of the study. Estimated β_{Mb} curves were then compared to the β_{Mb} calculated from measured titration data. Once satisfied that the model was accurate, it was then used to

calculate titration curves and generate β_{Mb} for species where Mb amino acid sequence was available but measured titration data was not.

Contribution of Mb to whole muscle non-bicarbonate buffering

Whole muscle non-bicarbonate buffering capacities ($\beta_{muscleNB}$) for mammalian species where [Mb] and Mb sequence are known were collected from literature sources or experimentally determined in this study. To quantify in a given species how much Mb contributes to $\beta_{muscleNB}$, [Mb] data was multiplied by the specific Mb buffer value (β_{Mb}) at pH 6.5 to give the total contribution of Mb to muscle non-bicarbonate buffering ($\beta_{muscleMb}$). This pH is in the middle of the range over which whole muscle buffer values are usually determined (pH 6.0 to 7.0) and also in the middle of the physiological pH range for human - and likely other mammalian - skeletal muscles between rest and maximal exercise (ca. pH 7.0 to 6.0, respectively Sahlin et al., 1976; Hermansen & Osnes, 1972; Robergs et al., 2004). $\beta_{muscleMb}$ can then be expressed as a percentage of $\beta_{muscleNB}$. This data was then combined with information about the concentration of His-related dipeptides for each species where data could be found. A specific buffer capacity for each dipeptide (balenine, anserine and carnosine) was determined in the same way as for β_{Mb}, using experimentally measured p*K*a values (**Table. 2.3**; Baltes, 1971).

Table 2.3 p*Ka* values of ionisable groups used for Mb net surface charge calculations. Net charge and buffer value have been calculated for each residue based on the p*Ka* shown below.

Residue	p*Ka*	Ref	Protein derivative	Species	Temp	Ionic strength (M)	Net Charge at pH 6.5	Buffer value at pH 6.5
His 8	6.12	1	MetMb	MW/HS	25°C	0.1	0.29	0.48
His 12	6.49	2	MetMb	SW	25°C	0.2	0.49	0.58
His 24	<4.5	3	MbCO	SW	35°C	0.002	<0.01	<0.02
His 34	6.60	4	Various		25°C		0.56	0.57
His 35	5.52	1	MetMb	DfSW	25°C	0.1	0.09	0.20
His 36	7.89	2	MetMb	SW/Horse	25°C	0.2	0.96	0.09
His 48	5.59	2	MetMb	SW/Horse	25°C	0.2	0.11	0.22
His 64	<5	3	MbCO	SW	35°C	0.002	<0.03	<0.07
His 66	6.60	4	Various		25°C		0.56	0.57
His 81	6.91	2	MetMb	SW/Horse	25°C	0.2	0.72	0.46
His 82*	6.60	4	Various		25°C		0.56	0.37
His 88	7.10	This study	MetMbCN	Sheep	25°C	0.1	0.80	0.57
His 91	6.60	4	Various		25°C		0.56	0.57
His 93	<5	3	MbCO	SW	35°C	0.002	<0.03	<0.07
His 97	5.63	3	MbCO	SW	35°C	0.002	0.12	0.24
His 113	5.69	2	MetMb	SW/Horse	25°C	0.2	0.13	0.27
His 116	6.75	2	MetMb	SW/Horse	25°C	0.2	0.64	0.53
His 119	6.49	2	MetMb	SW/Horse	25°C	0.2	0.49	0.58
His 121	6.60	4	Various		25°C		0.56	0.57
His 124	6.60	4	Various		25°C		0.56	0.57
His 128	5.53	1	MetMb	Sea lion	25°C	0.1	0.10	0.20
His 140	6.60	4	Various		25°C		0.56	0.57
His 152	6.10	1	MetMb	6 Delphs./HS	25°C	0.1	0.28	0.47
Amino terminus Val	7.52	2	MetMb	SW	25°C	0.2	0.91	0.18
Amino terminus Gly	7.81	5	MetMbCN	Horse	36°C	0.1	0.95	0.10
Haem proprionate	2.90	6	MetMb	SW	25°C	0.1	-1.00	0.00
Carboxyl terminus	3.30	4	Various		25°C		-1.00	0.00
Lys	10.50	4	Various		25°C		1.00	0.00
Arg	12.30	4	Various		25°C		1.00	0.00
Asp	3.50	4	Various		25°C		-1.00	0.00
Glu	4.20	4	Various		25°C		-1.00	0.01
Tyr	10.30	4	Various		25°C		0.00	0.00
Cys	6.80	4	Various		25°C		-0.33	0.51
Balenine	6.30	7			22°C	0.1	1.00	0.55
Carnosine	7.04	7			22°C	0.1	1.00	0.40
Anserine	6.83	7			22°C	0.1	1.00	0.50

1 Bothelo et al., 1978
2 Kao et al., 2000
3 Bashford et al., 1993
4 Pace et al., 2099
5 Wilbur & Allerhand 1977
6 Friend & Gurd 1979
7 Baltes 1971

* Indicated to be fully charged at pH 5.7 (Cheng & Schoeborn, 1991)
MW = minke whale HS = harbour seal
SW = sperm whale DfSW = dwarf sperm whale
Sea lion = Californian sea lion
6 Delphs = common dolphin, bottlenose dolphin, pilot whale, common porpoise, Amazon river dolphin, Dall's porpoise

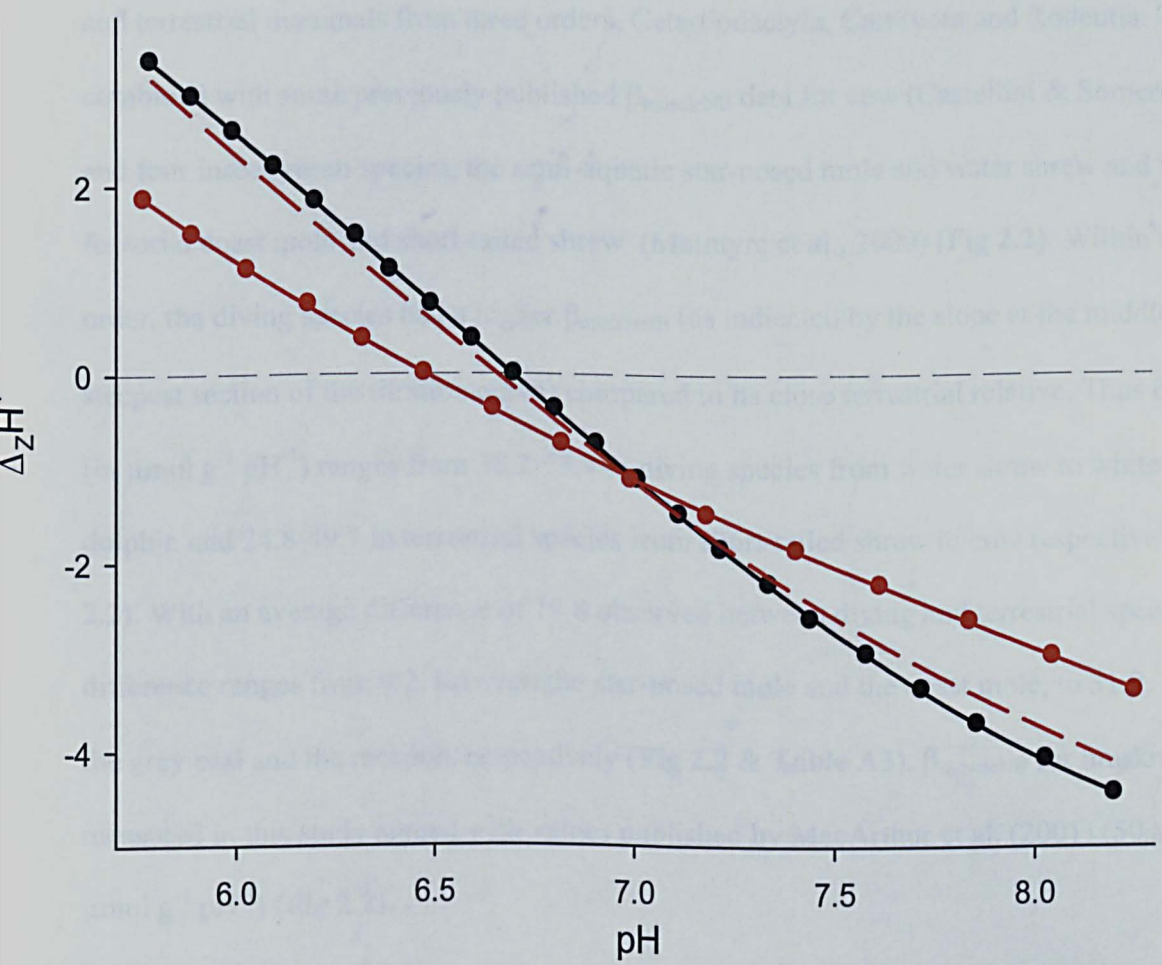


Figure 2.1 Acid-base titration curve for purified MetMbCN of sheep (black dots) and pig (red dots), 25°C, 0.1 M KCl, [Mb] = 0.099 mM and 0.100 mM for sheep and pig, respectively. The solid lines through the dots are polynomial curves fitted through the experimental data. The dashed red line is based on a non-linear iterative curve fit that determined the unknown p*K*a of His 88 that was necessary to fit the sheep titration curve by adding to the pig titration curve the individual titration curves of His 88, His 113 and His 116 minus a charged Lys.

RESULTS

Experimental acid-base titrations were carried out on whole muscle tissues of diving and terrestrial mammals from three orders, Cetartiodactyla, Carnivora and Rodentia. This was combined with some previously published $\beta_{muscleNB}$ data for cow (Castellini & Somero, 1981) and four insectivoran species, the semi-aquatic star-nosed mole and water shrew and the strict fossorial coast mole and short-tailed shrew (McIntyre et al., 2000) (**Fig 2.2**). Within each order, the diving species had a higher $\beta_{muscleNB}$ (as indicated by the slope at the middle, steepest section of the titration curve) compared to its close terrestrial relative. Thus $\beta_{muscleNB}$ (in $\mu mol\ g^{-1}\ pH^{-1}$) ranges from 38.2-73.4 in diving species from water shrew to white-beaked dolphin and 24.8-49.7 in terrestrial species from short-tailed shrew to cow respectively (**Fig 2.2**). With an average difference of 19.8 observed between diving and terrestrial species. The difference ranges from 9.2, between the star-nosed mole and the coast mole, to 31.9, between the grey seal and the raccoon, respectively (**Fig 2.2** & **Table A3**). $\beta_{muscleNB}$ for muskrat measured in this study agreed with values published by MacArthur et al. (2001) (50.8-56.9 $\mu mol\ g^{-1}\ pH^{-1}$) (**Fig 2.2**).

The major components of $\beta_{muscleNB}$ are free and protein-bound His and His related dipeptides (Abe, 1995). A strong positive correlation between $\beta_{muscleNB}$ and Mb concentration has been shown previously (Castellini & Somero, 1981). In order to ascertain the contribution of $\beta_{muscleMb}$ to $\beta_{muscleNB}$, this study first compared Mb amino acid sequences for diving and terrestrial mammals from four orders, Carnivora, Cetartiodactyla, Insectivora and Rodentia. Twenty six species have had their Mb nucleotide sequence identified for the first time in this study and these are highlighted with an asterisk in **Figures 2.3A-D**. Nucleotide sequences are given in **Figure A1**. In five species; short-tailed shrew, star-nosed mole, coast mole, American mink, and grey squirrel, the first twenty eight nucleotides have been influenced by the primer used for PCR. The primer in question is the rodent primer (**Table 2.2**) taken

from Bianchi et al., (2005) which overlaps the coding region from the UTR. Tissue samples from these species either ran out or subsequent tissues were too degraded to determine the 5' end, once a new primer had been established that did not influence sequencing. However the same rodent primer was also used to initially sequence the 5' end of the water shrew Mb. When the water shrew was re-sequenced after using the new general 5' end UTR primer, nucleotide sequence was not altered. This suggests that the nucleotide sequence for species closely related to the water shrew, short-tailed shrew, star-nosed mole and coast mole, would also be unaffected by the rodent primer. Available rodent nucleotide sequences, Norway rat, *Rattus norvegicus* and house mouse, *Mus musculus* are identical in these first twenty eight residues and the corresponding amino acid sequence (the first eight amino acids) is identical for all rodent sequences published to date (**Table A1**). The Mb sequence of the grey squirrel is identical to mouse and rat in the first 28 nucleotides, therefore suggesting that the sequence identified here for the grey squirrel is correct at the 5' end. With the exception of the phocid seals the first eight amino acids for carnivorans are identical, suggesting that the sequence for the American mink is also correct. The first eight amino acid residues of the Mb protein are highly conserved across all the mammalian species observed in this study (**Table A1**), and any changes observed do not influence the objectives of this study. Therefore the amino acid residues for the five species mentioned above will be taken as correct for the remainder of this thesis.

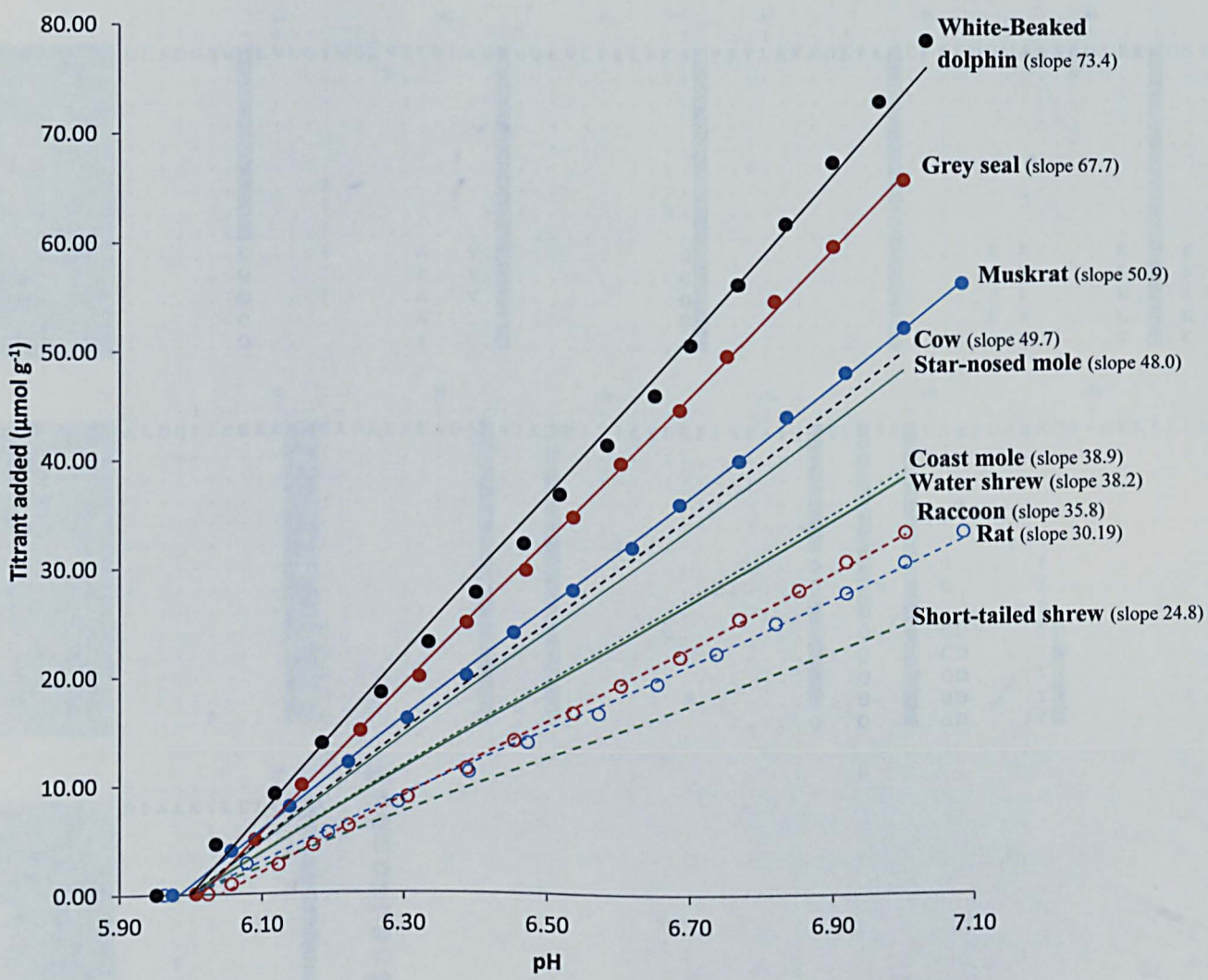


Figure 2.2 Titration of whole muscle tissue homogenates with NaOH. Open circles indicate terrestrial species, filled circles indicate diving species. Colour indicates phylogenetic order; black Cetartiodactyla, blue Rodentia, red Carnivora and green Insectivora. Black dashed line indicates previously published whole muscle non-bicarbonate buffering ($\beta_{muscleNB}$) capacity for cow (Castellini & Somero, 1981). The solid and dashed green lines indicate published $\beta_{muscleNB}$ for star-nosed mole and coast mole (McIntyre et al., 2002), water shrew and short-tailed shrew (Gusztak, 2008) .

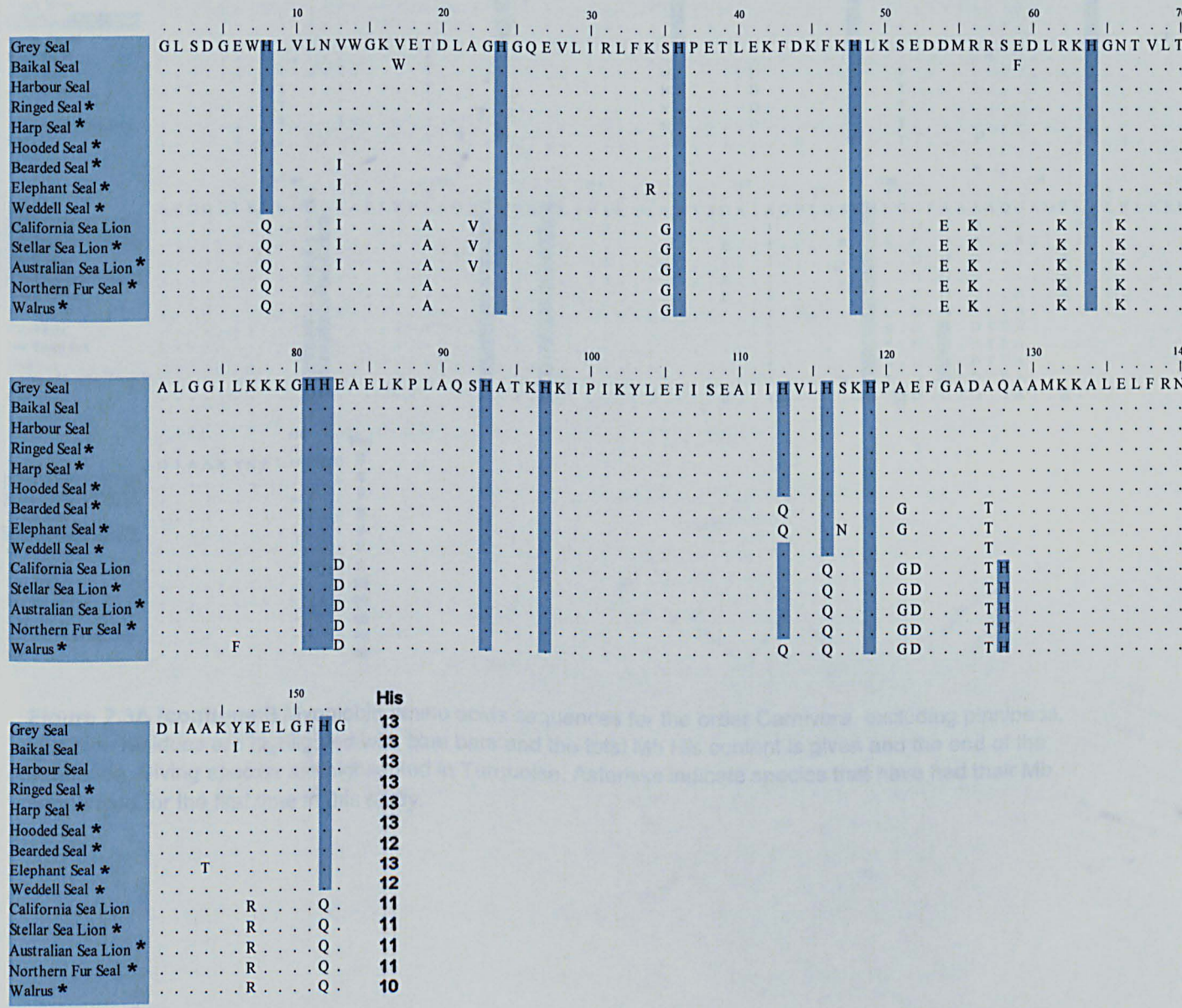


Figure 2.3A Myoglobin amino acids sequences of pinnipeds. Histidine residues are highlighted with blue bars and the total Mb His content given and the end of the sequence. Diving species are highlighted in Turquoise. Asterisks indicate species that have had their Mb sequenced for the first time in this study.

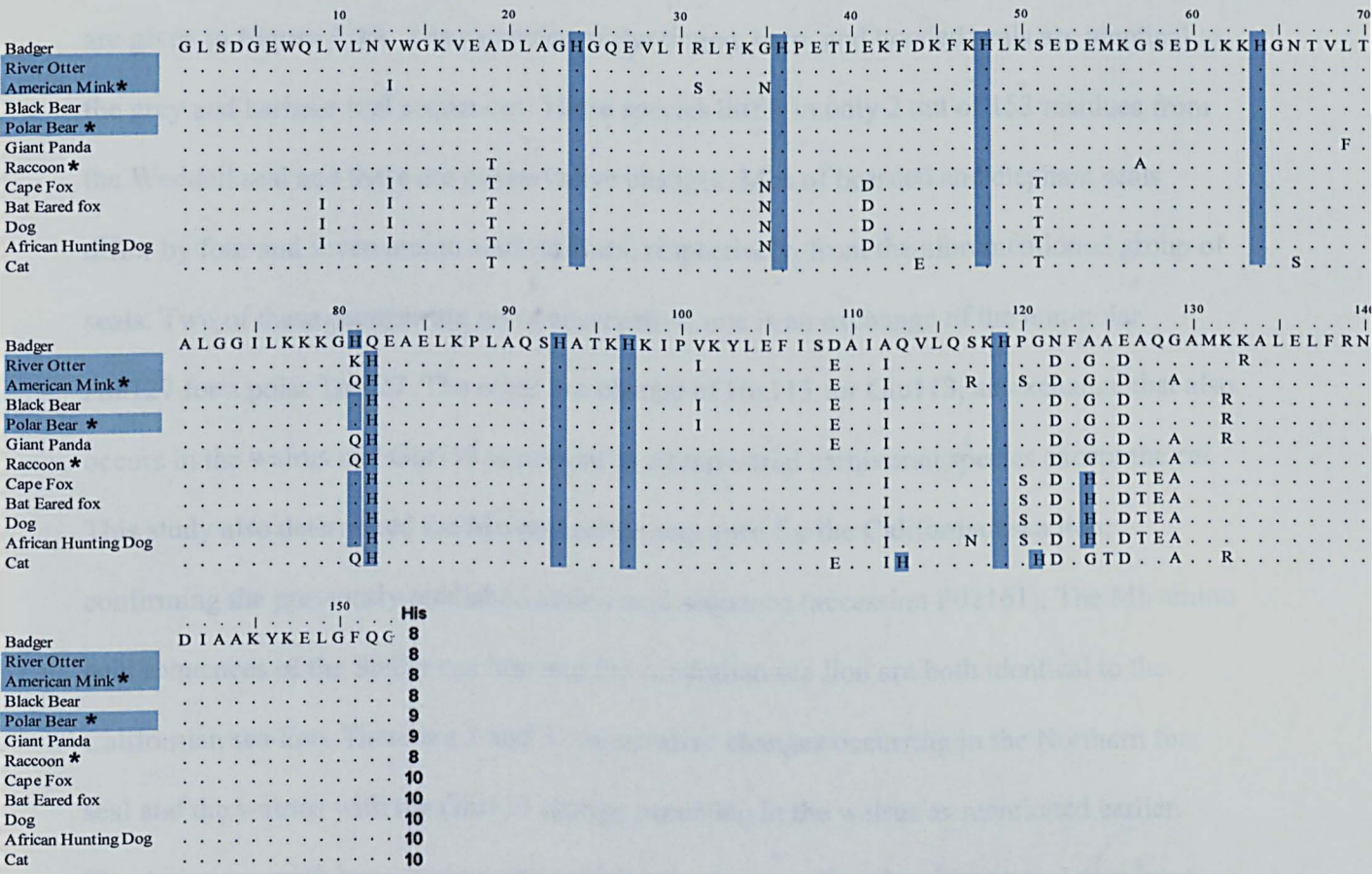


Figure 2.3A (continued) Myoglobin amino acids sequences for the order Carnivora, excluding pinnipeds. Histidine residues are highlighted with blue bars and the total Mb His content is given and the end of the sequence. Diving species are highlighted in Turquoise. Asterisks indicate species that have had their Mb sequenced for the first time in this study.

The Mb protein sequences for the twelve carnivoran species sequenced in this study are given in **Figure 2.3A**. The sequence of the ringed, harp, and hooded seals are identical to the grey and harbour seal sequences. These species differ in only 2 out of 153 residues from the Weddell seal and these are conservative changes. Mbs of bearded and elephant seals differ by four and seven amino acid residues, respectively, from the aforementioned group of seals. Two of these changes are non-conservative, one is an exchange of the non-polar Ala127 for a polar Thr127. The other is a change of His113 for Glu113, an exchange that also occurs in the walrus and Glu113 is present in all terrestrial carnivoran species except the cat. This study also determined the Mb nucleotide sequence for the Californian sea lion, confirming the previously published amino acid sequence (accession P02161). The Mb amino acid sequences of the Steller sea lion and the Australian sea lion are both identical to the Californian sea lion. There are 2 and 3 conservative changes occurring in the Northern fur seal and the walrus, with the Glu113 change occurring in the walrus as mentioned earlier. The American mink has 9 amino acid substitutions compared to the river otter, *Lutra lutra*, sequence (Accession P11343), 4 are conservative and the remaining non-conservative changes include: Arg31-Ser31, Gly35-Asn35, Lys81-Gln81, Ser117-Arg117 and Pro-Ser120, from river otter to American mink respectively. The raccoon shows 7 amino acid substitutions compared to the river otter, 5 conservative substitutions plus Ala19-Thr19 and Lys81-Gln81 from the river otter to raccoon respectively. The polar bear Mb protein sequence is identical to the black bear, *Ursus americanus*, sequence, and has 3 conservative substitutions compared to the giant panda, *Ailuropoda melanoleuca*, and 1 non-conservative substitution of Gln81-His81 from giant panda to polar bear respectively. Diving species within the order Carnivora generally show a trend towards increasing the number of His residues within their Mb protein. The phocid seals (**Fig 2.3A**) have thirteen His residues, the highest number of Mb His residues in the order Carnivora, with a range of 8-13 His seen

within the remaining species in the order (**Fig 2.3A**). Interestingly His residues 8, 128 & 152 can differentiate phocid and otariid seals, as one group of seals will have a His while the other has a Gln residue at the aforementioned positions (**Fig 2.3A**). This suggests an independent evolutionary pathway of the Mb protein of these two lineages of seals.

New sequences in the order Cetartiodactyla are shown in **Figure 2.3B**, including; pygmy hippo, Bowhead whale, Sowerby's beaked whale, Northern bottlenose whale, narwhal, white beaked dolphin and Risso's dolphin. The bowhead whale Mb is similar to the Mb in other baleen whales observed in this study showing 5 conservative substitutions compared to other baleen whales. The beaked whales all have very similar Mbs. The Sowerby's beaked whale has an identical Mb protein sequence to the Stejneger's and Hubb's beaked whale, while the Northern bottlenose whale has only 1 conservative substitution compared to these species. The dolphin species observed in this study all have very similar Mbs, too. The two newly sequenced species, white-beaked dolphin and Risso's dolphin, are identical and have 1 conservative substitution compared to the bridled, *Stenella attenuata*, saddleback, *Delphinus delphis* and bottlenose dolphins (**Fig2.3B**).

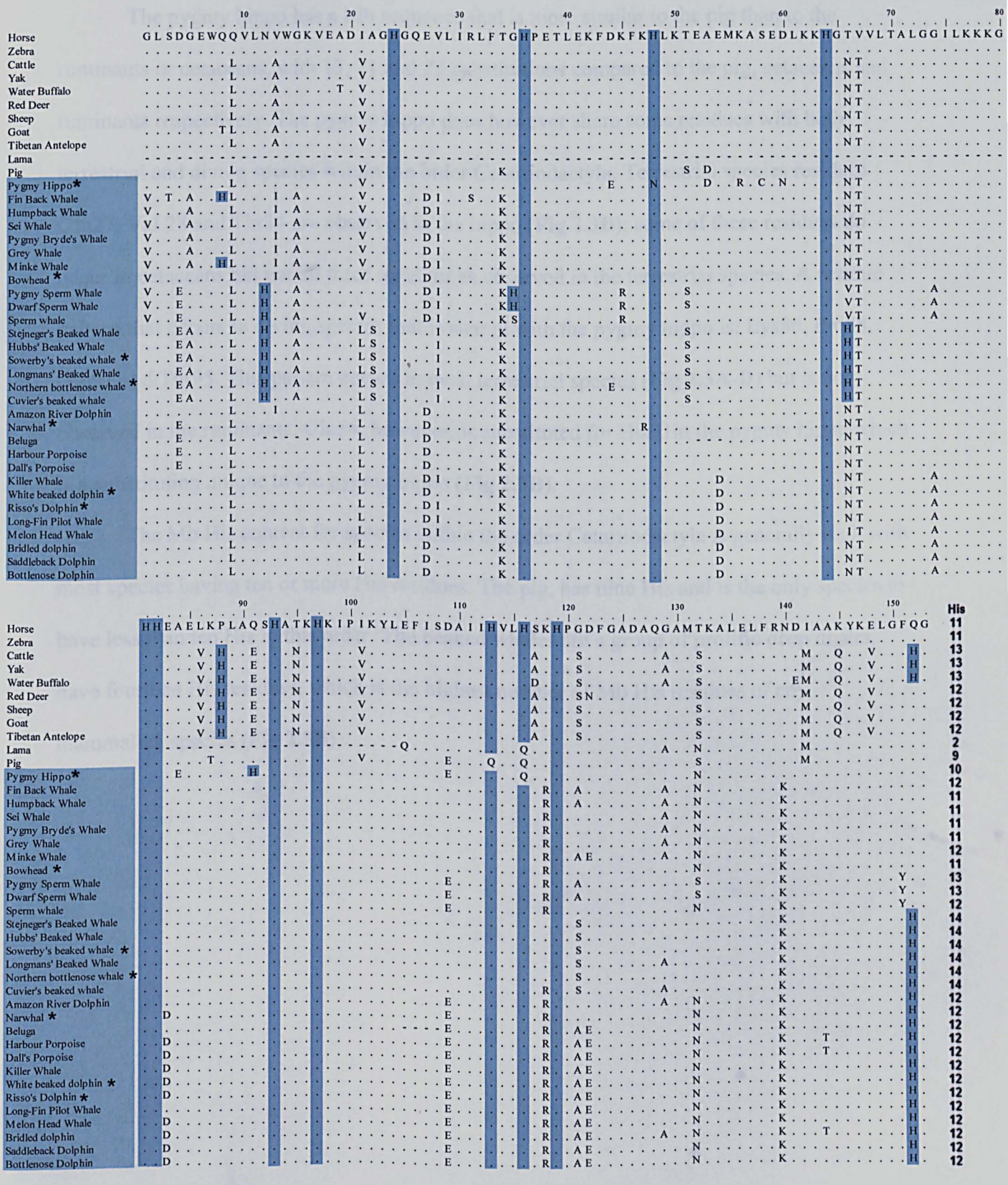


Figure 2.3B Myoglobin amino acid sequences for the order Cetartiodactyla. Histidine residues are highlighted with blue bars and the total Mb His content is given at the end of the sequence. Diving species are highlighted in Turquoise. Asterisks indicate species that have had their Mb sequenced for the first time in this study.

The pygmy hippo has a Mb sequence that is more similar to the pig than to the ruminants or cetaceans, with 13, 21 and 23 substitutions compared to the pig, cetaceans and ruminants respectively. The pygmy hippo does however share some residues with both terrestrial and diving species within the order Cetartiodactyla. Terrestrial species residues Glu27, Val 28 and Thr34 are observed in the hippo (**Fig 2.3B**); some of these residues do occur in cetaceans but not all three together as observed in the terrestrial species. A residue pattern that occurs in diving species that is shared with the pygmy hippo is; Lys86, Pro88, Gln91 and Thr95. This pattern occurs in most terrestrial species (**Fig 2.3A-D**) but is not observed in the ruminants. Gln88, however, is substituted for His91in the pygmy hippo which is a substitution unique to the pygmy hippo (**Fig 2.3B**).

The Mb His content for species within the order Cetartiodactyla is generally high with most species having ten or more His residues. The pig, has nine His and is the only species to have less than ten His in this order. The beaked whales are a group of prolific deep divers, have fourteen His residues, which is the highest number of Mb His residues of any mammalian species (**Fig 2.3B**).

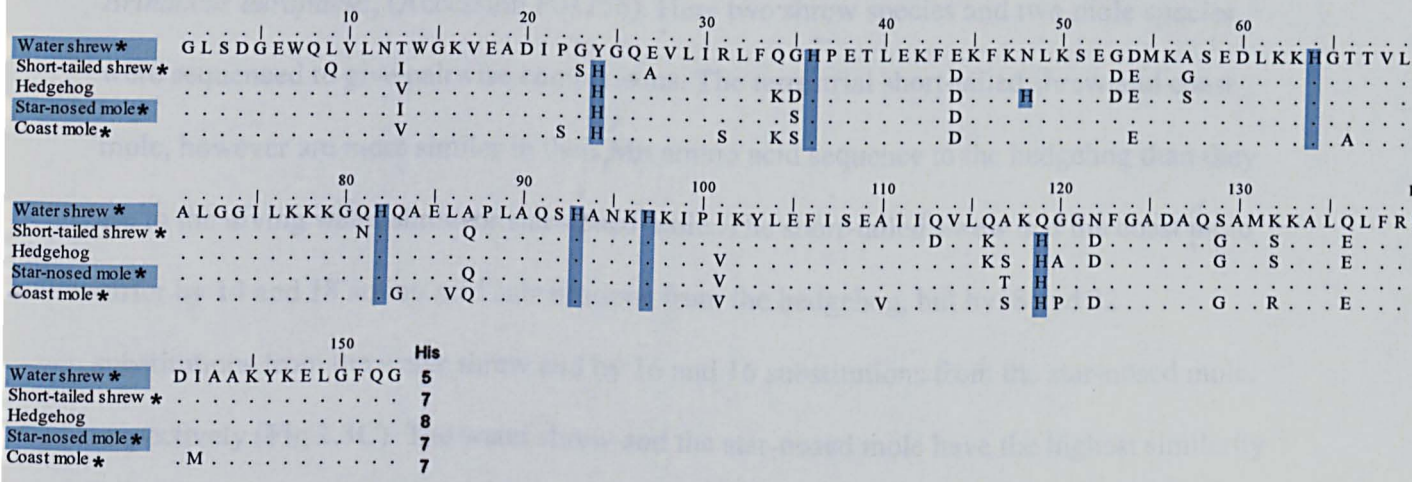


Figure 2.3C Myoglobin amino acid sequences for the order Insectivora. Histidine residues are highlighted with blue bars and the total Mb His content is given at the end of the sequence. Diving species are highlighted in Turquoise. Asterisks indicate species that have had their Mb sequenced for the first time in this study.

Four insectivoran species have newly sequenced Mbs in this study (**Fig 2.3C**), previously only one insectivoran species had a known Mb protein sequence, the hedgehog, *Erinaceus europaeus*, (Accession P01256). Here two shrew species and two mole species were sequenced to give pairwise comparisons. The terrestrial short-tailed shrew and coast mole, however are more similar in their Mb amino acid sequence to the hedgehog than they are to the diving water shrew or star-nosed mole. The short-tailed shrew and the coast mole differ by 14 and 18 amino acid substitutions from the hedgehog, but by18 and 22 substitutions from the water shrew and by 16 and 16 substitutions from the star-nosed mole, respectively (**Fig 2.3C**). The water shrew and the star-nosed mole have the highest similarity with only 8 amino acid substitutions between them and three of those are conservative changes (**Fig 2.3C**). One striking similarity is that both diving insectivoran species, water shrew and star-nosed mole, have a reduction in the number of strongly negatively charged residues compared to the terrestrial species (see **Chapter 3** for a discussion of Mb charge). Mb His content in insectivorans is generally low ranging from 5-8, with no discernible trend seen between diving and terrestrial species. The diving water shrew has five His residues, the lowest number in this order. The hedgehog has eight His which is the highest number with all other species in this order all having seven His residues (**Fig 2.3C**).

This study has newly sequenced one new rodent Mb, the grey squirrel (**Fig 2.3D**) it is most similar to the kangaroo rat with 16 amino acid substitutions between them, with 6 of these being conservative changes and it has the same number of His as the mole rat, *Spalax carmeli* and the mouse, *Mus musculus*.

Diving species within the Rodentia generally show a trend towards increasing the number of His residues within their Mb protein. Mb His ranges from 6-11 in the order Rodentia, with the diving species having at least three additional His residues compared to the terrestrial species (**Fig 2.3D**).

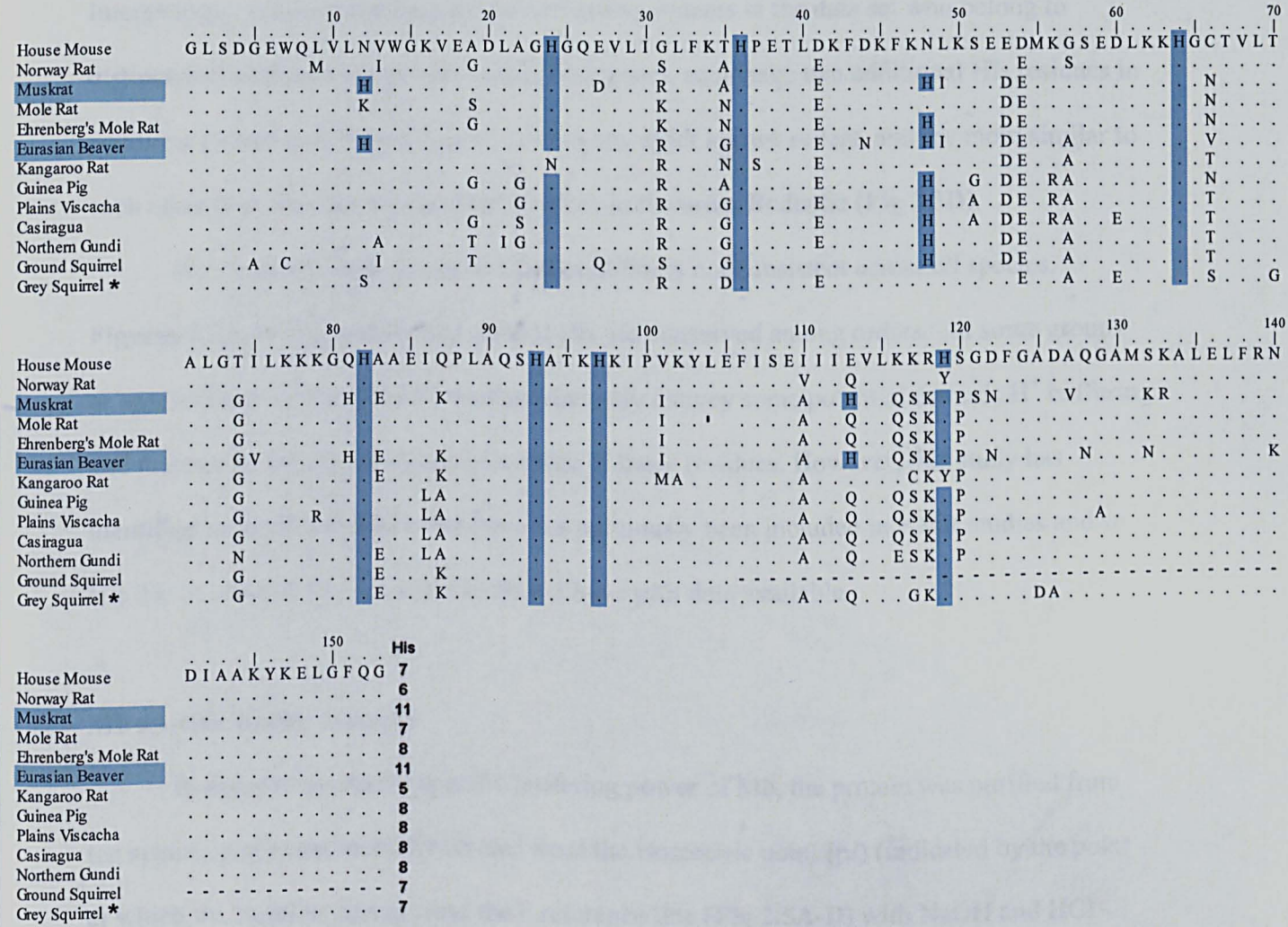


Figure 2.3D Myoglobin amino acids sequences for the order Rodentia. Histidine residues are highlighted with blue bars and the total Mb His content is given and the end of the sequence. Diving species are highlighted in Turquoise. Asterisks indicate species that have had their Mb sequenced for the first time in this study.

Interestingly, muskrat and beaver, the two diving rodents in the data set who belong to distinctively different sub groups within their order, each have two additional His residues in positions 12 and 113, which do not occur in any other known rodent, and are more similar to each other than they are to any other member in the order Rodentia (**Fig 2.3D**).

However, the positioning of additional His is not consistent across all species, **Figures 2.3A-D** above show that several His are conserved among orders, but some groups or species have unique Mb His residues that may convey some potential towards H^+ buffering and this can be inferred from the p*K*a value of those residues. However, this study has identified seven His residues that have not previously been included in NMR studies and so His 34, 35, 66, 88, 91, 121 and 124 do not have p*K*a data available.

Mb specific buffer capacity

In order to assess the specific buffering power of Mb, the protein was purified from ten species and experimentally titrated from the isoelectric point (p*I*) (indicated by the point at which the titration curves cross the 0 reference line (**Fig 2.5A-D**) with NaOH and HCl between approximately pH 5.8 and 8 (**Fig 2.5**). The variability of the titration experiments was assessed by conducting replicates for cow on three individual animals. The mean measured Mb charge and standard deviation at pH 6.0, 6.5 and 7.0 was 3.83 ± 0.38, 1.89 ± 0.34 and 0.07 ± 0.28, respectively. The mean Mb buffer value and standard deviation was 3.74 ± 0.10, 3.97 ± 0.17, and 3.81 ± 0.08, for pH 6.0, 6.5 and 7.0 respectively. There is greater variability surrounding the mean charge measurement which may be accounted for by partial deamidation occurring within the Mb of individual 1. This is indicated by constant offset of half a charge throughout the pH range 6.0-7.0 (**Fig 2.4**). Since the buffer value is determined by the slope of the line and all three individuals have a similar slope then there is less variation surrounding the means of measured β_{Mb} (**Fig 2.4**).

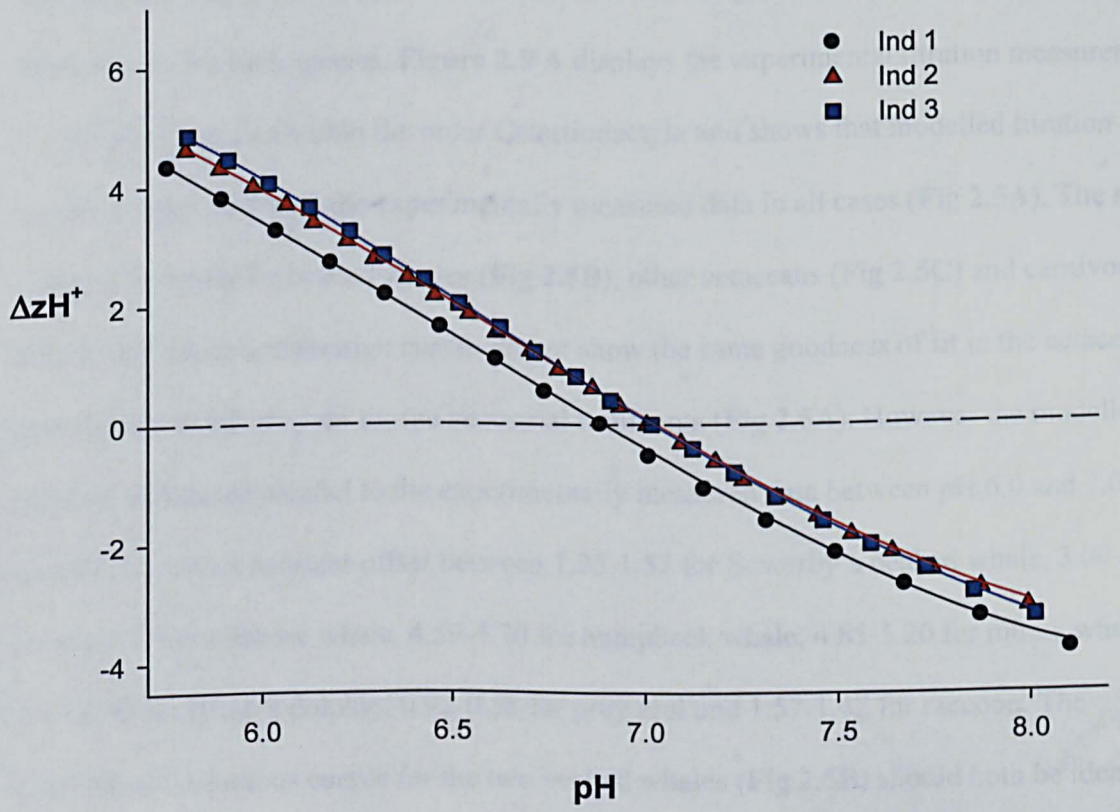


Figure 2.4 Experimental titration curves of purified cow MetMbCN for three individuals. Titration was conducted at 25°C and at 0.1M KCl. A constant downward offset can be observed in individual 1 of approximately half a charge between pH 6.0 and 7.0, which could be explained by partial deamidation.

It was also necessary to make a reliable model for estimating Mb titration curves based on the sum of contributions of charged residues. These models are assessed in **Figure 2.5** by comparing the modelled titration estimate and the goodness of fit to the measured titration data for each species. **Figure 2.5 A** displays the experimental titration measurements for terrestrial species within the order Cetartiodactyla and shows that modelled titration curves fit very well with the experimentally measured data in all cases (**Fig 2.5A**). The same analysis is shown for beaked whales (**Fig 2.5B**), other cetaceans (**Fig 2.5C**) and carnivorans (**Fig 2.5D**). Modelled titration curves do not show the same goodness of fit in the cetaceans and carnivorans as they do for the terrestrial ruminants (**Fig 2.5A**). However the modelled titration curves are parallel to the experimentally measured data between pH 6.0 and 7.0, in every case, with a constant offset between 1.25-1.57 for Sowerby's beaked whale, 3.00-3.52 for Northern bottlenose whale, 4.59-4.70 for humpback whale, 4.85-5.20 for minke whale, 1.10-0.83 for Risso's dolphin, 0.92-0.58 for grey seal and 1.57-1.42 for raccoon. The experimental titrations curves for the two beaked whales (**Fig 2.5B**) should both be identical, as the here for the first time reported amino acid sequences of the Mbs of Sowerby's beaked whale and the Northern bottle-nose whale do not differ in the composition of their ionisable amino acid residues. However, there is a constant offset between them of almost two charges over the pH range 6-7, with the northern bottle-nosed whale, having a lower p*I* than the Sowerby's beaked whale. This offset can be explained by deamidation as all samples apart from those in **Fig 2.5A** have been obtained from stranding or trapping events, and have experienced a certain amount of decomposition prior to tissue extraction. This phenomenon of deamidation could also account for the difference between the modelled titration curves compared to experimentally measured data for all of the species in **Fig 2.5C and D**.

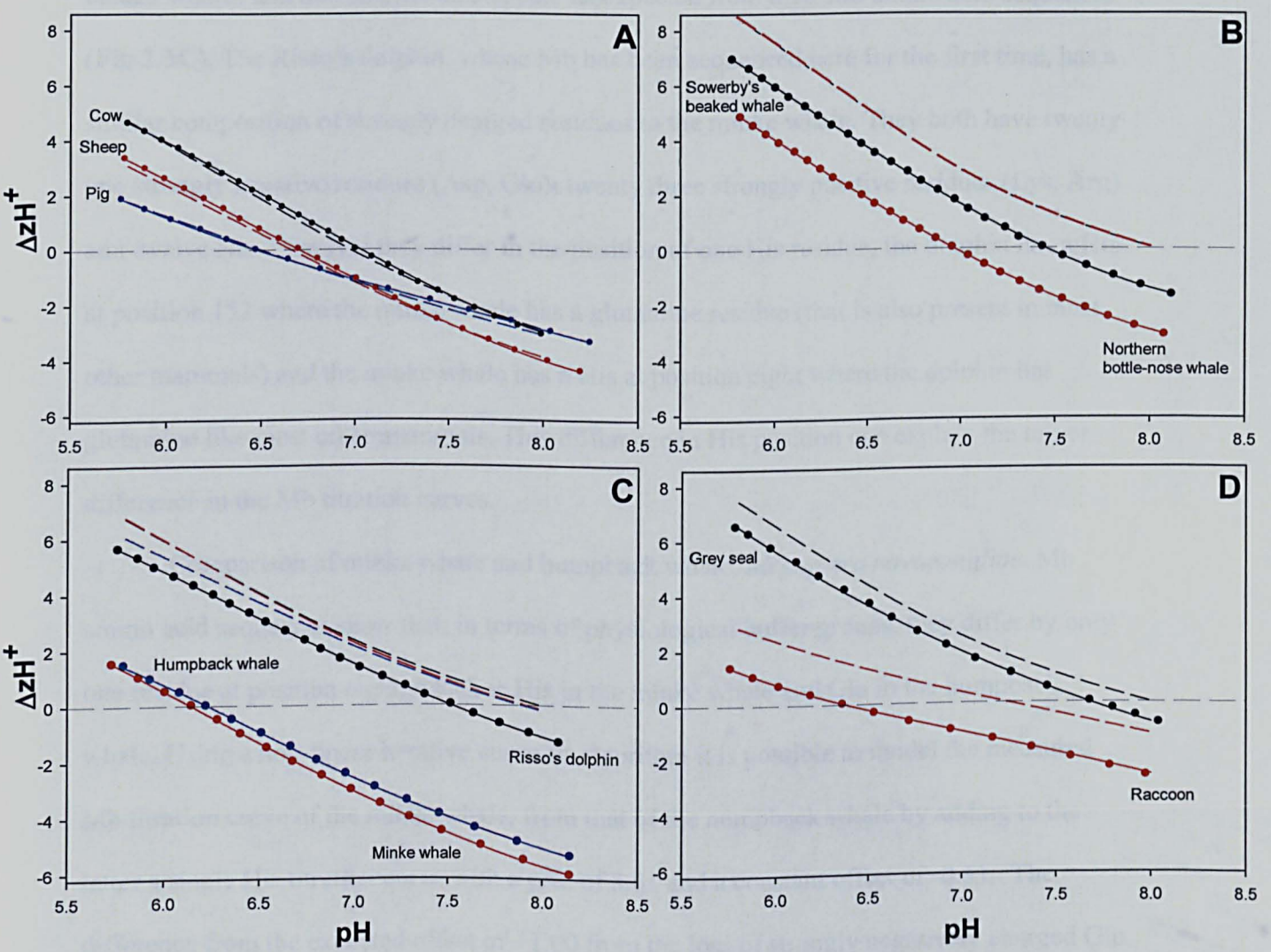


Figure 2.5 Experimental titration of purified MetMbCN protein for ten species of Cetartiodactyla and Carnivora. Experimental data is shown with dots and solid lines, modelled titrations curves are indicated with dashed lines of the same colour. (Titrations were conducted with MetMbCN at 25°C in 0.1M KCl)

Apart from the constant offsets just discussed, experimental titration curves of two baleen whales and one dolphin also appear as expected from their Mb amino acid sequences (**Fig 2.5C**). The Risso's dolphin, whose Mb has been sequenced here for the first time, has a similar composition of strongly charged residues to the minke whale. They both have twenty one strongly negative residues (Asp, Glu), twenty three strongly positive residues (Lys, Arg) and twelve His. However they differ in the position of one His residue, the dolphin has a His at position 152 where the minke whale has a glutamine residue (that is also present in most other mammals) and the minke whale has a His at position eight where the dolphin has glutamine like most other mammals. This difference in His position can explain the minor difference in the Mb titration curves.

Comparison of minke whale and humpback whale, *Megaptera novaeangliae*, Mb amino acid sequences show that, in terms of physiological buffer groups, they differ by only one residue at position eight, which is His in the minke whale and Glu in the humpback whale. Using a non-linear iterative curve fit algorithm it is possible to model the measured Mb titration curve of the minke whale, from that of the humpback whale by adding to the latter a single His titration curve with a p*K*a of 5.81 and a constant offset of -0.61. The difference from the expected offset of +1.00 from the loss of strongly negatively charged Glu residue can again be explained by deamidation. Given the large range of experimentally determined p*K*a values from < 4.5 to 7.8, which can be adopted by specific His residues in Mb (**Table 2.3** above), and given differences in ionic strength during the measurements, the estimated p*K*a of 5.81 for His 8 in this study is reasonably close to the p*K*a of 6.1 measured for this His in the same species in an early NMR study (Bothelo et al., 1978).

Mb Titration curves for the two carnivoran species (**Fig 2.5D**) again show a good match between the shapes of the experimental and the modelled curves, despite different degrees of negative vertical offsets that can be attributed to deamidation of the native

proteins. Taken the modelled curves as good approximations for the titration behaviour of native Mb in the living animal clearly indicates a higher p*I* in the grey seal, compared to the raccoon. This trend of diving mammals having a higher *pI* than their close terrestrial relatives is observed in all estimated titration curves (**Fig 2.5A-D**). Divers also appear to have steeper titration curves compared to their close terrestrial relatives. Thus the pig (**Fig 2.5A**) and the raccoon have the shallowest curves (**Fig 2.5D**) whereas the beaked whales have the steepest curves (**Fig 2.5B**). The slope of the Mb titration curve at any one pH is equal to the buffer value for the protein at that pH. This indicates therefore that β_{Mb} in divers is higher than in their terrestrial relatives. As mentioned previously, in species whose tissues could not be sampled immediately after death (**Figure 2.5 B-D**) there is a constant negative vertical offset of the experimentally measured titration curve relative to the estimated titration curve. However, this does not mean that the slope of the titration curves should be different. Therefore the modelled titration curves should be a good indicator of β_{Mb} over the physiological pH range. Correlations of measured β_{Mb} and modelled β_{Mb} are shown in **Figure 2.6A-C**. The line plotted is a line of identity and shows that modelled data accurately represents the experimentally measured β_{Mb} across the pH range 6.0 to 7.0, which covers the expected range of physiological intracellular pH values in mammalian muscles (Sahlin et al., 1976; Hermansen & Osnes, 1972; Robergs et al., 2004).

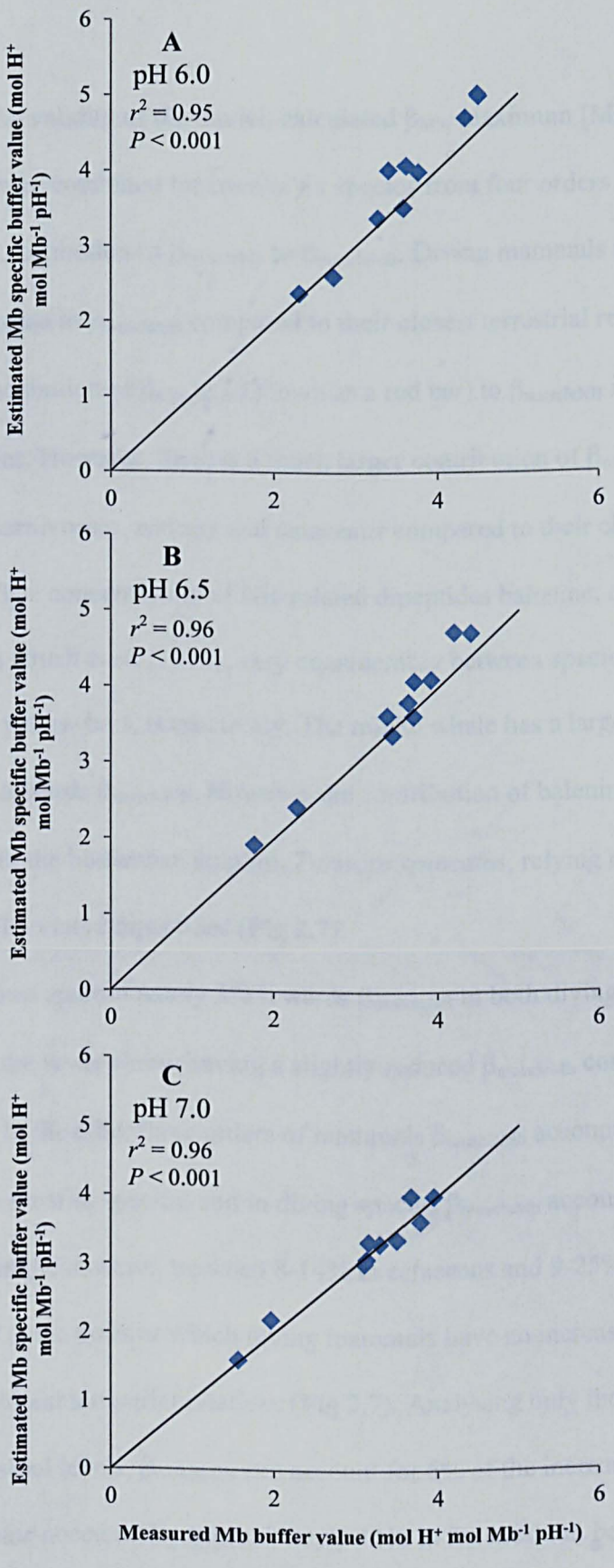


Figure 2.6 A strong positive correlation shown between measured Mb buffer values and estimated Mb specific buffer values (mol H^+ mol Mb^{-1} pH^{-1}), in cow, sheep, pig, Sowerby's beaked whale, Northern bottlenose whale, humpback whale, Risso's dolphin, minke whale, grey seal and raccoon. Line shown is a line of identity. Values spanning the physiological pH range have been calculated at; panel A pH 6.0, panel B pH 6.5 and panel C pH 7.0.

Accepting the validity of the model, calculated β_{Mb}, maximum [Mb] and $\beta_{muscleNB}$ data (**Table A2 & A3**) were combined for twenty six species from four orders of mammals in order to assess the contribution of $\beta_{muscleMb}$ to $\beta_{muscleNB}$. Diving mammals in each of the four orders show an increase in $\beta_{muscleNB}$ compared to their closest terrestrial relatives (**Fig 2.7**). In insectivores the contribution of $\beta_{muscleMb}$ (shown as a red bar) to $\beta_{muscleNB}$ is similar in diving and terrestrial species. However, there is a much larger contribution of $\beta_{muscleMb}$ towards $\beta_{muscleNB}$ in diving carnivorans, rodents and cetaceans compared to their close terrestrial relatives (**Fig 2.7**). The concentration of His-related dipeptides balenine, carnosine and anserine taken from Crush et al. (1970), vary considerably between species, as indicated by the blue, green and yellow bars, respectively. The minke whale has a large contribution from balenine (blue bar) towards $\beta_{muscleNB}$. However the contribution of balenine is smaller in the dolphin species, with the bottlenose dolphin, *Tursiops truncatus*, relying more on $\beta_{muscleMB}$ contribution than His-related dipeptides (**Fig 2.7**).

Mb contributes approximately 3% towards $\beta_{muscleNB}$ in both diving and terrestrial insectivorans, with the water shrew having a slightly reduced $\beta_{muscleMb}$ contribution of approximately 2%. In the other three orders of mammals $\beta_{muscleMb}$ accounts for between 0.4-2% of $\beta_{muscleNB}$ in terrestrial species, and in diving species $\beta_{muscleMb}$ accounts for approximately 5% in the muskrat, between 8-14% in cetaceans and 9-25% in pinnipeds. Also indicated in **Fig 2.7** is the point at which diving mammals have an increase in $\beta_{muscleNB}$ compared to their closest terrestrial relatives (**Fig 2.7**). Analysing only the increase in $\beta_{muscleNB}$ from terrestrial levels, $\beta_{muscleMb}$ can account for 6% of the increase in the water shrew, 16% in the star-nosed mole, approximately 11% in the muskrat, between 13-32% in cetaceans and between 21-45% in pinnipeds. These results show that the combined increase

of muscle [Mb] and β_{Mb} in several species of mammalian divers significantly contributes to the observed increase in whole muscle non-bicarbonate buffer values.

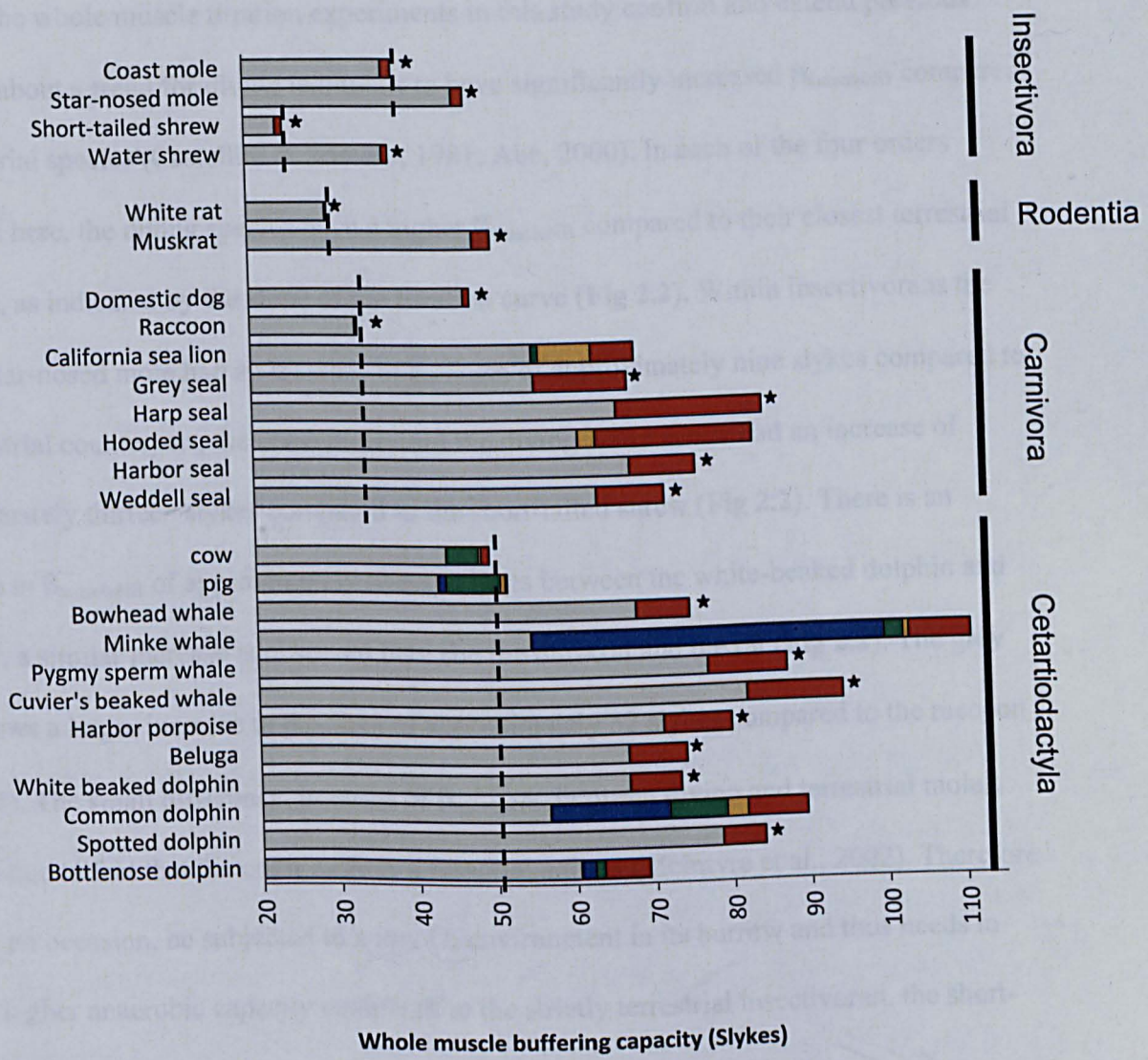


Figure 2.7 Measured whole muscle non-bicarbonate buffering capacity (slykes). A slyke is defined as the µmol titrant added g^{-1} pH^{-1} unit (between pH 6.0 -7.0). Black dashed lines indicate the level of buffering capacity in the closest terrestrial relatives to diving species. Values for Mb contribution towards buffering were calculated in this study and other buffering components were taken from; Crush et al., 1970 and Castellini & Somero 1981, and are highlighted by the colours in the bar as: = Mb = Anserine = Carnosine = Balenine = Others Stars indicate that concentration of His-related dipeptides (Anserine, Caarnosine & Balenine) is unknown.

Discussion

The whole muscle titration experiments in this study confirm and extend previous findings about a trend for diving mammals to have significantly increased $\beta_{muscleNB}$ compared to terrestrial species (Castellini & Somero, 1981; Abe, 2000). In each of the four orders observed here, the diving species have a higher $\beta_{muscleNB}$ compared to their closest terrestrial relatives, as indicated by the slope of the titration curve (**Fig 2.2**). Within insectivorans the diving star-nosed mole had an increase in $\beta_{muscleNB}$ of approximately nine slykes compared to its terrestrial counterpart, the coast mole; and the diving water shrew had an increase of approximately thirteen slykes compared to the short-tailed shrew (**Fig 2.2**). There is an increase in $\beta_{muscleNB}$ of approximately twenty slykes between the white-beaked dolphin and the cow, a similar increase is observed between the muskrat and the rat (**Fig 2.2**). The grey seal shows a larger increase in $\beta_{muscleNB}$ of approximately 32 slykes compared to the raccoon (**Fig 2.2**). The small difference, in terms of $\beta_{muscleNB}$, between diving and terrestrial moles, may be due to fact that the coast mole is a fossorial animal (McIntyre et al., 2002). Therefore it may, on occasion, be subjected to a low O_2 environment in its burrow and thus needs to have a higher anaerobic capacity compared to the strictly terrestrial insectivoran, the short-tailed shrew, which has the lowest $\beta_{muscleNB}$ of all mammals observed here.

Intracellular non-bicarbonate buffering in muscle tissue occurs predominately due to the uptake of protons by the imidazole group of His residues in proteins, free L-histidine and His-related dipeptides (Abe, 2000). In this study the Mb amino acid sequences have been analysed for eighty two mammalian species, finding that diving mammals have a higher quantity of His residues in their Mb sequence than their closest terrestrial relatives in 6 out of 14 lineages of investigated divers (**Fig 2.3A-D** and **Table A2**). The diving lineages latter are comprised of two lineages occurring in insectivorans, one in musteloids, one in ursids, two within seals, six within cetartiodactylans and two within rodents. Those species where Mb

His content is higher than in the close terrestrial relatives tend to be the proficient deep diving species, the phocid and otariid seals, and the sperm and beaked whales, but this trend is also seen in the diving rodents, muskrat and beaver (**Table A2**). Those species where Mb His content is the same or reduced compared to close terrestrial relatives tend to be semi-aquatic species, the water shrew, star-nosed mole, American mink, river otter, polar bear and pygmy hippo, but also include the baleen whales, porpoises and dolphins (**Table A2**). The baleen whales, porpoises and dolphins in fact have high Mb His content but this is masked by all members of Cetartiodactyla having a high Mb His content (**Table A2**). The other diving species where Mb His content is low generally have low skeletal muscle [Mb] and do not have long dive durations (**Table A7**). The evolution of diving in all of these species may well be recent, as it has been shown for the polar bear and sea otter, *Enhydra lutris*, (Berta et al., 2006).

As previously mentioned, within the order Cetartiodactyla the Mb His content is high for all species, with most members having ten or more His. The baleen whales tend to have eleven His residues, which is one less than most ruminant species (**Fig 2.3B**). The members of Delphinoidea all have twelve His residues and their positions are conserved between them. In fact they have highly similar Mb amino acid sequences showing, above 90% similarity across all delphinoid species (**Fig 2.3B**), which may reflect their recent rapid evolution (McGowen et al., 2009). The sperm whales and the bovid species both have thirteen His, although the positioning of these residues is not conserved for some of the residues, suggesting that this is not the result of inheritance from their last common ancestor. The highest number of His occurs in the deep diving beaked whales, a group that has fourteen His, with one unique His at position 66, which is asparagine in most other species of this order (**Fig 2.3B**). This study also finds that the ruminants have a His that is unique to them at position 88, which is a proline residue in all other mammals (**Fig 2.3A-D**). The pygmy hippo,

one of the species that has had its Mb amino acid sequence identified in this study, is particularly interesting in that hippopotamids are regarded as the closest living relatives to cetaceans (Boisserie et al., 2005; Gatesy 1997). It has ten His, including a unique His at position 91, which is occupied by Glu in most other mammalian species (**Fig 2.3A-D**). This indicates that the last common ancestor of the pygmy hippo and cetaceans may have had a similarly low Mb His content that only increased after the whale lineage diverged from the hippo lineage.

Within the orders of Carnivora and Rodentia, there is an obvious trend for increased Mb His content in the diving species (**Fig 2.3A & D**). The two independently evolved lineages of diving rodents have eleven His which is at least three more His residues than their close terrestrial relatives (**Fig 2.3D**). His positions within these two species are conserved between them both, which is remarkable, given their relatively distant evolutionary relationship. This indicates that certain sites in the Mb protein are much more favourable for a His substitution than others. Within the order Carnivora diving has evolved three times (**Fig 4.1**). In Ursidae (bears) and the Mustelidae (weasel family) divers show no trend regarding His compared to their close terrestrial relatives; in fact the Mb amino acid sequence for the polar bear is identical to that of the black bear (**Fig 2.3A**). The river otter and American mink also have an identical His content compared to the other musteloids (**Fig 2.3A**). The canines and the one feline representative in this study all have a high His content (10 residues) (**Fig 2.3A**), this may be linked to their need for a high $\beta_{muscleNB}$ due to their adapted methods of locomotion, prolonged periods of fast endurance running in the canines or fast bursts of locomotion in the felines (Castellini & Somero, 1981). The Mb His content of pinnipeds is high in all cases. The otariid seals all have eleven His and most of the phocid seals have thirteen His, again the positioning of these His residues is not consistent across the species (**Fig 2.3A**). Phocid seal have His8, 116 and 152 which is Gln in the otariid seals, and

otariid seals have His128 which is Gln in phocid seals. This suggests an independent evolution in the Mb protein after these two groups of species diverged. Which may be the result of the independent evolution of diving behaviour within the two groups, supported by the very different adaptations that have evolved within the two groups e.g. phocid seals swim with their hind limbs and tend to exhale before a dive, while otariid seals swim with their forelimbs and tend to inhale before a dive (Berta et al., 2006)

The functionality of any amino acid residue will depend on its positioning within the three-dimensional structure of a protein (Lesk, 2001). His is a unique amino acid residue in that it may function both in the hydrophobic interior and on the solvent exposed external surface of a protein (Betts & Russell, 2003). In Mb, internal His residues have contact with the haem group and can play a role in stabilising the structure of the protein. External His residues may have a functional role in increasing the $\beta_{muscleNB}$, as one of the two nitrogen atoms on the imidazole ring can undergo protonation (Abe, 2000), but this will depend on the p*K*a value of those external residues. The buffering power of any His residue is at its highest when the intracellular pH is equal to the p*K*a of that His residue (Davey, 1960), therefore changes away from the p*K*a of a residue will diminish its buffering potential. The p*K*a values for Mb His residues have been the subject of numerous NMR studies looking at the effects of temperature (Bhattacharya & Lecomte, 1997), the effects of salt concentrations (Kao et al., 2000) and the difference between Mb ligation and oxidation states (Cocco et al., 1992; Bashford et al., 1993). As a result the p*K*a for many of the His that occur in mammalian Mb have been characterised.

This study is the first to test whether increases in Mb His content affect the buffering power of the protein, $\beta_{Mb.}$ In order to achieve this, acid-base titrations were carried out on purified Mb from ten species (**Fig 2.5**). The results suggest that Mb His content is indeed closely linked to the buffer power of the protein. Titration curves of the deep diving beaked

whales and the grey seal show a higher β_{Mb} compared to all the terrestrial mammals observed here, as indicating by the steepness of the titration curves (**Fig 2.5A-D**). The Mbs of cow and sheep have one more His residue compared to the minke whale and the Risso's dolphin. Indeed between pH 6.5 -7 a steeper titration curve is observed for cow Mb compared to the two whale Mbs mentioned above (**Fig 2.5A & C**). However at a lower pH levels (approaching pH 6) minke whale Mb has a higher specific Mb buffer value compared to the cow and sheep. This increase in buffering may be the result of protonation in His8, at lower pH values. His8 is a residue that has been shown to have a p*K*a of 6.10 in the minke whale (Bothelo et al., 1981), which helps to explain why β_{Mb} in the minke whale surpasses that of an animal with a larger His content at lower pH values. This supports that the position and p*K*a of amino acid residue is important. The β_{Mb} of Risso's dolphin is greater than that of the cow and the sheep at a pH just below pH 6. This pH is at the very limit of the measured physiological range of human skeletal muscle (Sahlin et al., 1976; Hermansen & Osnes, 1972; Robergs et al., 2004), but may indeed be an intracellular level occurring in the dolphin because of the high anaerobic cost of burst locomotion (Castellini & Somero, 1981; Williams et al., 2000). Comparison of the two carnivorans used in this study shows that the grey seal, with an increase of five His residues, has a greatly increased β_{Mb} compared to its close terrestrial relative, the raccoon in this case. The evidence shown above suggests that Mb His content may indeed play a role in $\beta_{muscleNB}$. However the overall contribution of Mb His content is determined by the [Mb] within the muscle tissue.

This study has produced a method of calculating Mb net surface charge from the contribution of individual ionisable residues, and from this it is possible to estimate Mb titration curves. Estimated titration curves for each of the ten species were calculated, and were shown to accurately predict the measured titration of cow, sheep and pig Mb, shown by dashed lines (**Fig 2.5A**). For every other species observed in this study, measured and

estimated titration curves do not match as accurately. This may be due to deamidation. Deamidation is a chemical reaction that occurs when a protein degrades, affecting mainly glutamine and asparagine amino acid residues. It affects the amide groups on these residues by conversion to the acidic forms, glutamic acid and aspartic acid, and thereby results in the addition of a negative charge (Robinson & Robinson, 2004). This has been shown to occur at three amino acid positions during the sequencing of *Delphinus delphis* Mb (Wang et al., 1977). During the purification of sperm whale Mb, comparisons have been made regarding various fractions (IV, IIIB, IIIA and II), where fraction IV is the major component and the others are minor fractions (Garner et al., 1974). Shire et al., (1974a) quoting Garner et al., (1974) noted a two charged residue difference between the major component IV and the minor component II, corresponding to the deamidation of two asparagine residues. The addition of strong negative charges over the physiological pH range, results in a downward shift of the titration curve and thereby a reduction of the p*I* of the protein. This is seen quite clearly in the minke whale and humpback whale where estimated titrations based on the native amino acid sequence would predict a p*I* of approximately 8, when an experimentally measured p*I* was closer to 6 for both species (**Fig 2.5C**). A constant offset, between estimated and experimentally measured titration curves, was noted for both species. The offset would correspond to the deamidation of approximately five residues, which is more than previously observed during purification of the dolphin and sperm whale (Wang et al., 1977; Shire et al., 1974a). These two species probably had the highest amount of tissue degradation due to the time of death before tissue samples were collected. Tissues were collected from the minke whale approximately one week after the stranding event. Calculation of titration curves for the beaked whales were identical due to the species having the same composition of strongly charged residues and the same number and position of His residues. However the experimental titrations show that the Northern bottle-nosed whale has a reduction of almost

two fully charged residues compared to the Sowerby's beaked whale (**Fig 2.5B**). The constant offset between estimated and measured titration curves for both the Sowerby's beaked whale and Northern bottlenose whale suggest that deamidation has occurred to a similar degree to that seen in the dolphin and sperm whale (Wang et al., 1977; Shire et al., 1974a).

However as the estimated titration curves of all species are parallel to the measured titration curves, shown by constant offsets over the physiological pH range, the calculation of β_{Mb} will remain accurate, as it has been derived by calculating the slope on the titration curve at every pH throughout the physiological range. Plots of measured β_{Mb} against estimated β_{Mb} show how accurate the predicted β_{Mb} value can be, with an average (range) deviation from the line of identity of 0.25 (0.08-0.6), 0.19 (0.08-0.52) and 0.04 (0.02-0.33) at pH 6.0, 6.5 and 7.0 respectively (**Fig 2.6**). Estimated values are slightly lower than measured values at pH 6.0 and 6.5 in the species with the highest β_{Mb}, the beaked whales and the grey seal (**Fig 2.6**). This could be explained by variation within p*K*a values for His residues among species. For example a maximum difference, between seven species, of 0.34 is observed in the p*K*a of His152, a residue with an average p*K*a of 6.10 (Bothelo et al., 1978). Similarly the maximum observed difference in p*K*a value of His81 is 0.5, when comparing horse and grey whale, *Eschrichtius robustus*, (Bothelo et al., 1978; Kao et al., 2000).

Satisfied that model predictions are good and that Mb amino acid composition can be used to accurately predict β_{Mb}. $\beta_{muscleMb}$ was then calculated for as many species as possible and expressed as a percentage of $\beta_{muscleNB}$ in order to quantify the contribution of Mb. **Figure 2.7** indicates that with the exception of the insectivorans, Mb contributes a much larger amount to $\beta_{muscleNB}$ in mammalian divers than in their closest terrestrial relatives (**Fig 2.7**). In the order Insectivora the contribution of $\beta_{muscleMb}$ to $\beta_{muscleNB}$ is similar between divers and their closest terrestrial relatives, and in fact in the water shrew the contribution is even

slightly smaller than in the short-tailed. This may be due to the muscle composition of these small mammals. Skeletal muscles of terrestrial shrews consist almost exclusively of type II (fast twitch) fibres (Peters et al., 1999), which usually contain less Mb than type I (slow twitch) fibres (Peters et al., 1999). If this also applies to semi-aquatic shrews and even other members of the insectivorans, this may explain the relatively modest increase in Mb concentration in the skeletal muscles of the water shrew and star-nosed mole compared to their terrestrial relatives (**Table A7**). Clearly, given a low muscle [Mb], it would be futile to increase the Mb His content as the effect on $\beta_{muscleNB}$ would be minimal. It appears that insectivoran divers have used some other mechanism in order to achieve the elevated $\beta_{muscleNB}$ observed in these species. Perhaps there is a large increase in the concentration of His-related dipeptides? But so far these have not been measured in insectivores.

It should be pointed out that the absolute levels of $\beta_{muscleNB}$ attained in diving insectivorans are relatively modest compared to seals and whales (**Fig 2.7**). Emmet and Hochachka (1981) have shown that the maximal activity per unit muscle mass of lactate dehydrogenase, a marker enzyme for anaerobic metabolic capacity, decreases with increasing body mass across several mammalian species. Furthermore, the values for the single shrew species included in their study were more than two-fold lower than expected for their body mass based on the regression line for all investigated mammals (Emmett & Hochachka, 1981). Exceptionally low capacities for anaerobic metabolism have now been confirmed for two other shrew species, including the pygmy shrew, *Suncus etruscus*, one of the smallest existing mammalian species (Peters et al., 1999). Although there is currently no comparable information on diving shrews or other insectivorans, a generally low capacity for lactic acid production in the insectivoran skeletal muscle may have reduced the selection pressure for elevated $\beta_{muscleNB}$ in this order, explaining the relatively smaller increase in $\beta_{muscleNB}$ in diving insectivorans compared to diving mammals in other orders.

In all other diving species there is a substantial increase in contribution of $\beta_{muscleMb}$ to $\beta_{muscleNB}$ compared to terrestrial species, this is due to the increase in both β_{Mb} and [Mb] (**Table A2 & A3**). **Figure 2.7** highlights the variability in the contribution of His-related dipeptides to $\beta_{muscleNB}$, with some species, such as minke whale and common dolphin, relying quite substantially on them, yet in other species, such as bottlenose dolphin and hooded seal, their contribution is less than that of $\beta_{muscleMb}$.

This study suggests that between 6-45% of the increase in $\beta_{muscleNB}$ seen in mammalian divers compared to terrestrial species can be accounted for by the increase in $\beta_{muscleMb}$ (**Fig 2.7**). Differences between species observed here, may be explained by fibre type, as in shrews as discussed above, or by the dive behaviour of the individual species. Higher $\beta_{muscleNB}$ is seen in animals capable of long distance anaerobic, or burst locomotion (Castellini & Somero, 1981), such as dolphins and porpoises. A similar result is found for $\beta_{muscleMb}$, with the more active deeper diving phocid seals appearing to be more reliant on $\beta_{muscleMb}$ compared to the sea lion (**Fig 2.7**). $\beta_{muscleMb}$ (**Table A3**) is consistently lower in otariid seals compared to phocid seals. Similarly in the deeper diving pygmy sperm whale and Cuvier's beaked whale $\beta_{muscleMb}$ appears to contribute more towards $\beta_{muscleNB}$ than in other cetacean species.

Conclusion

In conclusion, the present study has determined 25 novel mammalian Mb amino acid sequences and demonstrates a general trend towards an increased Mb His content in 6 out of 14 investigated lineages of mammalian divers compared to their terrestrial counterparts. Comparison of the acid base titration curves of 10 mammalian Mbs with their primary sequence and known p*K*a values of ionisable groups in Mb have then been used to develop a model that accurately predicts the specific Mb buffer value, β_{Mb}, of any Mb from its primary

sequence. Together with data on muscle Mb concentration, calculation of $\beta_{muscleMb}$ has allowed for the first time quantification of the contribution of Mb towards $\beta_{muscleNB}$ in diving and terrestrial mammals and to compare it with the contribution of specific His-dipeptides that have been found to be elevated in muscles of some divers (Crush et al., 1970; Abe, 2000). The data shows that, contrary to previous reports, Mb in some species contributes a substantial proportion to the increased muscle non-bicarbonate buffer value generally seen in diving mammals. Thus, the elevated β_{Mb} due to three extra His residues in Mb of true seals, together with increases in [Mb], has been shown to increase $\beta_{muscleNB}$ by up to forty five per cent above the level of $\beta_{muscleNB}$ in their close terrestrial relatives.

This study provides novel insights into how cumulative substitutions on the molecular surface of Mb, away from the active site of the haem group, can have a profound adaptive effects on the physiological properties conveyed to the whole animal.

Chapter 3 - Increased myoglobin net charge in mammalian divers: A mechanism to aid high concentration?

Introduction

Protein self-association is a problem with both biochemical and medical concerns. Protein aggregates can form as a result of mis-folding or as a consequence of high protein concentration; and these self-associations occur more frequently in partially folded intermediates (Shiraki et al., 2002). Protein aggregates can either be soluble or insoluble, soluble aggregates tend to be small peptides or proteins, recently these have been shown to occur more frequently than previously thought and are closely associated with neural misfunction, more so than the larger amyloid fibrils (Rajagopalan et al., 2011). These small soluble aggregates may then lead to the formation of larger insoluble aggregations, at which point precipitation can occur, if this occurs *in vivo* then serious medical problems can occur. Aggregation of misfolded bA4 amyloid protein and its associated deposition in brain tissue is responsible for Alzheimer's disease (Joachim and Selkoe, 1992). Other structured deposits (Amyloid fibrils), such as fibrillar a-synuclein Lewy bodies, are associated with Parkinson's disease (Lansbury, 1999) and fibrillar Huntingtin nuclear inclusions are responsible for Huntington's disease (Scherzinger et al., 1997). Another form of spherical aggregate called a spherulite, has been observed to occur in insulin with implications in type II diabetes (Krebs et al., 2008). As a result, mechanisms of aggregation are being studied extensively. Insoluble amyloid-fibril aggregates seem to form more readily in proteins consisting mainly of β sheets, because hydrophobicity of helical structures helps to prevent fibril formation (Fandrich et al., 2003). High protein concentrations lead to the formation of amyloid-fibrils (Fink, 1998), and high protein concentration has also been shown to help the formation of aggregates in proteins that do not readily form fibrils (Rumen & Appella, 1962).

During a dive any mammalian diver must carry enough oxygen to support the duration of a dive. Oxygen can be stored within myoglobin (Mb) in the skeletal muscles of

diving mammals (Kanatous & Mammen, 2010). A hallmark of mammalian divers is an elevated level of skeletal muscle Mb with concentrations of up to thirty times higher than those of their close terrestrial relatives (Irving 1939; Scholander 1940). This raises the question of how mammalian divers avoid the problem of Mb aggregation at the high concentrations needed to provide enough oxygen for long dives.

Any protein is least soluble at its iso-electric point (p*I*), this is also the point where the protein has zero net surface charge. If the protein net surface charge is increased or decreased then the solubility of the protein increases (**Fig 3.1A**). The net surface charge of a protein is governed by the p*K* of the ionisable residues on its surface (Tanford, 1962). The p*K*a value of a residue provides information about the ionisation state of the amino acid residue. Thus, at a pH identical to its p*K*a value, half of a given ionisable residue will be protonated. Therefore the residue will contribute half a charge towards the overall net charge of the protein. Strongly positively charged residues, arginine (Arg) and lysine (Lys) have high p*K*a values and at high pH will be neutral, as pH decreases these residues become protonated and will have a full positive charge within the physiological pH range. Equally, strongly negatively charged residues aspartate (Asp) and glutamate (Glu) are negatively charged at high pH and take up a proton becoming neutral at very low pH. They will therefore be fully negatively charged at physiological pH. Also strongly charged are the amino terminal (N-term) amino group, which is positively charged, and the carboxyl terminus (C-term) which is negatively charged. Residues that are not as strongly charged, such as the neutral to positively charged histidine (His) and the neutral to negatively charged cysteine (Cys), will become protonated or give up a proton, respectively, within the physiological pH range. Tyrosine (Tyr) is a negatively charged residue with a high p*K*a value therefore meaning it will be neutral in the physiological pH range (**Fig 3.1B**).

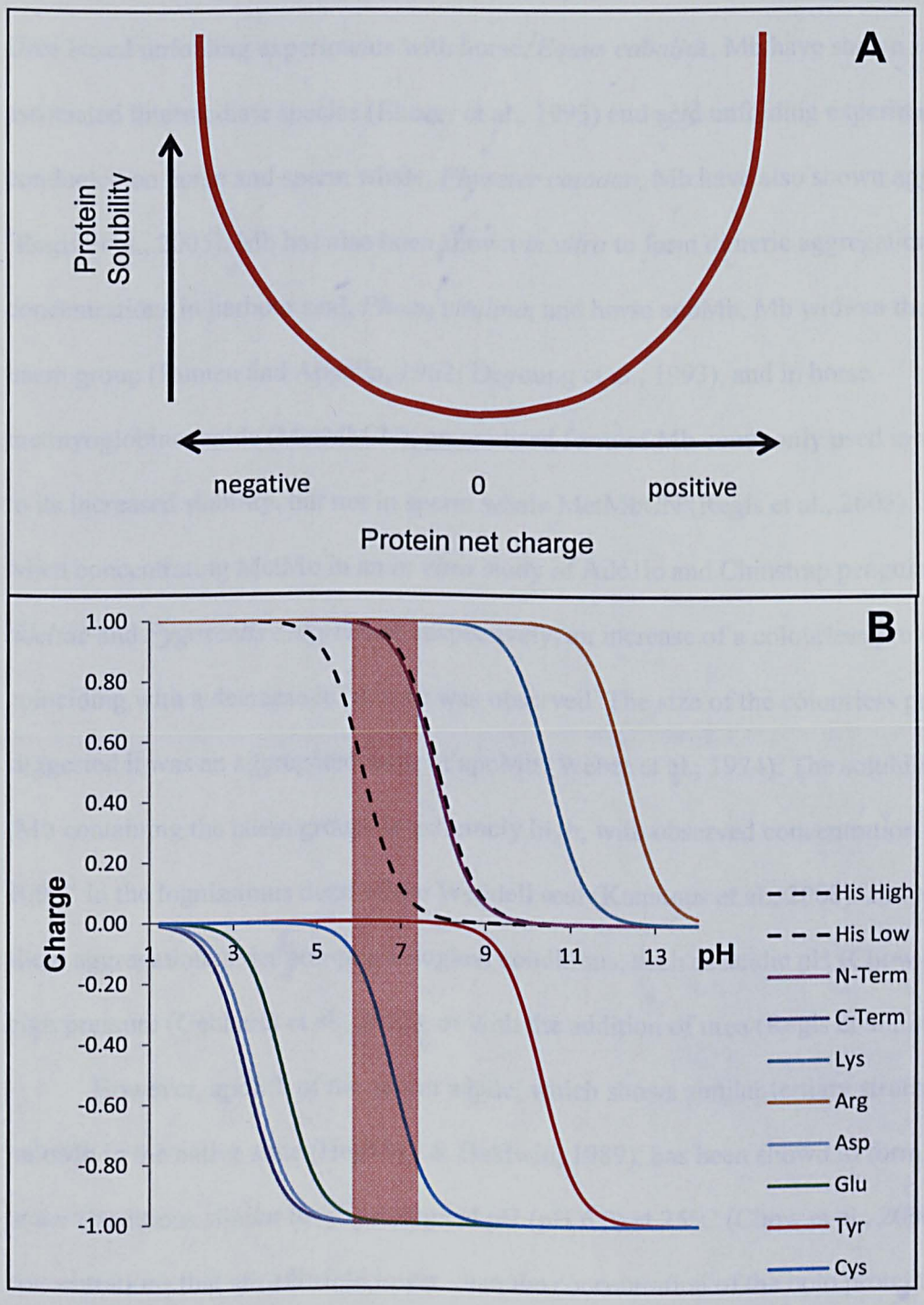


Figure 3.1: A) Schematic representation of the increase in protein solubility as protein net charge is increased or decreased from the p*I*. **B)** Ionisation states of ionisable amino acid residues. The red bar indicates physiological pH and the dotted black lines represent representative His residues in Mb with a low and a high p*K*a value, respectively.

Even though Mb is a mostly helical protein it has been shown to form aggregates. Urea based unfolding experiments with horse, *Equus caballus*, Mb have shown self-associated intermediate species (Eliezer et al., 1993) and acid unfolding experiments conducted on horse and sperm whale, *Physeter catodon*, Mb have also shown aggregations (Regis et al., 2005). Mb has also been shown *in vitro* to form dimeric aggregations at high concentrations in harbour seal, *Phoca vitulina*, and horse apoMb, Mb without the associated haem group (Rumen and Appella, 1962; Deyoung et al., 1993), and in horse metmyoglobincyanide (MetMbCN), an oxidised form of Mb commonly used in research due to its increased stability, but not in sperm whale MetMbCN (Regis et al., 2005). Interestingly, when concentrating MetMb in an *in vitro* study of Adélie and Chinstrap penguins, *Pygoscelis adeliae* and *Pygoscelis antarcticus*, respectively, an increase of a colourless protein coinciding with a decrease in MetMb was observed. The size of the colourless protein suggested it was an aggregated form of apoMb (Weber et al., 1974). The solubility of holoMb (Mb containing the haem group) is extremely high, with observed concentration of over 7 g $100g^{-1}$ in the lognissimus dorsi of the Weddell seal (Kanatous et al., 2008), and tends only to show aggregation under non-physiological conditions, such as acidic pH (Chow et al., 2006), high pressure (Gebhardt et al., 2003), or with the addition of urea (Regis et al., 2005).

However, apoMb of the sperm whale, which shows similar tertiary structure to holoMb in the native state (Hughson & Baldwin, 1989), has been shown to form aggregates under conditions similar to physiological pH (pH 6.0) at 25°C (Chow et al., 2006), and at concentrations that are 100-fold lower than the concentration of the holo protein for sperm whale, namely 48.45 mg 100 g^{-1} muscle wet weight apoMb (Chow et al., 2006) compared to 7.0 g 100 g^{-1} holoMb (Sharp & Marsh, 1953). Investigations into the stability of apoMb have shown that this Mb precursor in diving mammals generally has higher resistance to acid

denaturation and urea unfolding than apoMb of terrestrial mammals (Hughson and Baldwin, 1989; Regis et al., 2005; Scott et al., 2000).

Regis et al. (2005) demonstrated that sperm whale MetMbCN is about one and a half times more resistant to urea unfolding compared to horse MetMbCN. They also found that horse MetMbCN forms aggregates at much lower concentrations compared to sperm whale. Thus, at twice the concentration leading to the formation of aggregates in horse MetMbCN, sperm whale still has MetMbCN in the single monomer form. The same study indicated that it was not differences in haem binding properties, but rather differences in the primary structure of Mb that conveyed a greater resistance to aggregation in mammalian divers compared to terrestrial species.

Recently, increased attention has been directed at the structural properties of a protein that determine its aggregation propensity. Trovato et al. (2007) developed a theoretical model to determine aggregation prone regions of proteins. When applied to sperm whale Mb the model predicted an increased propensity to aggregate in the G-helix region, residues 100-118. Indeed, horse apoMb has been shown to form aggregations involving the G-helix and these aggregates have led to the formation of amyloid fibrils in non-physiological conditions (Fandrich et al., 2003).

In order to prevent aggregation occurring because of high concentration, then it makes sense that the solubility of a protein must be increased. Shaw et al. (2001) demonstrated that protein solubility is affected by the net surface charge of a protein. By directly mutating the primary sequence of ribonuclease Sa, an enzyme involved in RNA degradation, these authors increased the content of positively charged residues within the amino acid sequence, thereby raising the net surface charge of the protein. They found that the mutations altered the iso-electric point (p*I*) of the protein, the point at which a protein has zero charge and is least soluble, and that they increased its solubility at different pH values compared to the native

protein. McLellan (1984) compared Mb protein sequences from 13 species of cetacean and horse and showed that differences in amino acid sequence were associated with differences in electrophoretic mobility in native polyacrylamide gels, a method of separating similar sized proteins on the basis of surface charge of the protein. Differences in mobility and therefore charge were explained by the composition of charged residues in the amino acid sequences of the different Mbs.

This study compares Mb primary sequences of a large number of mammalian species with the aim to identify specific amino acid changes that only occur in the Mb protein of diving mammals. The specific hypothesis is that diving species from multiple lineages will have an increase in the net surface charge of their Mb protein compared to terrestrial mammals, and that this increase in net surface charge would convey a greater aggregation resistance in the holo protein or its precursor at high concentrations, by means of electrostatic repulsion.

Methods

The study of Mb net surface charge requires the knowledge of Mb amino acid sequence for as many mammalian species as possible. Published amino acid sequences were obtained from the national centre for biotechnology information (NCBI) and from the gene index project (TGI formerly TIGR) see appendix **Table A.1** for details. Mb amino acid sequences for twenty six species were determined in this study by PCR of cDNA and Sanger sequencing (see **Chapter 2** for details). Mb net surface charge was determined in **Chapter 2,** however the data is analysed in detail in this chapter.

Charge estimation

Mb net surface charge was calculated according to the protocol in **Chapter 2.** Briefly protein charge was estimated, from the estimated charge contribution of each ionisable residue in the amino acid sequence. The charge of each residue at every pH value ranging from 4-11 with increments of 0.1 was calculated according to the formula

$$S = [H^+]^n/([H^+]^n+a^n) \qquad \textbf{Equation 2.2}$$

where $[H^+]$ is the hydrogen ion activity 10^{-pH}, a is 10^{-pKa} and n refers to a cooperativity constant. As the cooperativity constant was not available for all residues, and when cooperativity constants were included in initial analysis the difference it made to charge calculations in the physiological pH range were minor, unity was assumed for n for all ionisable groups. Experimental p*K*a values determined for each specific residue in Mb were used where possible. An average value for that type of residue in folded proteins was used if experimental data was not available (**Table 2.3**)

Mb Purification

The purification of muscle tissues from eight species, namely Sowerby's beaked whale, *Mesoplodon bidens*, Northern bottlenose whale, *Hyperoodon ampullatus*, Risso's dolphin, *Grampus griseus*, Humpback whale, *Megaptera novaeangliae*, Minke whale, *Balaenoptera acutorostrata*, Cow, *Bos taurus*, Sheep, *Ovis aries* and Pig, *Sus scrofa,* was carried out as described in **Chapter 2**. Briefly, tissues were thawed and homogenised on ice using an Ultra-Turrax T25 basic, in cold 20 mM phosphate buffer in a ratio of 1 g muscle tissue per 5 ml of buffer. The samples were centrifuged at 10,500 *g* for 20 min at 4°C and the supernatant filtered, before pH adjustment back to its starting pH.

A two-step approach of cation exchange followed by gel filtration was used, removing the need for initial ammonium sulphate precipitation and therefore reducing loss of protein (O'Brien 1992). Cation exchange was conducted at room temperature on a column containing SP Sepharose fast flow (Sigma) at a flow rate of 2 ml min^{-1}. Elution of Mb was achieved through the addition of 40 mM NaCl. The collected Mb fractions were concentrated using Amicon Ultra-15 centrifugal filter unit (Millipore). Mb was separated from any remaining proteins by size exclusion gel filtration chromatography using a Hi-Load 16/60 Superdex 75 prep grade column (GE healthcare) equilibrated with 100 mM KCl using a flow rate of 0.5 ml min^{-1} at a temperature of 4°C.

Native polyacrylamide gel electrophoresis (PAGE)

Native PAGE was performed using a 9% polyacrylamide gel with a 2-morpholinoethanesulfonic acid (MES) buffer at pH 6.0 in a 0.3 M final concentration, pH 6.0 was used to ensure that all samples would run towards the cathode. 5 µl of purified Mb samples (~0.1mM) from the eight species mentioned above was converted to MetMbCN and readied for Native PAGE, with the addition of 1µl 10X modified Drabkin's reagent,

consisting of $NaHCO_3$ (11.9 mmol l^{-1}), $K_3[Fe(CN)_6]$ (0.61 mmol l^{-1}) and KCN (0.77 mmol l^{-1}) (Völkel & Berenbrink 2000), and 5 µl 40% glycerol, 0.2 M MES pH 6.0 loading buffer and incubated at room temperature for 5 min. 5µl of treated protein sample was loaded to the gel and electrophoresis carried out at 200 V for 50 min, on ice with stirring of electrophoresis buffer, in a Bio-Rad Mini-PROTEAN II gel system. Gels were then stained with EZBlue™ gel staining reagent (Sigma) according to manufacturer's guidelines. Electrophoretic mobility was taken as the distance travelled through the native gel, measured to the nearest mm. Electrophoretic mobility was then plotted against calculated net charge at pH 6.0, and to test for a significant correlation, accounting for phylogenetic relatedness using phylogenetically independent contrasts as described below.

Phylogenetic Independent Contrasts

Maximal Mb content was collected for as many mammalian species as possible (**Table A3**). These data were then tested for a correlation with calculated net charge at pH 6.5 by standard ordinary least squares regression as calculated using Excel. Data was trimmed by removing closely related species that had identical Mb protein sequences, leaving only the species with the highest Mb content. The data set was then transferred to the mesquite software package (Maddison & Maddison, 2010) and regression analysis was corrected for phylogenetic relationships using Felsenstein's (1985) phylogenetically independent contrasts (PIC), within the added software module Phenotypic Diversity Analysis Programs (PDAP):PDTREE (Midford et al., 2005).

PIC analysis corrects for statistical non-independence in correlational analyses of two continuous valued characters in groups of hierarchically related species by taking their phylogenetic relationship (**Fig 1.1**) into account. Branch lengths were set to Pagel's arbitrary branch lengths. Statistically independent values are computationally created for internal

nodes and standardised contrasts are performed with this information. Diagnostic plots of absolute values of standardized contrasts versus their standard deviations were used to confirm the suitability of branch lengths in the analysis of the trimmed data set. Phylogenetically independent regression was performed by producing scatter plots of the standardised contrasts with an ordinary least squares regression forced through the origin.

RESULTS

A summary of the number of strongly charged residues and the calculated Mb net surface charge at pH 6.5 is given for mammals in **Table 3.1**. The pattern of calculated charge shown here is almost identical across a range of pH from 6.0 to 7.0 and thus only charge calculated at pH 6.5 is shown, as this is a mid-point in the physiological pH range observed for the exercising skeletal muscle in humans (Sahlin et al., 1976). Currently there is no data available for exercising muscle pH in diving species. **Table 3.1** indicates that almost all diving mammals have an increased Mb net positive charge at pH 6.5 compared to their closest terrestrial relatives. In phocid and otariid seals Mb net charge is usually double that of the terrestrial carnivorans. Similarly Mb net charge in cetaceans is almost double that of their closest terrestrial relatives the Artiodactyla, except for the red deer, *Cervus elaphus*, which has a higher estimated Mb net charge compared to other artiodactyls. The Mb net surface charge of diving insectivorans and rodents is more than double that of their close terrestrial relatives, more than a four-fold increase in Mb net charge is shown between the Eurasian beaver, *Castor fiber*, and its closest relative the kangaroo rat, *Dipodomys ordii* (**Table 3.1**).

Within the insectivorans the number of strongly positively charged residues is identical among all species observed, with the exception of the hedgehog, *Erinaceus europaeus*, that has one additional strongly positively charged residue. The Mb His content in insectivorans is generally low compared to other mammals and there is no trend in His content among diving and terrestrial species. However, there is a difference in the content of strongly negatively charged residues whereby the diving species, water shrew, *Sorex palustris*, and star-nosed mole, *Condylura cristata*, have a reduction in the amount of these strongly negatively charged residues (**Table 3.1**). This indicates that diving insectivorans have increased Mb net charge by reducing the content of strongly negatively charged residues.

Table 3.1 Number of ionisable amino acid residues and estimated Mb net surface charge at pH 6.5 for mammals observed in this study. Semi-aquatic species are highlighted in green and prolific divers are highlighted in blue. Asterisks indicate species whose Mb has been sequenced for the first time in this study

Species	Glu	Asp	Σ strong -ve	Lys	Arg	Σ strong +ve	His	Net charge @ pH 6.5		
Water shrew*	12	6	18	19	2	21	5	2.65	Insectivora	
Short-tailed shrew*	12	9	21	19	2	21	7	0.15		
Hedgehog	13	9	22	20	2	22	8	0.27		
Star-nosed mole*	11	7	18	19	2	21	7	3.15		
Coast mole*	13	7	20	19	2	21	7	1.16		
Grey seal	14	8	22	19	5	24	13	4.34	Phocidae	
Baikal seal	13	8	21	19	5	24	13	5.34		
Harbour seal	14	8	22	19	5	24	13	4.34		
Ringed seal*	14	8	22	19	5	24	13	4.34		
Harp seal*	14	8	22	19	5	24	13	4.34		
Hooded seal*	14	8	22	19	5	24	13	4.34		
Bearded seal*	14	8	22	19	5	24	12	4.21		
Weddell seal*	14	8	22	19	5	24	13	4.34		
Elephant seal*	14	8	22	18	6	24	12	4.21		
California sea lion *	13	9	22	21	4	25	11	4.22	Otariidae	
Steller sea lion *	13	9	22	21	4	25	11	4.22		
Australian sea lion*	13	9	22	21	4	25	11	4.22		
Northern fur seal*	13	9	22	21	4	25	11	4.22		
Walrus*	13	9	22	21	4	25	10	4.08		
Raccoon*	13	9	22	21	2	23	8	1.27	Musetlidae	
Badger	14	7	21	21	2	23	8	2.43		
American mink*	14	8	22	21	2	23	8	1.27		
River otter	14	8	22	21	3	24	8	2.27		
Black bear	14	8	22	20	3	23	9	1.99	Ursidae	
Polar bear*	14	8	22	20	3	23	9	1.99		
Giant panda	14	8	22	20	3	23	8	1.27		
Cape fox	13	10	23	21	2	23	10	1.54	Canidae	
Bat-eared fox	13	10	23	21	2	23	10	1.54		
Dog	13	10	23	21	2	23	10	1.54		
African hunting dog	13	10	23	21	2	23	10	1.54		
Cat	15	7	22	20	3	23	10	1.97		
Horse	13	8	21	19	2	21	11	1.76	Artiodactyla	
Zebra	13	8	21	19	2	21	11	1.76		
Alpaca (incomplete)	2	4	6	4	1	5	2			
Pig	14	8	22	19	2	21	9	-0.01		
Cow	13	8	21	18	2	20	13	1.84		
Yak	13	8	21	18	2	20	13	1.84		
Water buffalo	14	8	22	18	2	20	13	0.85		
Goat	13	8	21	18	2	20	12	1.56		
Sheep	14	7	21	17	2	19	12	0.56		
Tibetan antelope	13	8	21	18	2	20	12	1.56		
Red deer	13	7	20	18	2	20	12	2.56		

Species	Glu	Asp	Σ strong -ve	Lys	Arg	Σ strong +ve	His	Net charge @ pH 6.5		
Pygmy hippo*	16	6	22	18	3	21	10	0.25		
Fin back whale	12	9	21	20	2	22	12	3.01		Cetacea
Humpback whale	12	9	21	20	3	23	11	3.72		
Grey whale	12	9	21	20	3	23	11	3.72		
Pygmy Bryde's whale	12	9	21	20	3	23	11	3.72		
Sei whale	12	9	21	20	3	23	11	3.72		
Minke whale	13	8	21	20	3	23	12	4.01		
Bowhead*	12	9	21	20	3	23	11	3.72		
Pygmy sperm whale	14	7	21	19	4	23	13	4.31		
Dwarf sperm whale	14	7	21	19	4	23	13	4.31		
Sperm whale	14	7	21	19	4	23	12	4.22		
Stejneger's beaked whale	14	7	21	21	2	23	14	5.10		
Hubbs' beaked whale	14	7	21	21	2	23	14	5.10		
Sowerby's beaked whale*	14	7	21	21	2	23	14	5.10		
Longmans' beaked whale	14	7	21	21	2	23	14	5.10		
Northern bottlenose whale*	15	6	21	21	2	23	14	5.10		
Cuvier's beaked whale	14	7	21	20	3	23	14	5.10		
Amazon river dolphin	13	8	21	20	3	23	12	4.04		
Narwhal*	13	8	21	19	4	23	12	4.04		
Beluga (incomplete)	12	6	18	20	3	23	12			
Harbour porpoise	14	7	21	20	3	23	12	4.05		
Dall's porpoise	14	7	21	20	3	23	12	4.05		
Killer whale	12	9	21	20	3	23	12	4.04		
White beaked dolphin*	12	9	21	20	3	23	12	4.04		
Risso's dolphin*	12	9	21	20	3	23	12	4.04		
Long-fin pilot whale	13	8	21	20	3	23	12	4.04		
Melon head whale	12	9	21	20	3	23	12	4.04		
Bridled dolphin	12	9	21	20	3	23	12	4.04		
Saddleback dolphin	12	9	21	20	3	23	12	4.04		
Bottlenose dolphin	12	9	21	20	3	23	12	4.04		
House mouse	13	9	22	20	2	22	7	-0.18		Rodentia
Norway rat	14	7	21	20	2	22	6	0.33		
Muskrat	13	8	21	20	3	23	11	3.61		
Mole rat	13	8	21	21	1	22	7	1.16		
Ehrenberg's mole rat	13	8	21	20	1	21	8	0.27		
Kangaroo rat	15	8	23	20	2	22	5	-1.66		
Eurasian beaver	14	6	20	21	2	23	11	4.62		
Plains viscacha	13	8	21	17	4	21	8	0.27		
Casiragua	14	7	21	18	3	21	8	0.27		
Guinea pig	13	8	21	18	3	21	8	0.27		
Northern gundi	15	8	23	19	2	21	8	-1.72		
Grey squirrel*	16	7	23	21	2	23	7	0.17		
Ground squirrel (incomplete)	9	6	15	16	0	16	7			

All carnivorans have the same number of strongly negatively charged amino acids in their Mb sequences, with the exception of the canines that have one additional strongly negatively charged residue compared to the other carnivoran species. In terms of strongly positively charged amino acids all terrestrial species have twenty three residues. With the exception of the semi-aquatic American mink, *Neovison vison* and polar bear, *Ursus maritimus*, there is a trend in the diving carnivorans to increase the number of strongly positively charged residues. The river otter, *Lutra lutra*, and phocid seals all have twenty four residues and the otariid seals have twenty five strongly positively charged residues, which is the highest number of this type of residue in all mammals (**Table 3.1**). All mustelids have an identical Mb His content which is low compared to other carnivoran species. The ursids have one additional His compared to the mustelids but this is still lower than the canines and the one member of the felines, who all have ten His residues which is classified as a high number by this study. There is a trend to increase Mb His content in the more prolific divers, the otariid and phocid seals having eleven and twelve His, respectively, which is high compared to the other carnivorans (**Table 3.1**). It appears that the diving carnivorans increase their Mb net charge by increasing both the amount of strongly positively charged residues and in the case of the seal families by also increasing the Mb His content.

The members of the order Cetartiodactyla have an almost identical quantity of strongly negatively charged residues, with all terrestrial species having either twenty one or twenty two residues, with the exception of the red deer that has twenty. All diving species within this order have twenty one strongly negatively charged residues. The terrestrial species have twenty strongly positively charged residues, with the exceptions of the sheep, *Ovis aries*, and pig, *Sus scrofa*, that have nineteen and twenty one respectively. All cetaceans, however, have twenty three strongly positively charged amino acids, which is an increase of at least two residues compared to their close terrestrial relatives. Mb His content is high

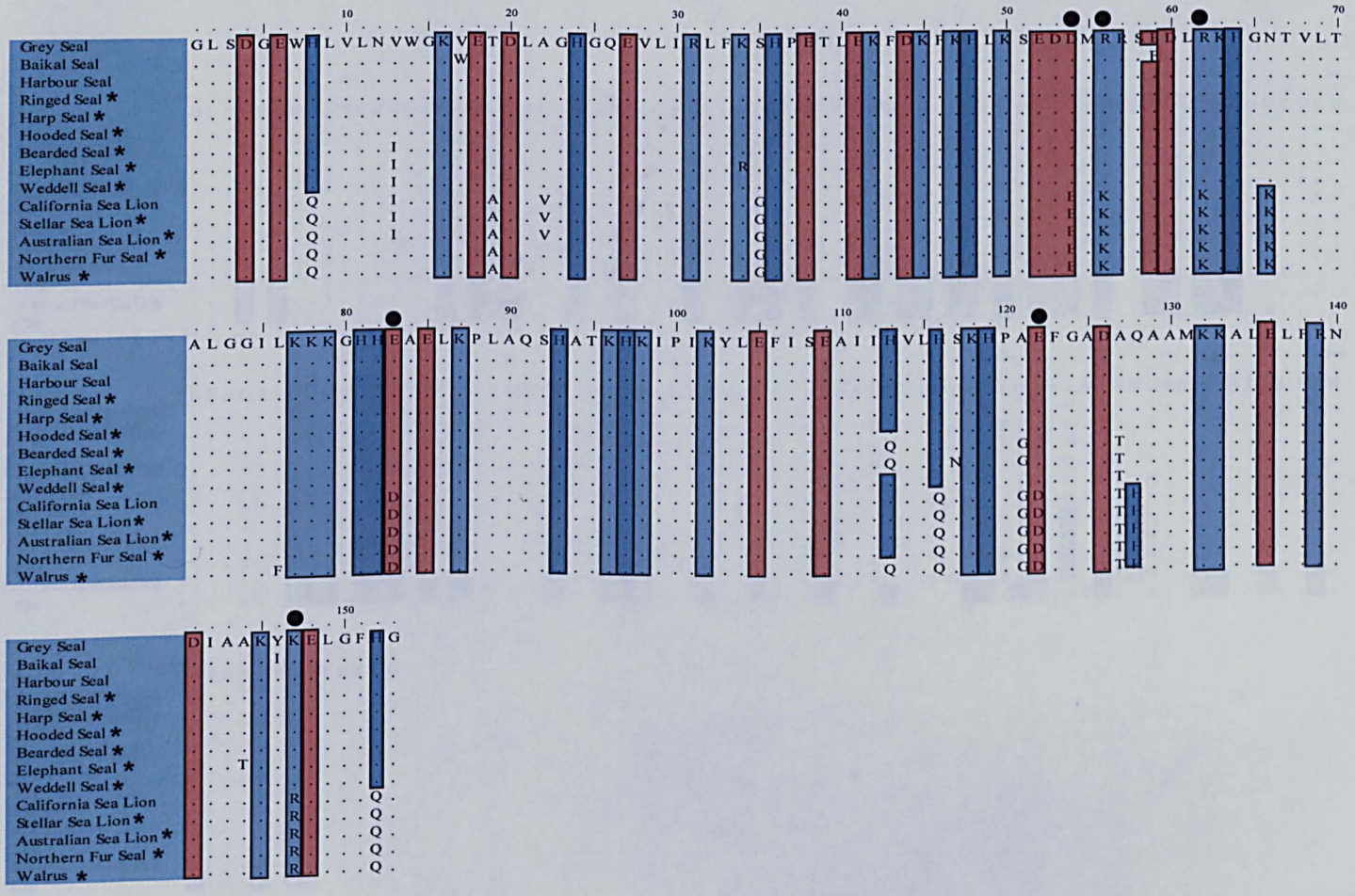
among all species within the order Cetartiodactyla, with the terrestrial species having either twelve or thirteen His residues, excluding the most basal member of the order investigated here, the pig that has nine. This compares to between eleven and fourteen His residues in the diving artiodactyls. The lowest number of His in the latter group is observed in the baleen whales and the highest His content of all mammals is seen in the deep diving beaked whales. This analysis suggests that diving species in the order Cetartiodactyla have increased Mb net charge by increasing the number of strongly positively charged residues and by having a high number of His, too. The exception to this is the pygmy hippo, *Choeropsis liberiensis*, which shows quantities of charged residues more similar to levels observed in terrestrial species and only ten His. The pygmy hippo also has a low estimated Mb net surface charge compared to most members of this order (**Table 3.1**).

Within the order Rodentia, terrestrial species generally have the lowest Mb net charge, a charge that is similar to the terrestrial insectivorans. The terrestrial rodents have between twenty one and twenty three strongly negatively charged residues and a similar number of strongly positively charged amino acids. While the diving species have twenty or twenty one strongly negatively charged residues they both have one additional strongly positively charged residue giving them twenty three. The Mb His content in terrestrial rodents is also very low, ranging between five in the kangaroo rat and eight, which is the same quantity as seen in the mustelids. The diving rodents have an increased Mb His content with eleven His residues in both beaver and muskrat, which is three more than in most other rodent Mbs (**Table 3.1**).

Amino acid sequence alignments for all the mammals involved in this section of the study are given in **Figures 3.2A-D**. The figure highlights that Mb is a highly conserved protein among mammalian species, and that the charged residues are extremely well conserved. Two examples of this are firstly the residues of the A-helix, Asp4, Glu6, Lys16,

Glu18 and Asp20, which are conserved in every species observed here. Second, the residues following the proximal His93 in the F-G Helix regions, Lys96, His97, Lys98, Lys102, Glu105 and Glu109 are found in nearly every species observed here. Interestingly, Glu109 is not conserved among all species, however, where amino acid changes have occurred it has been replaced by an equally negatively charged Asp residue, namely in the badger, *Meles meles*, raccoon, *Procyon lotor*, the canines, the baleen and sperm whales and the members of the dolphin-porpoise super family, Delphinoidea (**Fig 3.2A-B**). Other charged residues are conserved throughout the protein as well, indicating that these strongly charged residues play an important role in the protein.

With so much of the Mb protein conserved, if sequence alterations occur it is likely that they may have an important physiological role. **Table 3.1** shows that within the order Insectivora diving species have an increased Mb net charge and this has probably been achieved through replacement of strongly negatively charged with neutral amino acids. **Figure 3.2C** highlights the fact that both distantly related divers, the water shrew and star-nosed mole, have indeed replaced three strongly negatively charged residues with neutral residues at positions 53, 122 and 136. They have also replaced a neutral serine, Ser132, with a strongly positively charged Lys132. Within other orders, mammalian divers appear to have increased Mb net charge by increasing the content of both strongly positively charged residues and Mb His content. The increases in strongly positively charged residues appear mainly to be increases in Lys. However these changes have not occurred at the same locations between the orders (**Fig 3.2A-D**). The diving rodents have replaced the neutral Asn87 with Lys87, the cetaceans replace neutral Thr24 and Asn140 with Lys. The diving carnivorans, however, have replaced the neutral Gly57 with Arg57, and the otariid seals replaced the neutral Asn66 with Lys66.



3.2A Phocid and otariid seal protein sequences. Diving species are highlighted in turquoise. Strongly negatively amino acids highlighted with red boxes, strongly positively charged residues are highlighted with blue boxes as are histidine residues. Filled circles indicate where alternate residues have evolved but charge has been maintained. Asterisks indicate sequences that have been produced by this study.

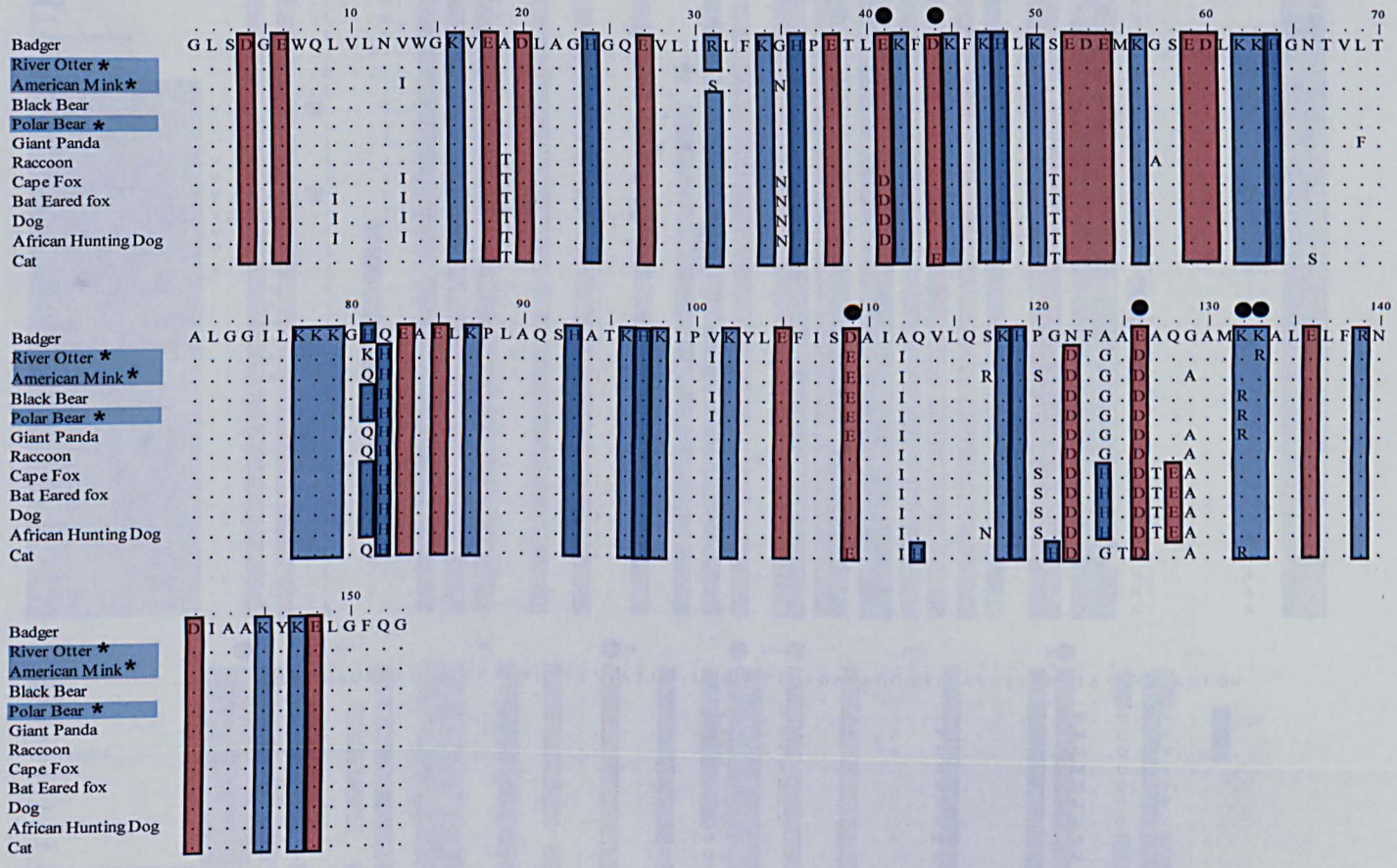


Figure 3.2A (Continued) Non-pinniped carnivoran protein sequences. Diving species are highlighted in turquoise. Strongly negatively charged amino acids highlighted with red boxes, strongly positively charged residues are highlighted with blue boxes as are histidine residues. Filled circles indicate where alternate residues have evolved but charge has been maintained. Asterisks indicate sequences that have been produced by this study.

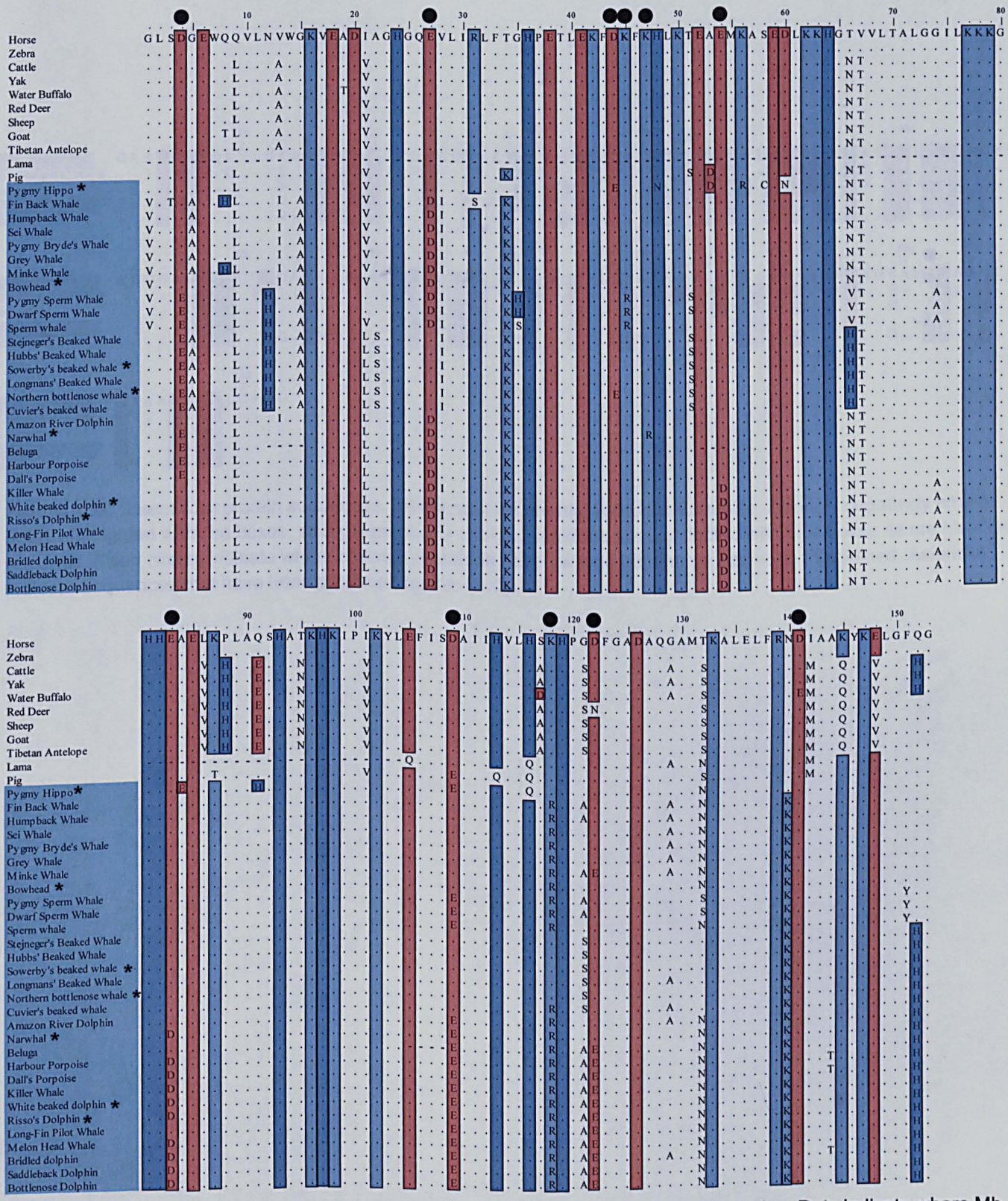


Figure 3.2B Cetartiodactyla protein sequences. Diving species are highlighted in turquoise. Dots indicate where Mb sequence is identical to the horse. Dashes indicate where sequence is unknown. Strongly negatively amino acids highlighted with red boxes, strongly positively charged residues are highlighted with blue boxes as are histidine residues. Filled circles indicate where alternate residues have evolved but charge has been maintained. Asterisks indicate sequences that have been produced by this study.

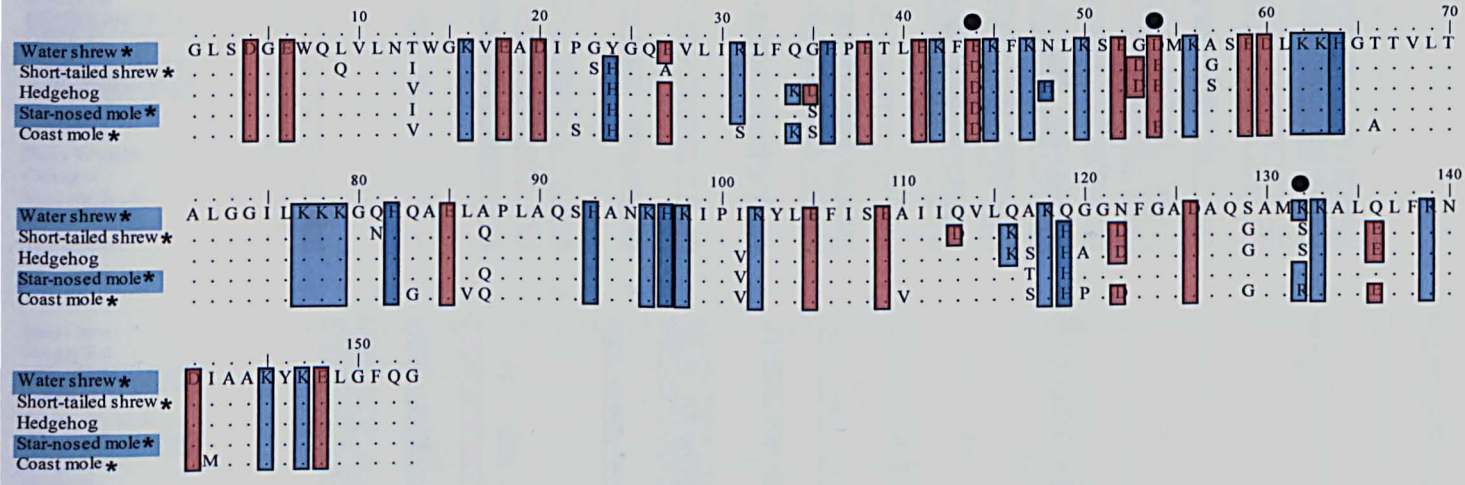


Figure 3.2C Insectivora protein sequences. Diving species are highlighted in turquoise. Strongly negatively amino acids highlighted with red boxes, strongly positively charged residues are highlighted with blue boxes as are histidine residues. Filled circles indicate where alternate residues have evolved but charge has been maintained. Asterisks indicate sequences that have been produced by this study.

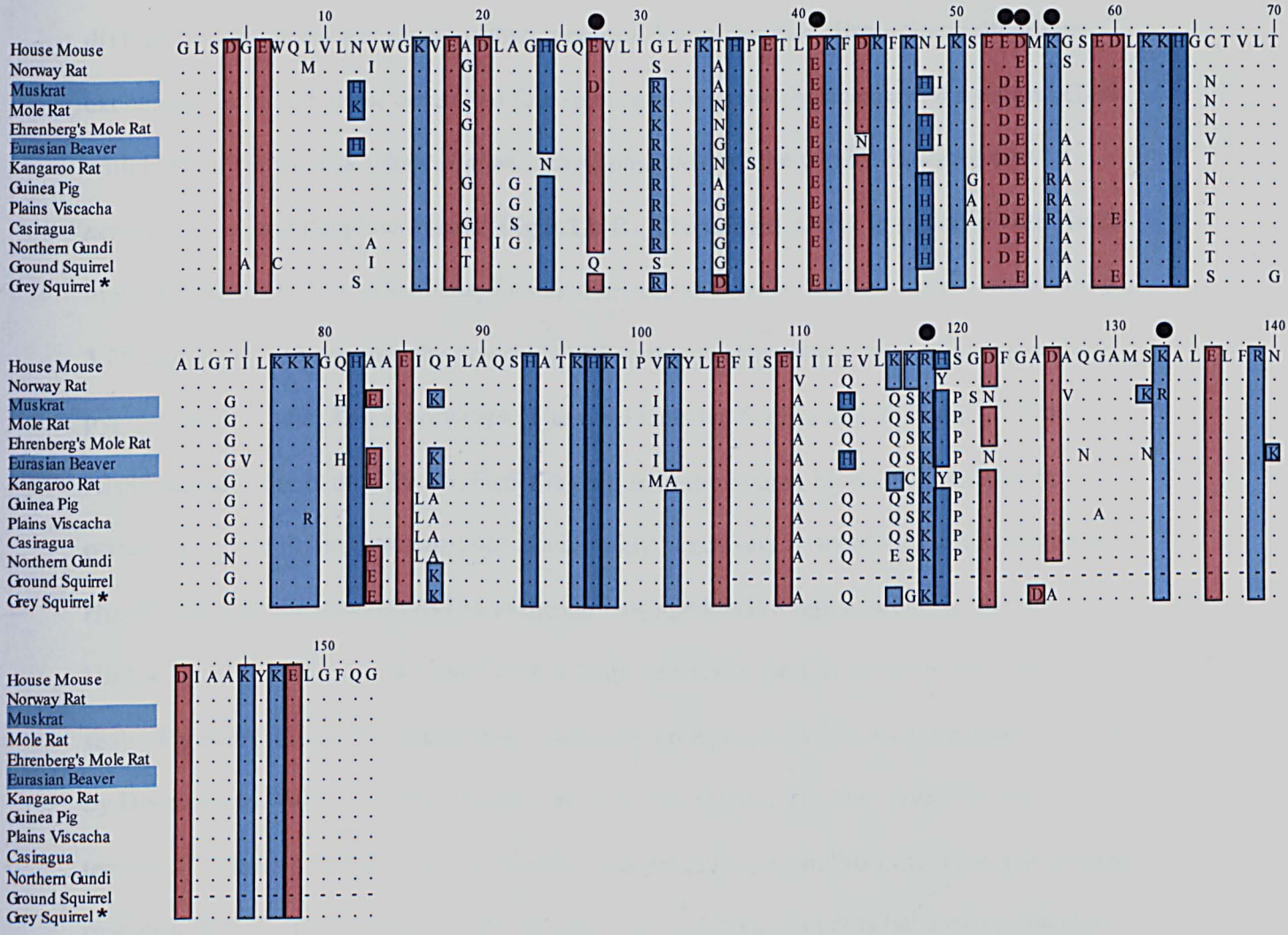


Figure 3.2D Rodentia protein sequences. Diving species are highlighted in turquoise. Dashes indicate where sequence is unknown. Strongly negatively amino acids highlighted with red boxes, strongly positively charged residues are highlighted with blue boxes as are histidine residues. Filled circles indicate where alternate residues have evolved but charge has been maintained. Asterisks indicate sequences that have been produced by this study.

One other amino acid change has occurred at position 66, this occurs in the deep diving beaked whales that replace either a neutral Val or Asn for His66. Some other His exchanges are noted, these occur in a few species such as the replacement of a neutral Gln8 with His8 in the finback whale, *Balaenoptera physalus*, minke whale, *Balaenoptera acutorostrata*, and the phocid seals (**Fig 3.2A-B**). The neutral Asn12 is replaced with His12 in the muskrat, *Ondatra zibethicus*, Eurasian beaver and the beaked and sperm whales (**Fig 3.2B** and **D**). His35 replaces the neutral Gly35 in the dwarf sperm whale, *Kogia simus*, and pygmy sperm whale, *Kogia breviceps*. The ruminants have a His that is unique to them, His88 replaced the neutral Pro88. His91 is unique to the pygmy hippo, this position is occupied by Gln in the cetaceans, but is a strongly negatively charged Glu in the ruminants. His124 replaces the neutral Gly124 in the canines and His128 replaces Gln in the otariids. His152 occurs in the beaked whales and dolphin species as well as in the bovids and phocid seals (**Fig 3.2A, B** and **D**). Interestingly there are three positions that are potentially occupied by His residues within the G-helix, these are His113, 116 and His119. Insectivorans, terrestrial rodents and terrestrial carnivorans and the diving mustelids only have one of these residues, His119. The cat, *Felis catus*, otariid seals and diving rodents have two of these G-helix residues, His113 and His119, otariid seals also have a third His residue in close proximity, His128, a His that is unique to those species. The biggest increase is observed in the phocid seals and the members of Cetartiodactyla. All of these species have all three of the G-helix His residues (**Fig3.2A-D**).

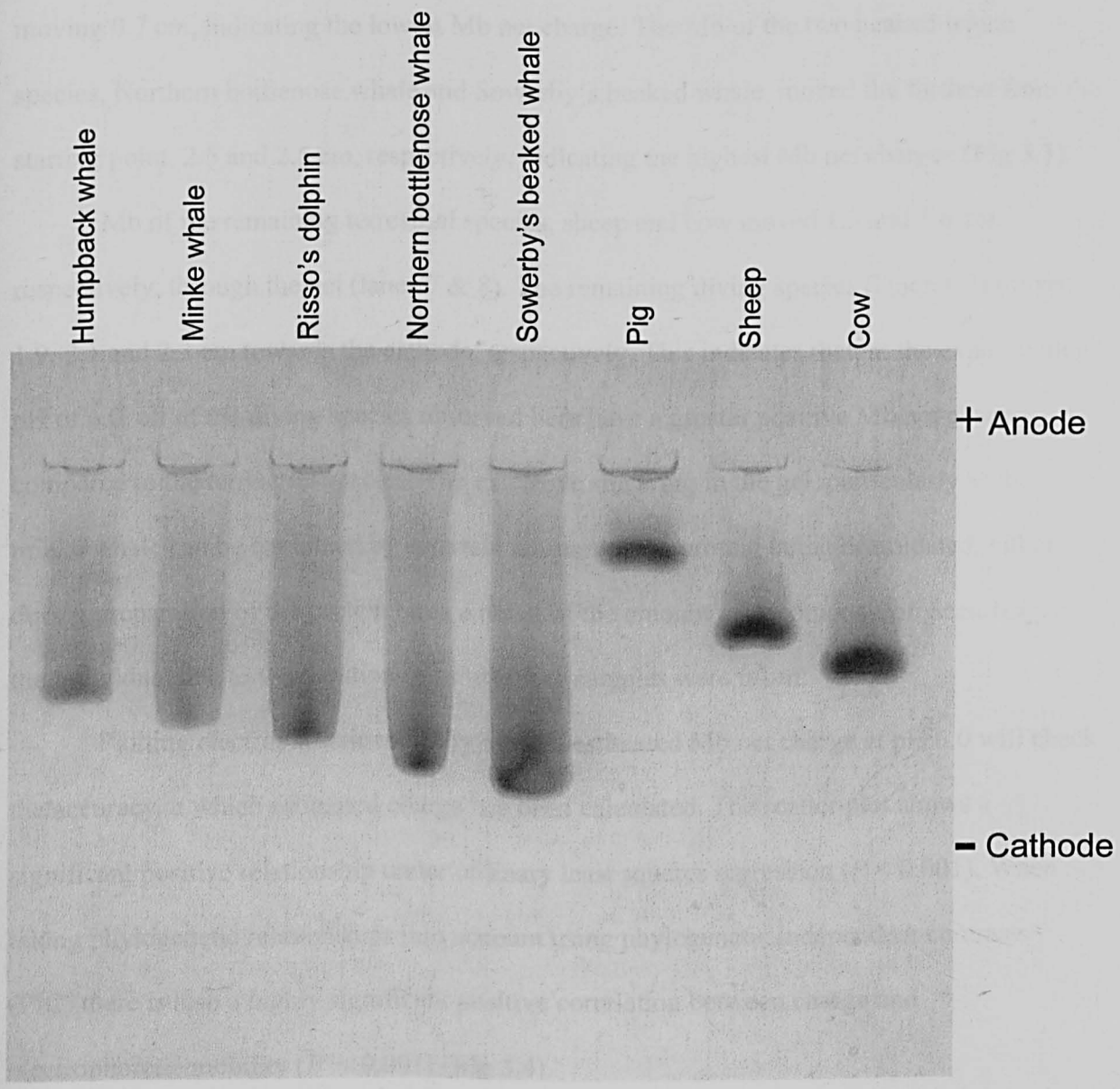


Figure 3.3 Gel electrophoresis of purified metMbCN under native PAGE conditions at pH 6.0. Gel was ran for 50 min at 200V. Cetacean species are: Lane 1 Humpback whale; 2 Minke whale; 3 Risso's dolphin; 4 Northern bottlenose whale; 5 Sowerby's beaked whale. Artiodactyl species are: Lane 6 Pig; 7 Sheep; 8 Cow. Extensive smearing is presumably due to deamidation.

The native PAGE image (**Fig 3.3**) shows that at pH 6.0 the purified MetMbCN for cetaceans observed in this study, travelled further towards the cathode than their closely related terrestrial counterparts. Pig Mb travelled most slowly of all the mammalian Mbs, moving 0.7 cm, indicating the lowest Mb net charge. The Mb of the two beaked whale species, Northern bottlenose whale and Sowerby's beaked whale moved the furthest from the starting point, 2.5 and 2.6 cm, respectively, indicating the highest Mb net charges (**Fig 3.3**).

Mb of the remaining terrestrial species, sheep and cow moved 1.3 and 1.6 cm, respectively, through the gel (lanes 7 & 8). The remaining diving species (lanes 1-3) moved 1.9, 2.1 and 2.3 cm towards the cathode, respectively. This indicates that, at the experimental pH of 6.0, all of the diving species observed here have a greater positive Mb net charge compared to the terrestrial species. The extensive smearing in the gel, particularly in the minke whale can be explained by a certain amount of the protein being deamidated, either during preparation of the protein or as a result of the amount of decomposition occurring in the individual due to the duration of time before samples were taken.

Plotting electrophoretic mobility against estimated Mb net charge at pH 6.0 will check the accuracy at which estimated charge has been calculated. The scatter plot shows a significant positive relationship under ordinary least squares regression ($P < 0.001$). When taking phylogenetic relationships into account using phylogenetic independent contrasts (PIC) there is also a highly significant positive correlation between charge and electrophoretic mobility ($P < 0.001$) (**Fig 3.4**).

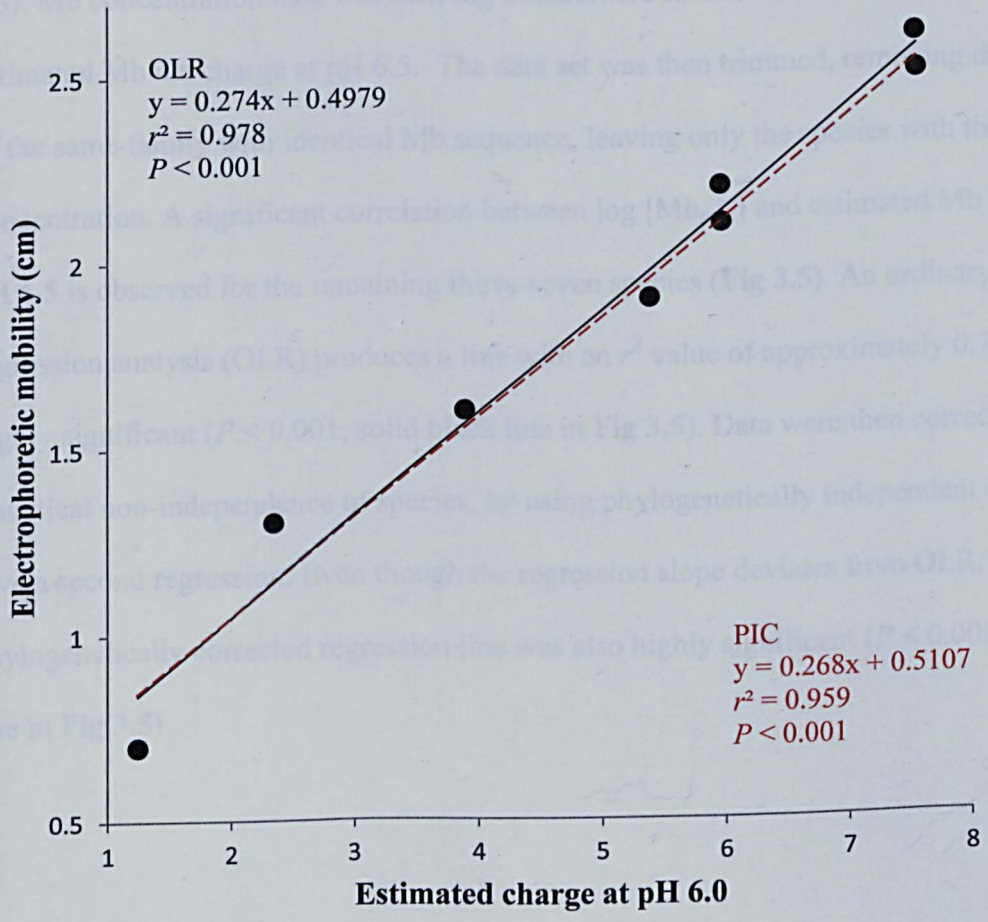


Figure 3.4. Significant positive correlation between electrophoretic mobility and estimated Mb net charge at pH 6.0. The terrestrial artiodactyls and cetaceans are the same as those described in figure 3.3. The black line indicates ordinary least squares regression (OLR) and the red dashed line is the phylogenetically corrected regression (PIC).

With the accuracy of estimated Mb net charge proven, this study set out to identify any link between Mb net charge and maximum Mb concentrations ($[Mb_{max}]$). Mb concentration data was taken for forty-eight species from various literature sources (**Table A3**). Mb concentration data was then log transformed to the base 10 and correlated with estimated Mb net charge at pH 6.5. The data set was then trimmed, removing data for species of the same family with identical Mb sequence, leaving only the species with the highest Mb concentration. A significant correlation between log $[Mb_{max}]$ and estimated Mb net charge at pH 6.5 is observed for the remaining thirty-seven species (**Fig 3.5**). An ordinary least squares regression analysis (OLR) produces a line with an r^2 value of approximately 0.72 that is highly significant ($P < 0.001$; solid black line in **Fig 3.5**). Data were then corrected for statistical non-independence of species, by using phylogenetically independent contrasts, to give a second regression. Even though the regression slope deviates from OLR, the phylogenetically corrected regression line was also highly significant ($P < 0.001$; red dashed line in **Fig 3.5**).

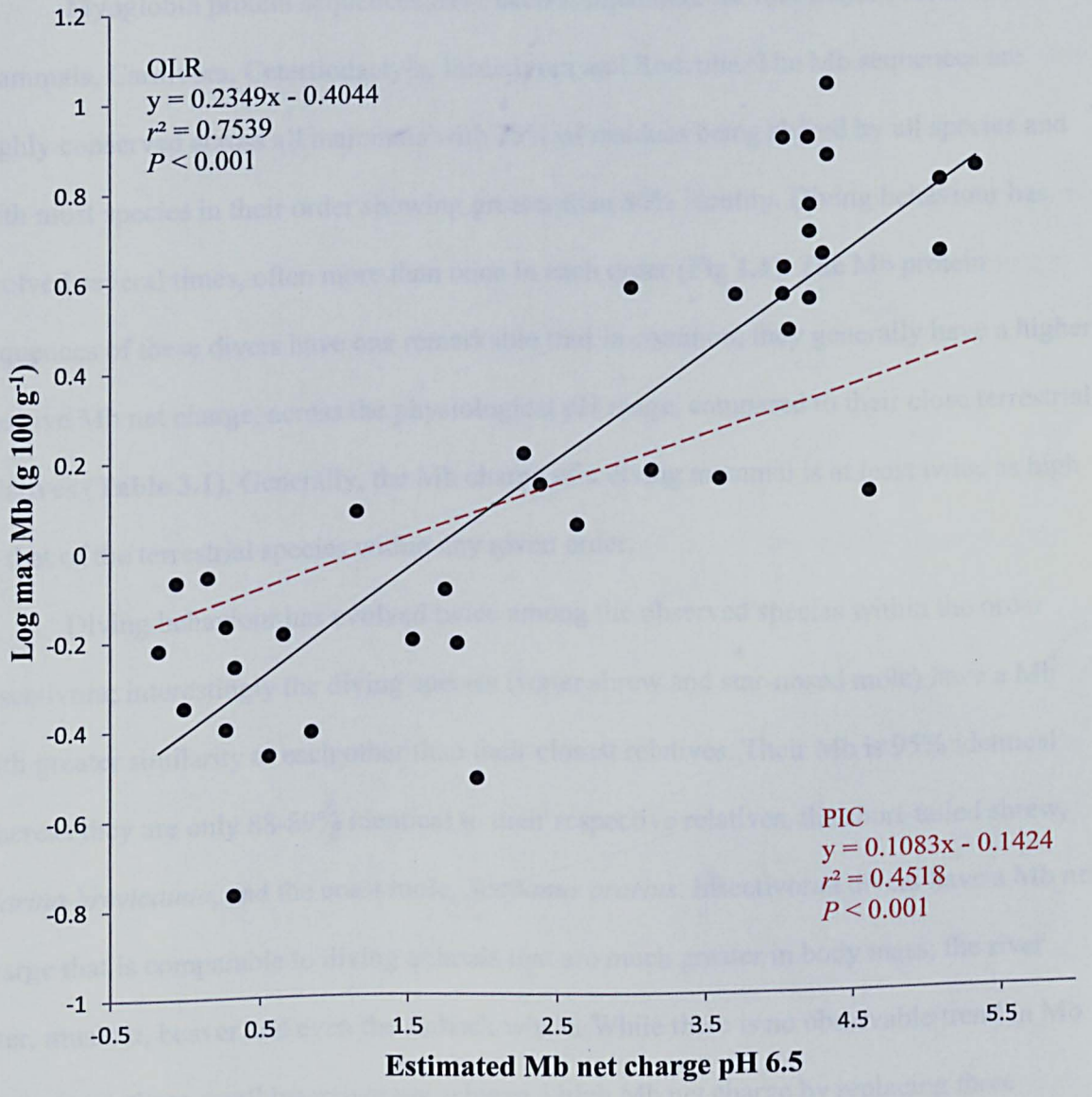


Figure 3.5 Significant positive correlation between Log maximum Mb concentration and estimated Mb net charge at pH 6.5 for terrestrial and diving mammals. The black line indicates ordinary least squares regression (OLR) and the red dashed line is phylogenetically corrected regression (PIC). Both regression calculations show a significant relationship at the 1% level.

Discussion

Estimated Mb net charge – Sequence comparisons

Myoglobin protein sequences have been studied here for four major orders of mammals, Carnivora, Cetartiodactyla, Insectivora and Rodentia. The Mb sequences are highly conserved across all mammals with 75% of residues being shared by all species and with most species in their order showing greater than 80% identity. Diving behaviour has evolved several times, often more than once in each order (**Fig 1.1**). The Mb protein sequences of these divers have one remarkable trait in common; they generally have a higher positive Mb net charge, across the physiological pH range, compared to their close terrestrial relatives (**Table 3.1**). Generally, the Mb charge of a diving mammal is at least twice as high as that of the terrestrial species within any given order.

Diving behaviour has evolved twice among the observed species within the order Insectivora; interestingly the diving species (water shrew and star-nosed mole) have a Mb with greater similarity to each other than their closest relatives. Their Mb is 95% identical whereas they are only 88-89% identical to their respective relatives, the short-tailed shrew, *Blarina brevicauda*, and the coast mole, *Scapanus orarius*. Insectivoran divers have a Mb net charge that is comparable to diving animals that are much greater in body mass; the river otter, muskrat, beaver and even the finback whale. While there is no observable trend in Mb His content, these small insectivorans achieve a high Mb net charge by replacing three strongly negatively charged residues with neutral ones, unlike other diving species. Only once do they replace a neutral residue with a strongly positively charged one, Ser132 to Lys132. This replacement of strongly negatively charged residues helps to increase the overall positive net surface charge of the protein.

Within the order Carnivora diving behaviour has evolved at least three times among the observed species. Of these only the phocid & otariid seals and the river otter rely heavily

on diving behaviour in order to forage, although the river otter will turn to alternative food sources if the availability of fish is low (Wise et al., 1981). The remaining divers, the American mink and polar bear are the only carnivoran species observed here that do not follow the trend of having a higher Mb net charge than their closest terrestrial relatives. This may be due to a limited reliance upon diving, either as a means of foraging or escape. The polar bear generally forages on ringed and bearded seals above the sea ice during the winter months and turns to alternative sources of food during the summer (these are not necessarily aquatic sources) (Dyck and Romberg, 2007). Therefore they do no rely on diving in order to forage, they have been observed swimming and diving, hunting for artic charr (Dyck and Romberg, 2007) but dive durations are much shorter than one would expect from allometry, for an animal the size of a polar bear an average maximal dive duration of 3.5 min would be expected (Halsey et al., 2006), however, the observed maximal dive duration is approximately 39 s (Dyck and Romberg, 2007; see **Chapter 5** for discussion of dive capacity). The American mink is a generalised forager when in a lake environment, preying on fish, birds and mammals in equal proportions; however, in a river environment foraging tends to be more on mammals (Darwin, 1859; Wise et al., 1981). Foraging underwater for fish tends to occur more during the winter months. From spring and during the summer they tend to forage on land, therefore they do not rely greatly on diving for a large part of the year (Wise et al., 1981). The river otter has a reasonably high charge compared to terrestrial mammals (**Table 3.1**) but its Mb charge is almost identical to the protein net charge seen in the badger, its closest terrestrial relative. This may not be because the charge for the river otter is low but in fact that the charge for the badger is higher than expected, which may be due to its lifestyle. Thus, animals that have adapted to a burrowing existence such as the badger and the coast mole, or to high altitude such as the yak, *Bos grunniens*, or Tibetan antelope, *Pantholops hodgsonii*, also have higher positive Mb net charges (**Table 3.1**).

Adaptation to these types of environment would also expose species to low oxygen conditions and may have triggered the evolution of high Mb net charge, as seen in the divers.

The diving species of the order Carnivora generally have a high content of positive residues within their Mb amino acid sequence (**Table 3.1**). Twenty three positive residues is comparable to diving species in the orders Cetacea and Rodentia. However diving carnivorans have a tendency to increase the strongly positively charged residues even further, by replacing Gly57 with Arg57. An additional replacement of the neutral Asn66 with Lys66 occurs in the otariid seals. According to protein structure 1MBO (Phillips, 1980), viewed using jmol version 12.0.41, this residue is in an external position and would therefore be ideally suited to contribute to repulsion of other positively charged molecules. Lys66 is notable as only one other group of species has a positively charged residue at this position; the deep diving beaked whales have also replaced the neutral Asn66 this time with His66. All other species do not have charged residues at this location. His residues are interesting in this study because at physiological pH they have a partial charge, whose strength depends upon their individual p*K*a values (Pace et al., 2009). Both phocid and otariid seals have an elevated number of His residues, which add to the increase in Mb net positive charge caused by strongly positively charged Arg and Lys seen in these species.

Interestingly the charges observed in phocid seals and otariid seals are almost identical (**Table 3.1**). The amino acid sequences of phocid seals are more than 95% identical. The sequences of otariid seals are 97% or more identical, with 88% identities between the two groups (**Fig 3.2A**). However the charged residues that are responsible for obtaining high charge differ between the two groups. Positions marked with a filled circle show where alternate residues have been used to obtain the same charge (**Fig 3.2A**). Arnason et al., (2006) indicate that the last common ancestor of Phocidae and Otariidae existed approximately 33 million years ago (MYA). The existence of these residues, coupled with evolution of His that

are not consistent across all diving carnivorans, indicates that the elevated Mb charge has evolved by different mechanisms in the two groups at some time after the Phocidae-Otariidae split. Phocid and otariid seals have also developed different adaptations towards diving, for example the otariid seals use the forelimbs for swimming while the phocids use their hind limbs. Another notable difference between the two species is that otariid seals inhale before a dive whereas the phocid seals exhale before diving, allowing their lungs to collapse during deep dives and then relying solely on oxygen stored in the blood and muscle tissues (Berta et al., 2006).

Within the order Rodentia diving and terrestrial mammals have a similar number of strongly negatively charged residues. The diving species follow the same trend as the carnivorans in increasing Mb net charge, they have replaced the neutral Asn 87 with Lys 87 and there is a trend towards increasing the Mb His content. Divers have a high Mb His content, eleven residues, which is comparable to otariid seals and baleen whales, and at least three more His residues than their close terrestrial relatives. The additional His residues replace neutral Asn12 with His12, Asn48 with His48 and Gln113 with His113. All of these residues in combination increase the Mb net charge of diving species.

Of all the species within the order Cetartiodactyla for which Mb sequences are available there is only one semi-aquatic species, the pygmy hippo. This species has the lowest Mb net charge of all the divers observed within this study (**Table 3.1**). Pygmy hippos may spend a lot of time in water and are capable of moving proficiently underwater. However, they tend to forage at night time on terrestrial vegetation (Fox & Meyers, 2000) and so may not require great adaptation to an aquatic existence. Dive times for any hippo species are very difficult to confirm and a reputable source for hippo dive durations has not been found during this study. Un-confirmed reports suggest very short dives for an animal of its size. Since Mb concentrations are high in proficient divers and Mb correlates well with charge, the fact that

the pygmy hippo does not have long dive durations and therefore does not require large oxygen stores may explain why pygmy hippo Mb has a low charge.

Cetaceans also increase the number of strongly positively charged residues to increase the Mb net charge, replacing neutral residues with strongly positively charged Lys at positions 24 and 140 (**Table 3.1**). The replacement of these two residues can alone account for the increase in charge observed in baleen whales. In toothed whales the additional Mb net positive charge comes from replacing neutral residues with His (**Fig 3.2B**). The Mb His content is high in all members of Cetartiodactyla. Interestingly the numbers of strongly positively and negatively charged residues in artiodactyl species is equal (**Table 3.1**) and therefore any net charge on the Mb protein is the result of charge contribution from His residues. This may account for the high Mb His content within these terrestrial mammals. The ruminants have replaced neutral Pro88 for His88, which is a substitution unique to the ruminants only. The p*K*a of His88 has been calculated for the first time in this study using a non-linear iterative curve fit algorithm. This computational estimation involved matching the experimental titration curve of pig MetMbCN, plus 3 extra His (two with known pKa) and minus one Lys to match the experimental titration curve of sheep MetMbCN (**Fig 2.1**, see **Chapter 2** for further details). The high p*K*a value of 7.06 attributed to His88 in this study suggests that it may contribute strongly towards Mb net charge within the physiological range. Molecular modelling of sperm whale Mb has shown that Pro88 in fact interacts with the backbone of the Mb chain producing a kink in the conformation (Lesk & Chothia, 1980). Molecular modelling in this study of cow Mb has shown that the exchange of Pro88 with His88 enables the same kink to be present in the conformation (data not shown).

Replacement of neutral Asn12 with His12 is observed in the sperm whales, beaked whales and the diving rodents. This residue coupled with Lys140 and Ile142 are thought to aid in stabilizing the A and H helices in apoMb protein (Scott et al., 2000). Also identified as

stabilizing substitutions are changes from Gly to Ala at positions 5 and 129 (Scott et al., 2000). The replacement of Gly5 with Ala5 only occurs in the baleen and beaked whales (**Fig 3.2B**). Gly is known as a helix breaker and therefore removal of this residue will add stability to the A-helix (Scott et al 2000). The Gly-Ala change at position 129, occurs in many species including; all baleen whales, Longman's and Cuvier's beaked whales (*Indopacetus pacificus* and *Ziphius cavirostris*, respectively), two more distantly related dolphin species, namely the Amazon river dolphin, *Inia geoffrensis*, and the bridled dolphin, *Stenella attenuata*, the bovid species, all carnivoran species (excluding the bears, river otter and badger) and the rodent plains viscacha, *Lagostomus maximus*, (**Fig3.2A-D**), suggesting that this is a common residue used to increase the stability of the H-helix.

Minke, finback whales and phocid seals have replaced neutral Gln8 with His8, this residue has a p*K*a of 6.1 (**Table 2.2**) which would contribute half a charge towards Mb net charge at the low end of the physiological pH range. One other residue of interest occurs in bovid species, toothed whales (except for sperm whales) and phocid seals, this is the replacement of neutral Gln152 with His152. Again this residue has a p*K*a value (**Table 2.2**) that would contribute towards overall Mb net positive charge. Intriguingly the species with the most His and the highest Mb net charge are the beaked whales (**Table 3.1**), members of this group are responsible for some of the deepest and longest dives recorded: the Cuvier's beaked whale has an average maximum dive time of almost 1.5 hr (Halsey et al., 2006).

One very interesting trend is the tendency to increase His residues within the G-helix. There are three possible His locations within this region, His113, His116 and His119. Most terrestrial species have only one of these His, usually His119. The otariid seals, canines and the diving rodents all have two of these three His residues. The phocid seals and the diving members of Cetartiodactyla have all three of the G-helix His residues.

Trovato et al. (2007) developed a theoretical model to determine the aggregation properties of proteins. They found that sperm whale Mb theoretically showed a propensity to aggregate in the G-helix region, residues 100-118. Experimental evidence has found that horse apoMb could form aggregations leading to amyloid fibrils, in non-physiological conditions, and that these aggregates were concentrated to the G-helix in the unfolded protein (Fandrich et al., 2003). It's fascinating that there is a concentration of G-helix His residues within most groups of divers. It may be possible that a G-helix rich in His residues helps to prevent aggregation of apoMb during the folding process, when in high concentrations. Looking at the evidence in this study it is plausible that alterations in the amino acid sequence leading to an increase in protein net surface charge are responsible for mammalian divers having a Mb protein with an increased solubility, which would therefore allow a higher Mb tissue concentration.

Electrophoretic mobility

In order to test the validity of the calculated net charges Mb was purified for several species. Purified protein was electrophoresed on native polyacrylamide gel at pH 6.0. There is a very good correspondence between how far the proteins travelled in the gel and the estimated charge at pH 6.0 (**Fig 3.3 & 3.4**). A similar result was found in a study by McLellan (1984). This author identified that at physiological pH values the Mb proteins migrated in the exact same order as the proteins used in this study. The beaked whales travelling the furthest of the cetaceans, corresponding to the highest charge, then descending in order of distance travelled McLellan (1984) found the dolphins and porpoises, the minke whale and finally the humpback whale, with the terrestrial species (horse) travelling a much smaller distance.

This study finds that the purified Mb of three terrestrial species, pig, sheep and cow, can also be separated, and they follow the same pattern as predicted by their estimated charge. Even though similar species and pH values have been used in both studies, the calculated charges do not agree, with the values in this study being higher in all cases. One explanation for this is because different sources of p*K*a values have been used in calculating net charge. Small differences in p*K*a value can lead to larger difference in charge, depending on the p*K*a value and the pH used in calculation. For example if the p*K*a of a model His is increased by 0.2 from 6.6 to 6.8, then the charge of that residue at pH 6.0 is increased by 0.06. At pH 6.5 the same residue will increase in charge by 0.12. Considering a Mb sequence with 10-14 histidines, then small changes in the p*K*a values used in charge calculation could lead to quite large differences in overall estimated net charge of the protein.

However, the main explanation for differences in calculated net charge between this study and McLellan (1984) is that His 24, 36, 82, 93 and 97 were deemed un-titratable by the previous study and therefore not used for calculation. However, the present study uses more recent information for the values of p*K*a and finds that His 36 is titratable with a very high p*K*a (Bashford et al., 1993; Cocco et al., 1992; Kao et al., 2000), His 97 is titratable but has a low p*K*a value (Bashford et al., 1993). His 82 was found to be controversial in relation to its p*K*a value. Most studies suggest that this residue is not titratable and has therefore been ignored during calculations (Botelho and Gurd, 1978; Cocco et al., 1992; Bashford et al., 1993; Bhattacharya and Lecomte, 1997; Kao et al., 2000). However a few studies have suggested that this residue is in fact titratable, with a high p*K*a value (Cheng and Schoenborn, 1991).

Comparisons of calculated charge over a pH range from 5.8-8.5, were made with the experimental titrations of three terrestrial species, cow, sheep and pig (see **Chapter 2** for more details). Experimental titrations suggested that the preliminary calculations were

inaccurate to a small degree. Unfortunately as a definitive p*K*a value for His 82 has not been determined, this study had to attribute it with a model value of 6.6. When His 82 was included, calculated net charge corresponded more accurately with experimental findings, therefore supporting the estimated calculations performed in this study. The inclusion of these three His residues explains the differences seen in calculated net Mb charge in this study, compared to those generated by McLellan (1984).

Lanes 4 and 5 in **Figure 3.3** are from two beaked whales, the Sowerby's beaked whale and the Northern bottlenose whale. According to calculated net Mb charge at pH 6.0, they should both have moved through the gel at the same rate as they have equal charge. There is only one residue difference in the amino acid sequence, occurring at position 44 where the Sowerby's beaked whale has an aspartic acid residue and the northern bottlenose whale has a glutamic acid residue. It is unlikely that this single residue exchange will alter the shape and general backbone of the protein, especially considering both residues are equally negative in charge at physiological pH. Then this is unlikely to explain the reduced movement of the northern bottlenose whale Mb. A more likely explanation is that a certain amount of deamidation has occurred in the Mb of the northern bottlenose whale. Tissue samples obtained for diving mammals in this study were the result of stranding events. As a result a certain amount of decay had occurred to the animals. The decaying proteins may have had some aspect of deamidation, or been prone to deamidation during purification. Deamidation is a chemical process that affects two amino acid residues in particular, Asn and Gln. Deamidation of these residues, reduces them to Asp and Glu, therefore adding negative charges to a protein (Robinson & Robinson, 2004). The process of deamidation has been previously observed in Mb of several species: during the purification of opossum (*Didelphis virginiana*) Mb prior to sequencing, residue 81 was deemed to show deamidation in one of the fractions collected (Romero-Herrera and Lehmann, 1975). Deamidation has also been

noted during purification of shark (*Mustelus antarcticus*) Mb (Fisher et al., 1980), and Quinn (1973) found that multiple fractions of bovine Mb were the result of non-enzymatic deamidation. A study characterising the reaction of methyl acetimidate in sperm whale Mb highlighted that there are six amino acid positions where deamidation is common, five of which occur in Gln residues and one in Asn (DiMarchi et al., 1978a). Evidence has also shown that the initial sequencing of sperm whale Mb, resulted in incorrect sequence, and deamidation was thought to be involved in determining the incorrect sequence (Romero Herrera & Lehman, 1975)

Correlation of estimated net charge and Mb concentration

A strong positive correlation was found between the distance travelled in the native gel and the estimated net Mb charge by means of ordinary least squares regression. The r^2 value of 0.978 is significant at the 1% level (**Fig 3.4**) and shows a better agreement than that seen by McLellan (1984), although a smaller sample was used in this study. Investigation of this relationship was then taken further by analysing the data taking phylogenetic similarity in to consideration. Using Felsenstein's (1985) phylogenetically independent contrasts analysis a slight deviation is seen in the PIC regression line, but the analysis still shows a highly significant relationship between estimated Mb net charge and electrophoretic mobility (**Fig 3.4**). This suggests that the calculations used in this study are an accurate representation of *in vivo* Mb charge for the species investigated. The use of PIC in this analysis is important to improve the reliability of the data, by considering that some of the species used in the data set will be phylogenetically related and therefore not independent of each other (Halsey et al., 2006).

This study is the first to demonstrate a close correlation between estimated Mb net charge and $[Mb_{max}]$. $[Mb_{max}]$ data was taken from literature sources (**Table A3**) and

compared with estimated Mb net charge for as many species of mammals as possible. ORL found a significant positive correlation at the 1% (**Fig 3.5**). This data was also subjected to PIC analysis and found to have a significant correlation at the 1% level (**Fig 3.5**).

Mb stability and solubility

Regis et al. (2005) observed differences in both the stability and aggregation properties between horse and sperm whale apo/holo/MetMbCN. They found that the terrestrial horse had less stable Mb than sperm whale Mb. Similar stability studies have been conducted on apoMb for various species finding a 600-fold difference in the stability of sperm whale and pig apoMb when exposed to guanidinium chloride (Scott et al., 2000). Using site directed mutagenesis, of only a small number of amino acids, furthermore demonstrated that it is possible to lower the stability of sperm whale apoMb to resemble the stability seen in pig apoMb, and that the stability of native pig apoMb can be increased to reach the stability of native sperm whale Mb with just five site directed point mutations.

Whether it is resistance to acid or urea denaturation and no matter what pH conditions are used, or to which unfolding end point, diving species tend to have more stable apoMb than terrestrial species (Hughson and Baldwin, 1989; Scott et al., 2000; Regis et al., 2005;) and the stability is not based on the properties of haem binding (Regis et al., 2005). Scott et al, (2000) suggested that certain residues are important for stability of apoMb (residues 5, 12, 28, 51, 53, 74, 87, 140 and 142) and found that 5 mutations in pig Mb are necessary to improve stability to the levels seen in the sperm whale. Charge may affect the stability of the Mb protein by reducing unfolding and maintain stability in certain residues, such as the increase in His in the G-Helix. Regis et al, (2005) suggested that residues 15 and 74, are important for stability. However when they used a sperm whale mutants they found that in all

cases monomeric Mb was observed at test concentrations, suggesting that other residues are important for the improved solubility of Mb.

As suggested earlier high [Mb] could have an effect on solubility. Interestingly a recent study found that horse MetMbCN (holoMb protein with cyanide added to aid stability) formed aggregates at a concentration of 200 μM during size exclusion chromatography experiments, during which samples were left to incubate at 20°C for seven days, whereas sperm whale MetMbCN did not form aggregations (Regis et al., 2005). The value for MetMbCN aggregation formation of 200 μM in horse (corresponding to about 0.255 g Mb 100 g^{-1} wet muscle with a water content of 75%) is much lower than the [Mb] that has been observed in the animal, a value of 0.316 g 100 g^{-1} has been observed in Horse cardiac muscle (Obrien et al., 1992) and an even higher concentration of 0.705 g 100 g^{-1} was observed in skeletal muscle (Lawrie, 1950). The low [Mb] value for aggregation reported by Regis et al. (2005) may be due to the amount of time the sample had to form aggregations and would not be realistic in a natural setting. Nevertheless it highlights that the diving sperm whale MetMbCN did not form aggregates under the same conditions. In a continuation of this study, an ultracentrifugation experiment found, the sedimentation profile of sperm whale was best fitted as a single non-interacting species at 40 μM (0.051 g 100 g^{-1}), yet the horse MetMbCN best fitted a monomer-dimer profile at the same concentration and would only fit a single ideal non-interacting species at 20 μM (0.0255 g 100 g^{-1}) (Regis et al., 2005).

Aggregations of apoMb have been studied since 1962 when seal (*Phoca vitulina*) apoMb at a concentration of 6 mg ml^{-1} was found to form aggregations between pH 3 and 5.5. A maximum occurrence of polymerisation occurred between pH 4-5 (Rumen and Appella, 1962). Increases in protein concentration not only increased the amount of aggregations formed, but also increased the amount of polymerisation. This was taken to suggest that aggregates larger than dimers are found at higher concentrations (Rumen and

Appella, 1962). Interestingly Rumen and Appella did not find aggregations at physiological pH during their study. This may have been due to the low concentrations used (a max of 8 mg ml^{-1}) or due to the properties of seal apoMb. Phocid seals have the second highest charges of all mammals observed here. This may provide their apoMb molecules a resistance to aggregation by means of electrostatic repulsion, thus maintaining solubility at pH values above 5. Horse apoMb precipitation has been observed when saturated solutions of apoMb, in potassium phosphate buffer, were altered from pH 7.3 to 6.7 (Deyoung et al., 1993). This suggests that horse apoMb at saturating conditions will form non-soluble aggregates at physiological pH. The same study also showed that at 1M urea concentrations, increases in protein concentration from 21 to 25 mg ml^{-1}, will more than double aggregation formation as shown by the amount of particulate scatter.

Most studies showing aggregation of holoMb or apoMb have used conditions that would not be seen under physiological conditions, i.e. the inclusion of a denaturant such as urea or severely acidic conditions. However recent evidence has shown that apoMb of sperm whale can form aggregations under physiological conditions. Chow et al., (2006) showed that aggregations formed over a 14 h period, with 0.0 M urea and at pH 6.0 at a concentration of 38 μM (0.048 g $100g^{-1}$). This is a very long time and it is unlikely that a protein would be allowed to remain in an aggregated state for so long in a natural setting. However, this is a very low concentration to be forming aggregates, considering the holo protein has been observed at concentrations of 7.0 g 100 g^{-1} in the sperm whale (Sharp & Marsh, 1953).

Conclusion

Previous work has shown that both Mb and apoMb are susceptible to aggregation even at physiological conditions. This study suggests that improved Mb solubility in mammalian divers is achieved by charge changes in the amino acid sequence that lead to an increase in the overall net charge of the protein. These changes include increases in the number of His residues and strongly positively charged residues or decreases in the number of strongly negatively charged residues. A trend towards increasing the number of strongly positively charged Mb amino acid residues has evolved independently in seven lineages of divers involving cetaceans, carnivorans and rodents. Furthermore, two independent evolutions of a decrease in the quantity of strongly negatively charged residues have been observed in the diving insectivorans. This study proposes that His residues in the G helix, positions 100-118, are important in preventing Mb aggregation in mammalian divers.

Amino acid changes leading to increased protein charge would permit an increase in protein concentration, due to an increase in solubility by electrostatic repulsion. Any changes in protein structure that would enable higher concentrations of Mb would be evolutionarily advantageous, due to the increase in oxygen available during a dive. Therefore it seems that adaptation to diving involves not only increases in Mb oxygen stores that have been previously reported, but also qualitative changes to the protein to increase solubility by increasing net positive charge, which then allows higher Mb concentrations.

Chapter 4 – Evolutionary trends in Mb net charge and Mb specific buffer value

Introduction

Diving mammals are a diverse group of animals that rely heavily on an aquatic existence for all or part of their life's needs; they regularly submerge their complete body in water, during which time they rely on breath-hold diving. This may be associated with foraging or predator escape or in some cases the entire life of the animal may be spent in an aquatic environment. Divers exist within six of the major orders of mammals, including Monotremta, Sirenia, Insectivora, Carnivora, Cetartiodactyla and Rodentia. The oldest and the most diverse group of extant divers reside within the order Cetartiodactyla. This is an order comprised of nearly three hundred extant species (MacDonald, 2006), and over a third of these are cetaceans. Cetaceans have existed for approximately 55 million years and are one of two fully aquatic families of mammals, the other being Sirenia which comprises of two extant genera, the manatees and dugong (Berta et al., 2006). Fossil evidence has recently shown that cetaceans have evolved from an aquatic group of artiodactyl ruminant species known as raoellids (Thewissen et al., 2007). These animals are believed to have inhabited riverine areas and lakes and to have been waders that foraged on land. When threatened they are thought to have retreated into the water to escape, much like the modern day mouse-deer (Thewissen et al., 2007; Meijaard et al., 2010). Eventually a species evolved known as *Pakicetus*, which is thought to be the earliest known ancestor of modern whales, existing between 45-55 million years ago (MYA). This species is thought to have some similarities with modern whales such as improved underwater hearing, however, fossils show retention of many terrestrial traits (Thewissen et al., 2001). Within the order Carnivora fossil evidence indicates that a species called *Puijila darwini* is the earliest ancestor to modern pinnipeds (Rybczynski et al., 2009). This species from the early Miocene epoch, approximately 20-25 MYA, is a freshwater intermediate species that used four webbed limbs for movement. Other

morphological features support a semi-aquatic life style similar to modern pinnipeds that spend time on land to give birth, molt and recuperate after long dives (Berta et al., 2006). However, some evidence suggests that *Puijila darwini* consumed terrestrial and freshwater prey, based on what appears to have been stomach contents of a small rodent and duck, found with the skeleton. Therefore terrestrial and aquatic hunting suggests that *Puijila darwini* would have relied on land to a greater extent than modern pinnipeds (Rybczynski et al., 2009; Canadian Museum of Nature, 14/09/2011, http://nature.ca/puijila/aa_e.cfm).

Modern phylogenetic studies identify evolutionary trends and relationships by incorporating molecular information, and some of these have revealed insights that have changed our perception of the evolution of some species compared to that based upon morphological data alone. The most striking example was the revelation that chimpanzees, e.g. *Pan troglodytes* are more closely related to humans, *Homo sapiens*, than to other ape species (King & Wilson 1975; Satta et al., 2000). As well as providing information as to the relatedness between species, molecular information can give insights into how and when adaptive evolutionary events may have occurred between species. For example Berenbrink et al. (2005) used anatomical, biochemical and physiological information coupled with phylogenetics to show how and where oxygen (O_2) secretion into the swimbladder evolved in teleost fish, a major adaptation that led to multiple radiations into new environments.
This study explores the molecular evolution of certain traits of the myoglobin gene within mammalian divers. Mb is the main store of oxygen in muscle tissue and has a role in facilitating the diffusion of O_2 from the capillaries to the mitochondria (Wittenberg and Wittenberg, 2003). Increased O_2 stores can lead to an increase in dive durations (see **Chapter 5** for discussion of O_2 stores and diving), and are a hallmark of mammalian divers. Since diving species cannot utilise O_2 from the surrounding water, then aerobic metabolism is fuelled by O_2 reservoirs within the body. It then follows that anything affecting the quantity

or the function of Mb will likely be under changing selection pressures during the evolution of diving mammals.

Chapter 2 of this study has shown that Mb specific buffer capacity (β_{Mb}) together with increased Mb content ([Mb]) contributes to increased whole muscle non-bicarbonate buffering of H^+ in mammalian divers compared to terrestrial species. **Chapter 3** shows that Mb net charge is significantly increased in mammalian divers compared to terrestrial species, within the orders Carnivora, Cetartiodactyla, Insectivora and Rodentia. Here it is hypothesised that these adaptations aid divers to overcome problems of acidosis and protein solubility at high concentrations of Mb. If this hypothesis holds then one can assume that increased Mb net charge and β_{Mb} offer advantages that increase dive duration, by helping to increase the amount of O_2 available and to prolong any periods of sustained anaerobic metabolism. If we assume that longer dive durations increase the survivability of an animal, through providing greater escape and evasion strategies or by opening new niches of untapped resources, then increased β_{Mb} and Mb net charge will be under direct evolutionary pressure, through natural selection. This study will track amino acid substitutions within the Mb protein throughout the evolution of mammalian species within the orders Monotremata, Marsupialia, Afrotheria, Xenathra, Insectivora, Chiroptera, Carnivora, Perissodactyla, Cetartiodactyla, Lagomorpha, Rodentia and Primates. The aim is to identify where evolutionary changes in the net charge and specific buffer capacity of the Mb protein have occurred and if these changes are concentrated in diving species.

Methods

Phylogenetic tree construction

The Mb gene cannot be used by itself to generate an accurate phylogeny because of the highly conserved nature of the protein, with at least 65% amino acid identity being observed between the 125 species in this study. For this reason a composite phylogenetic tree **(Fig 4.1)** has been constructed from literature sources for species whose Mb amino acid sequence is known. In an attempt to obtain the most accurate phylogeny multiple sources have been used, and these have been multiple gene studies where possible. Higher level relationships have been taken from Murphy et al. (2001), Bininda-Emonds et al. (2007), Murphy et al. (2007) and Philips et al. (2009). Species level resolutions within subgroups have been taken as follows: Monotremata: Phillips et al. (2009), Marsupialia: Nilsson et al. (2010), Afrotheria and Xenarthra: Murphy et al. (2009) and Nishihara et al., (2009), Insectivora: Grenyer & Purvis (2003), Dubey et al. (2007) and Bininda-Emonds et al. (2007), Chiroptera: Eick et al. (2005), Teeling et al. (2005) and Gu et al. (2008), Carnivora: Flynn et al. (2005), Fulton & Strobeck (2005) Arnason et al. (2007), Higdon et al. (2007) and Schroder et al. (2009), Perissodactyla: Murphy et al. (2001), Agnarsson & May-Collado (2008), Prasad et al. (2008) and Hou et al. (2009), Cetartiodactyla: Murphy et al. (2001), Fernandez & Verba (2005), Bininda-Emonds (2007), Agnarson & May-Collardo (2008), Prasad et al. (2008) and McGowen et al. (2009), Lagomorpha: Murphy et al. (2001 & 2007), Rodentia: Montgelard et al. (2008) and Primates: Chatterjee et al.(2009).

Reconstruction of myoglobin amino acid sequence evolution

Mb protein sequence information was collected from databases **(Table A1)** or directly determined from nucleotide sequencing in this study (see **Chapter 2**) and aligned with BioEdit (Hall, 2001). The sequence alignment was then transferred to phylogenetic analysis

software, MacClade (Maddison & Maddison, 2005), and a phylogenetic tree was constructed to resemble those species relationships set out by literature sources above. Mb amino acid evolution was then reconstructed using maximum parsimony, using the 'trace character' function within MacClade. Maximum parsimony is a simplistic reconstruction method that traces characters, amino acids in this case, in such a way as to minimise the number of evolutionary changes. Comparisons have shown that when there are few changes involved parsimony gives similar results to other more complex reconstruction methods such as maximum likelihood and neighbour-joining (Kuhner & Felsenstein, 1994). Ambiguities (equi-parsimonious solutions) within the reconstruction were analysed using deltran and acctran resolving options in MacClade. These methods delay or accelerate character changes respectively. Where ambiguities could not be resolved by these methods, the amino acid codes were analysed for the most probable alteration with third codon position changes being more likely to occur than first codon position changes (Lesk, 2001). All amino acid changes were then traced using the 'trace all changes' function within MacClade (Maddison & Maddison, 2005), allowing analysis of where charged amino acid substitutions and histidine (His) substitutions occur within the evolution of the Mb protein in mammalian lineages. The net charge and specific buffer value of each ionisable residue has been calculated at pH 6.5 (**Table 2.3**) and the Mb net charge and β_{Mb} is known at the terminal branch, for each extant species, (**Table A2; A3**). Working backwards through the most parsimonious amino acid substitutions, it is possible to reconstruct an estimate of Mb net charge and β_{Mb} value at each node in the evolution of mammalian Mb.

Concentrated changes analysis

To identify whether increases in Mb net charge and β_{Mb} are concentrated in lineages where diving behaviour has evolved, the concentrated changes test as implemented in MacClade was used (Maddison & Maddison, 2005). This test uses a binary system for calculating the probability of gains and losses of a character on specified branches of the phylogenetic tree, given a total number of gains/losses for the whole tree and assuming that these are randomly distributed. Specified branches in this case were those where diving behaviour has evolved. For the purpose of this investigation Mb net charges were re-coded from continuous to binary values, using a high and low Mb net charge character state (net charge $\geq +2.2$ and $< +2.2$, respectively) . The same was done for β_{Mb} , where the threshold of $\geq$ and < 3.2 mol H^+ mol Mb^{-1} pH^{-1} was used for the high and low β_{Mb} category, respectively.

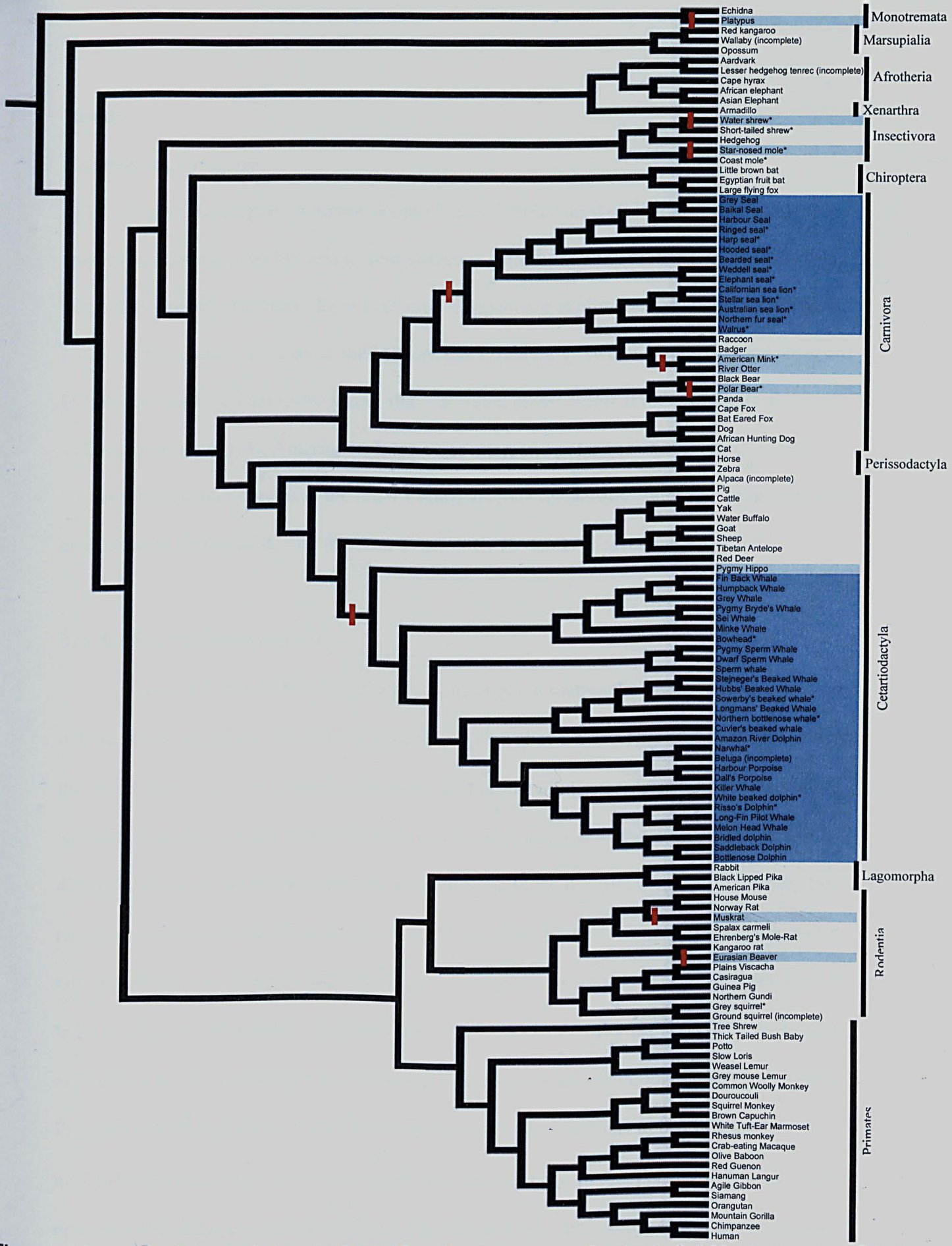


Figure 4.1 Composite phylogenetic tree constructed from literature sources. Species in dark blue boxes are proficient divers, those in light blue are semi-aquatic divers with shorter maximum dive durations. Red bars indicate where diving behaviour evolved in a parsimony reconstruction. Black bars indicate phylogenetic orders. Species highlighted with an asterisk were sequenced for the first time in this study

Results

Composite phylogeny

Figure 4.1 displays a composite phylogenetic tree based on literature sources for species that have known Mb amino acid sequence data. Parsimony reconstruction of diving behaviour indicates that there have been nine independent evolutions within the represented species. One evolution occurs in the Monotremata (platypus), two evolutions can be observed in Insectivora (American water shrew and star-nosed mole), while three evolutions are seen in Carnivora (pinnipeds, American mink + river otter, and polar bear. Only one evolution occurs within extant species in the order Cetartiodactyla (cetaceans + hippopotamus) and two evolutions of diving behaviour are seen in the order Rodentia (beaver and muskrat).

Mb specific buffering capacity

Figure 4.2 shows the evolution of β_{Mb} throughout mammalian lineages using the deltran resolving option. The acctran resolving option shows a similar pattern of events with the minor difference being that β_{Mb} increases earlier in evolutionary history in the Carnivora and Cetartiodactyla clades and in the Primates lineage, hence only the deltran resolve will be discussed in detail. Small discrepancies in the terminal taxa β_{Mb} and the nodal values are the result of synonymous substitutions involving Asp to Glu, both are negative residues however Glu contributes 0.01 towards buffering at pH 6.5 whereas Asp does not. The ancestral state is to have a β_{Mb} of 1.98 (mol H^+ mol Mb^{-1} pH^{-1}). This ancestral buffer property persists through evolution towards the lineages of Afrotheria, Insectivora, Chiroptera, Lagomorpha, Rodentia, Primates and through most of the Carnivora lineage, with deviations in β_{Mb} being observed near to the terminal branches, if seen at all. The ancestral β_{Mb} in fact persists unaltered in the opossum, *Didelphis virginiana*, aardvark, *Orycteropus afer*, hedgehog, *Erinaceus europaeus*,

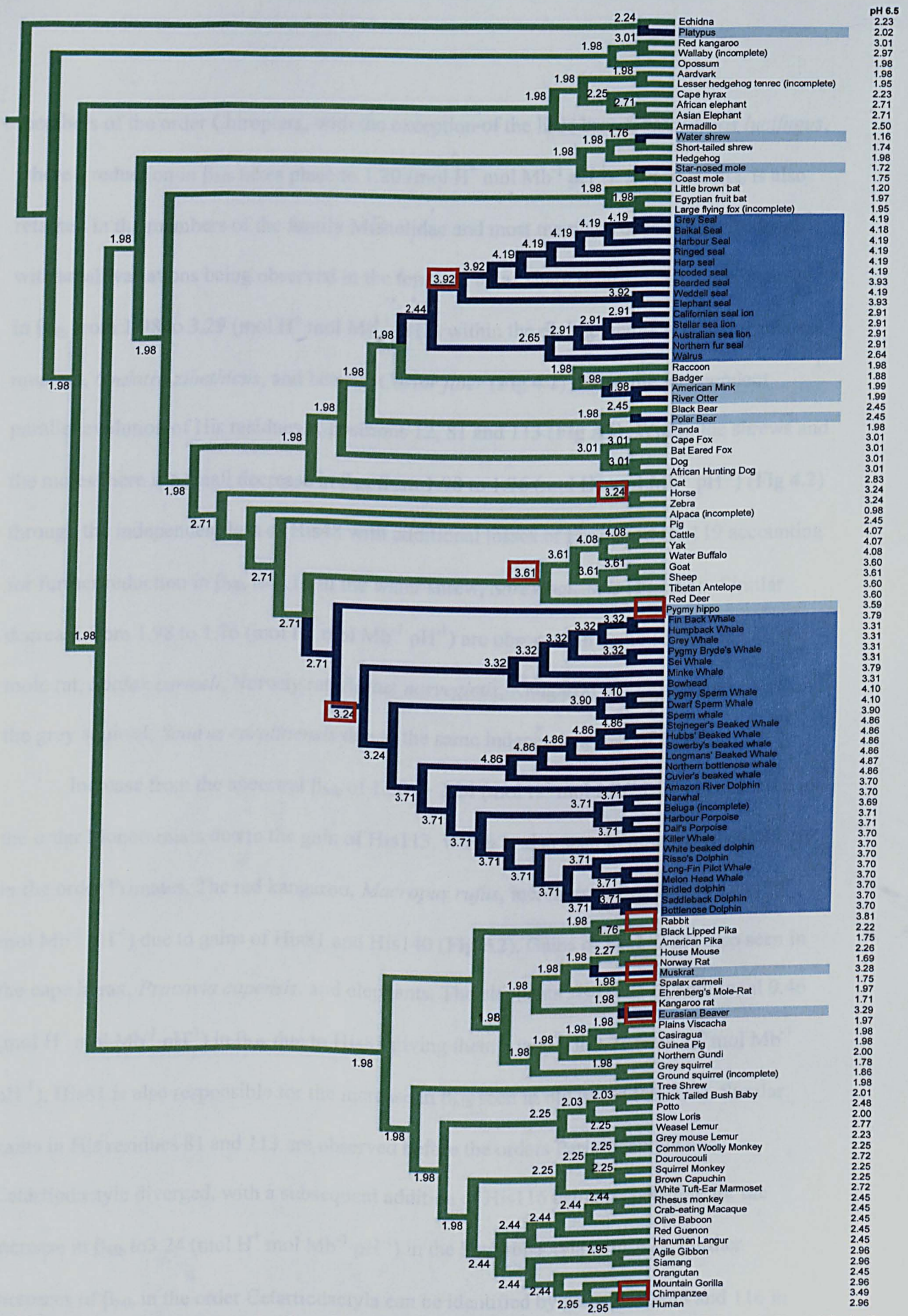


Figure 4.2 Maximum parsimony reconstruction of β_{Mb} at pH 6.5 using deltran resolving option. Values in the table indicate the β_{Mb} (mol H^+ mol Mb^{-1} pH^{-1}) of extant taxa. Values on branches show where alterations from the ancestral state occur and apply to subsequent nodes further changes are also shown. Blue boxes indicate diving species. Red boxes indicate β_{Mb} increases above 3.2 (mol H^+ mol Mb^{-1} pH^{-1}).

members of the order Chiroptera, with the exception of the little brown bat, *Myotis lucifugus*, where a reduction in β_{Mb} takes place to 1.20 (mol H^+ mol Mb^{-1} pH^{-1}). Ancestral β_{Mb} is also retained in the members of the family Mustelidae and most members of the order Rodentia with small variations being observed in the terminal taxa. There is however a large increase in β_{Mb} from 1.98 to 3.29 (mol H^+ mol Mb^{-1} pH^{-1}) within the diving members of Rodentia, the muskrat, *Ondatra zibethicus*, and beaver, *Castor fiber* (**Fig 4.2**) due to the independent parallel evolution of His residues at positions 12, 81 and 113 (**Fig A2**). Within the shrews and the moles there is a small decrease in β_{Mb} from 1.98 to 1.76 (mol H^+ mol Mb^{-1} pH^{-1}) (**Fig 4.2**) through the independent loss of His48 with additional losses of His24 and His119 accounting for further reduction in β_{Mb} to 1.16 in the water shrew, *Sorex palustris* (**Fig A2**). Similar decrease from 1.98 to 1.76 (mol H^+ mol Mb^{-1} pH^{-1}) are observed in some of the rodents, the mole rat, *Spalax carmeli*, Norway rat, *Rattus norvegicus*, Kangaroo rat, *Dipodomys ordii*, and the grey squirrel, *Sciurus carolinensis* due to the same independent loss of His48.

Increase from the ancestral β_{Mb} of 1.98 to 2.24 (mol H^+ mol Mb^{-1} pH^{-1}) is observed in the order Monotremata due to the gain of His113, which is also seen in new world monkeys in the order Primates. The red kangaroo, *Macropus rufus*, increases in β_{Mb} to 3.01 (mol H^+ mol Mb^{-1} pH^{-1}) due to gains of His81 and His140 (**Fig A2**). Gains of His113 are also seen in the cape hyrax, *Procavia capensis*, and elephants. The elephants also gain an additional 0.46 (mol H^+ mol Mb^{-1} pH^{-1}) in β_{Mb} due to His81 giving them a value of 2.71 (mol H^+ mol Mb^{-1} pH^{-1}), His81 is also responsible for the increase in β_{Mb} seen in old world Primates. Similar gains in His residues 81 and 113 are observed before the orders Perissodactyla and Cetartiodactyla diverged, with a subsequent addition of His116 being responsible for the increase in β_{Mb} to3.24 (mol H^+ mol Mb^{-1} pH^{-1}) in the Perissodactyla (**Fig A2**). Further increases of β_{Mb} in the order Cetartiodactyla can be identified by gains of His88 and 116 in the ruminants conveying a β_{Mb} of 3.61 (mol H^+ mol Mb^{-1} pH^{-1}) and His 152 which provides

the increase to 4.08 (mol H^+ mol Mb^{-1} pH^{-1}) in the bovid species. Within the baleen whales there is the same increase in His116, but also an additional contribution from the amino terminal residue valine which raises the β_{Mb} to 3.32 (mol H^+ mol Mb^{-1} pH^{-1}). This is also true for the sperm whales that have the same amino terminal residue, with the smaller dwarf and pygmy sperm whales, *Kogia simus* and *Kogia breviceps*, increasing their β_{Mb} to 4.10 (mol H^+ mol Mb^{-1} pH^{-1}) with a gain of His35 (**Fig A2**). The remaining toothed whales evolve a slightly different mechanism for increasing β_{Mb} using His116 and His152 giving a β_{Mb} of 3.71 (mol H^+ mol Mb^{-1} pH^{-1}), then after the beaked whales diverged from the dolphins and porpoises, the beaked whales gained His66 which increase their β_{Mb} to 4.86 (mol H^+ mol Mb^{-1} pH^{-1}) the highest value seen in any mammal observed in this study. **Figure 4.2** shows that there has been a gradual increase in β_{Mb} in the order Cetartiodactyla due to the replacement of neutral amino acids by one or two additional His residues (**Fig A2**). A similar pattern of evolution has been shown in pinniped β_{Mb}, whereas there is a sudden increase in β_{Mb} at or near the terminal branch in the other species with a high β_{Mb}, red kangaroo, canines, rabbit and diving rodents, this may be due to sampling not showing a gradual increase within the evolution of these species.

Within the order Carnivora increases in β_{Mb} are seen in the bears, canines and pinnipeds due to the gain of His81giving a β_{Mb} of 2.44 (mol H^+ mol Mb^{-1} pH^{-1}). In canines there is an additional increase of His124 raising the β_{Mb} to 3.01(mol H^+ mol Mb^{-1} pH^{-1}). Pinnipeds have evolved two different ways of further increasing β_{Mb} above the ancestral state. Otariid seals evolved gains of His128 taking β_{Mb} to 2.65 (mol H^+ mol Mb^{-1} pH^{-1}) and then His113 evolved after sea lions diverged from the walrus, *Odobenus rosmarus* increasing β_{Mb} to 2.91 (mol H^+ mol Mb^{-1} pH^{-1}). Phocid seals, on the other hand, evolved a gain of His116,

which increased their β_{Mb} to 3.92 (mol H^+ mol Mb^{-1} pH^{-1}), and a second gain of His152 that further increased β_{Mb} to 4.19 (mol H^+ mol Mb^{-1} pH^{-1}).

There are several cases in which diving species do not differ greatly from the close terrestrial relatives. These include divers within the orders Monotremata and Insectivora whose β_{Mb} is slightly lower compared to their closest terrestrial relatives, with reductions occurring in the terminal branches (**Fig 4.2**). Cetaceans have similar β_{Mb} to their close terrestrial relatives, the ruminants, and in the order Carnivora the polar bear and diving mustelids have the same β_{Mb} as their closest terrestrial relatives, while the pinniped seals and diving rodents have a greatly increased β_{Mb} compared to their closest terrestrial relatives. In summary a high value of β_{Mb} above 3.2 (mol H^+ mol Mb^{-1} pH^{-1}) has independently evolved 9 times, namely in phocid seals, perissodactyls, ruminants, pygmy hippo, cetaceans the rabbit (*Oryctolagus cuniculus*), the chimpanzee, and the two lineages of diving rodents muskrat and beaver (**Fig 4.2**).

Concentrated changes in β_{Mb}

Figure 4.2 showed that of the nine evolutions of β_{Mb} of 3.2 (mol H^+ mol Mb^{-1} pH^{-1}) or above, five occurred on branches of the tree where diving behaviour had already evolved (**Fig 4.2**). In order to test whether the observed increases in β_{Mb} were more concentrated in diving taxa than expected by chance, a concentrated changes test was conducted in MacClade (Maddison & Maddison, 2005). The test showed that the observed pattern was likely to occur by chance ($P = 0.161$). There is therefore no statistically proven link between an increase in β_{Mb} and the presence of diving.

Evolution of Mb net charge

The evolution of Mb net charge under the deltran resolving option is shown in **Figure 4.3.** Values on the branches show where alterations from the ancestral state occur and apply to all subsequent nodes unless further changes are shown. Choosing the acctran resolving option **(Fig A3)** a similar pattern of evolution in Mb net charge is seen, with slightly earlier increases in charge being observed in the Carnivora and Cetartiodactyla clade as well as in the Primate lineage, for this reason only the deltran tree will be discussed here.

The ancestral state for Mb net charge is a low charge of 0.27. Ancestral charge is retained in the hedgehog, the tree shrew, *Tupaia glis*, and four of the members of the order Rodentia, Ehrenberg's mole-rat, *Nannospalax ehrenbergi*, plains viscacha, *Lagostomus maximus*, casiragua, *Proechimys guairae*, and the guinea pig, *Cavia porcellus*. In these species there are numerous amino acid substitutions but the overall Mb net charge has been maintained, in the tree shrew none of the substitutions affect charge. In the rodents and the hedgehog, there are charge changes, most of these are synonymous, meaning that residues are replaced by a residue of the same type i.e. a negative residue is substituted with another negative residue. However they all show losses of either strongly positively or negatively charged residues, which are subsequently countered by a gain of either strongly positively or negatively charged residues **(Fig A2)**.

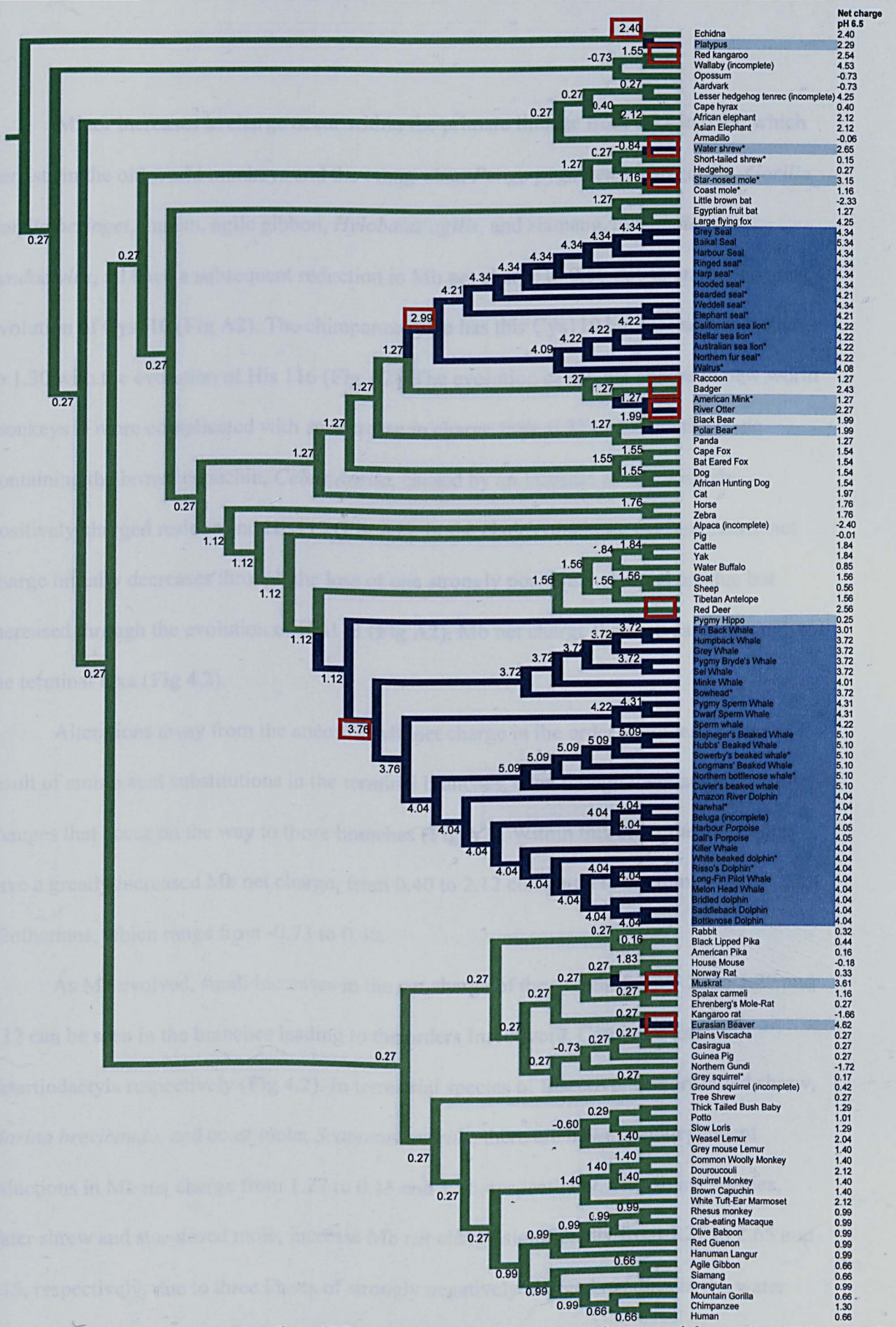


Figure 4.3 Maximum parsimony reconstruction of Mb net charge at pH 6.5 using deltran resolving option. Values on the branches indicate estimated net charge at pH 6.5 for each node. Blue boxes indicate diving species and blue branches show where diving behaviour evolved. Red boxes indicate where evolutions of high Mb net charge seen as those above 2.2 occur.

Minor increases in charge occur within the primate lineage from 0.27 to 0.99, which persists in the old world monkeys and the orang-utan, *Pongo pygmaeus*. The gorilla, *Gorilla gorilla beringei*, human, agile gibbon, *Hylobates agilis*, and siamang, *Symphalangus syndactylus*, all have a subsequent reduction in Mb net charge to 0.66 due to an independent evolution of Cys110 (**Fig A2**). The chimpanzee also has this Cys110 but increases net charge to 1.30 with the evolution of His 116 (**Fig A2**). The evolution of Mb net charge in new world monkeys is more complicated with an increase in charge from 0.27 to 1.40 in the clade containing the brown capuchin, *Cebus apella*, caused by an increase of one strongly positively charged residue and His113 (**Fig A2**). In the clade containing the lemurs Mb net charge initially decreases through the loss of one strongly positively charged residue but increased through the evolution of His113 (**Fig A2**), Mb net charge then increases through to the terminal taxa (**Fig 4.2**).

Alterations away from the ancestral Mb net charge in the order Afrotheria occur as a result of amino acid substitutions in the terminal branches, even though there are numerous changes that occur on the way to those branches (**Fig A2**). Within this order the elephants have a greatly increased Mb net charge, from 0.40 to 2.12 compared to the remaining afrotharians, which range from -0.73 to 0.40.

As Mb evolved, small increases in the net charge of the protein from 0.27 to 1.27 and 1.12 can be seen in the branches leading to the orders Insectivora, Carnivora and Cetartiodactyla respectively (**Fig 4.2**). In terrestrial species of Insectivora, short-tailed shrew, *Blarina brevicauda*, and coast mole, *Scapanus orarius*, there are however independent reductions in Mb net charge from 1.27 to 0.15 and 1.16, respectively. The diving species, water shrew and star-nosed mole, increase Mb net charge significantly from 1.27 to 2.65 and 3.15, respectively, due to three losses of strongly negatively charged residues in the water shrew and two losses of strongly negatively charged residues in the star-nosed mole at the

terminal branch (**Fig A2**). The terrestrial Carnivora species have slight increases in Mb net charge as the transition is made to the terminal branches, however, in every species apart from the badger, *Meles meles*, the Mb net charge never increase above 2.0. The diving species tend to show an increase in Mb net charge, with the exceptions being the polar bear, *Ursus maritimus*, whose Mb is identical to that of the black bear, *Ursus americanus*, and they therefore show the same Mb net charge of 1.99. The American mink, *Neovison vison*, also does not show an increase in Mb net charge above the 1.27 that evolved in primitive Carnivora species. The river otter has an increase in Mb net charge above 2.0 seen at the terminal branch. After the pinnipeds diverged from the Mustelidae their Mb net charge increased from 1.27 to 2.99. However, after the phocid-otariid divergence these species evolved independent mechanisms to achieve an increase in Mb net charge through the evolution of different His residues as outlined above, and gains of strongly positively or losses of strongly negatively charged residues such as Glu83 in the phocid seals and Asp83 in otariid seals (**Fig A2**).

A similar trend is seen in the order Cetartiodactyla where there is a small increase in Mb net charge from 1.12 to 1.56 in the ruminant lineage and a further increase to 1.86 in the bovine species, but all terrestrial species in this order do not evolve Mb net charge above 2.0 with the exception of the red deer, *Cervus elaphus*. However after the cetaceans diverged from the Hippopotamidae the Mb net charge increases greatly from 1.12 to 3.76. There is then a slight reduction in the baleen whales to 3.72, but all toothed whales go on to further increase their Mb net charge above the ancestral state, with the beaked whales having the highest Mb net charge of all mammalian species observed here (**Fig 4.3**). There is however a secondary reduction in Mb net charge in the pygmy hippo from the basal Cetartiodactyla charge of 1.12 to 0.25. This reduction is caused by loss of one strongly positively charged residue and His48, plus the addition of a Cys residue and His91 (**Fig A2**). **Figure 4.3**

highlights the fact that Mb net charge has evolved above the ancestral state in most species, and that having a Mb net charge of 2.2 or higher has tended to evolve through lineages where diving behaviour has also evolved.

Concentrated changes in Mb net charge

In order to test whether increases in high Mb net charge are more concentrated in diving taxa than expected by chance a concentrated changes test was conducted once again using MacClade (Maddison & Maddison, 2005). When tracing the evolution of Mb net charge, phylogenetic analysis shows eleven evolutions of Mb net charge of 2.2 or above, with eight of these occurring on branches where diving behaviour is present. The test found that these evolutions of high Mb net charge were not random and that evolution of high Mb net charge is in fact significantly concentrated in diving mammals ($P < 0.01$).

Discussion

Composite phylogenetic tree

The analysis of Mb protein evolution requires information regarding the relatedness of the species involved. Phylogenetic tree creation is outside the scope of this study and so the relationships between species have been taken from literature sources. To give the most accurate data about Mb evolution an accurate phylogenetic tree is needed. The phylogeny described here, compiled from literature sources, is the most reliable phylogeny available at this time. However, as the study of cladistics and molecular phylogenies is continually evolving and changing, there are alternative hypotheses regarding the relationships of some species. The support for these relationships depend upon the genes used to discover those relationships, for example using cytochrome b yields different mammalian relationships than those using mitochondrial genomes or nuclear genes (Hatch et al., 2006; Wada et al., 2007; Agnarsson & May-Collado, 2008; O'leary & Gatesy, 2008). For this reason this study has attempted to combine relationships from multiple gene studies where possible. However a few alternate resolutions of relationships are worth mentioning here. The resolution of the order Insectivora shown here describes a closer relationship between shrews and hedgehogs than to moles as supported by Bininda-Emonds et al. (2007) and Dubey et al. (2007), however a closer relationship between moles and shrews has been described by Grenyer & Purvis (2003), a study which was used here to ascertain species level relationships for the star-nosed mole, *Condylura cristata*. The study of Grenyer & Purvis (2003) shows strong Bremer support for the relationship between moles and shrews. In terms of the evolution of diving behaviour and the consequences this would have on patterns of Mb evolution, a close relationship between moles and shrews would make no difference, as there would still be at least two evolutions of diving behaviour in the order Insectivora due to the number of other, non-diving species in the respective families Soricidae and Talpidae.

The position of pinnipeds within Carnivora is uncertain, with mustelids and ursids being debated as the sister taxon to pinnipeds. Traditionally, pinnipeds and bears have been considered as sister taxa to the exclusion of mustelids. However, molecular studies have still found weak support for this relationship (Vrana et al., 1994; Delise & Strobeck 2005; Rybczynski et al., 2009). A relationship between pinnipeds as sister to mustelids to the exclusion of bears has grown with the incorporation of more molecular data (Flynn et al., 2005; Sato et al., 2006; Arnason et al., 2007; Fulton & Strobeck 2007). Confusion between relationships could stem from different methods of analysing the data, with Bayesian, maximum likelihood and parsimony showing differential support for the relationship of these three taxa (Delise & Strobeck 2005; Arnason et al., 2007).

The relationship of pinnipeds and mustelids as sisters to the exclusion of ursids is becoming more widely accepted and the strongest support for this comes from Schroder et al. (2009). This matter may be resolved with further evidence in future studies but for now, it has no real impact on the evolution of molecular properties of Mb shown in this study. In terms of both β_{Mb} and Mb net charge the pattern of evolution will remain the same as the basal member of Musteloidae, the raccoon, *Procyon lotor*, and Ursidae, the giant panda, *Ailuropoda melanoleuca*, both have identical β_{Mb} and Mb net charge, suggesting that Mb properties were highly conserved through its evolution in Carnivora, with changes only occurring in the terminal taxa (**Fig 4.2; 4.3**).

There has also been some debate over the positioning of the Perissodactyla, with some studies suggesting a close relationship with Carnivora (Murphy et al., 2007; Arnason et al., 2008) and others suggesting the relationship shown in this study (Murphy et al., 2001; Agnarsson & May-Collado 2008; Prasad et al., 2008 and Hou et al., 2009). Prasad et al. (2008) have shown weak support for the positioning of the horse no matter which relationship was suggested, however they say that it generally tends towards a sister group relationship

with Cetartiodactyla. Hou et al. (2009) showed stronger evidence for an alliance of Perissodactyla with Cetartiodactyla, however, they could not exclude the possibility of a relationship with the horse and dog. Recent retroposon analysis of short interspersed nuclear element (SINE) insertions has grouped the horse with the order Chiroptera (Nishihara et al., 2009), suggesting that methodology is important for understanding the evolution of Perissodactyla. The addition of Perissodactyla at the base of the order Carnivora would cause an ambiguity in the evolution of β_{Mb} which could lead to an increase in β_{Mb} followed by a subsequent reduction or it would remain as it is shown, with the increase being observed only in the branch leading to the terminal group, depending upon whether deltran or acctran resolves are being used. A small increase in Mb net charge would be seen earlier if Perissodactyla was the sister group of the order Carnivora. However the positioning of Perissodactyla would not influence the overall pattern in the evolution of molecular Mb properties.

There has been some debate over positioning of certain members of the order Cetartiodactyla, including the positioning of the minke whale, *Balaenoptera acutorostrata* as a sister taxon to the fin-back whale, *Balaenoptera physalus*, and humpback whale (Sasaki et al., 2005). This would affect the evolution of β_{Mb} and Mb net charge because His8 would only have one evolution. The relationships shown in this study has been taken from one of the most recent studies that have used the largest number of mitochondrial and nuclear genes and included SINE insertions into the analysis (McGowen et al., 2009). The study used here highlighted that lack of resolution within Balenopteroidea and Delphinoidea is the result of rapid evolution and lack of data within river dolphins and claims to have resolved confusion within baleen whale evolution with Bayesian analysis and under parsimony and maximum likelihood. There were a few conflicting resolution compared with the tree shown here, but these had weak support (McGowen et al., 2009).

There is still some debate surrounding basal evolution in the order Rodentia (Montgelard et al., 2008; Blanga-Kanfi et al., 2009), however, any new resolution within this order will unlikely influence the pattern of events described in this study as βMb and Mb net charge have followed similar evolutionary trends in most rodents (**Fig 4.2; 4.3**). According to the study by Chatterjee et al. (2009) there is still debate over the placement of the common-woolly monkey, *Lagothrix lagotricha*, and whether it should be placed at the base of the clade containing the lemurs. Resolving issues within the order Primates will not, however, affect the general pattern of evolution of Mb as the major sub-groups in this order follow similar trends of evolution (**Fig 4.2; 4.3**) and thus will not be considered further.

Evolution of Mb specific buffering

Chapter 2 of this study showed that β_{Mb} contributes more to whole muscle non-bicarbonate buffering in several mammalian divers compared to terrestrial species due to the His content of the Mb protein and the increased [Mb]. This chapter has shown that the evolution of increased β_{Mb} through alterations in His content is not limited to diving mammals (**Fig 4.2**). Conversely, although increases from the ancestral β_{Mb} occur in most divers, this is not the case in the semi-aquatic diving insectivorans and mustelids. Next to proficient divers, high values of β_{Mb} are also observed in terrestrial species including the red kangaroo, *Macropus rufus*, horse, *Equus caballus*, zebra, *Equus burchellii*, ruminants, the rabbit and the chimpanzee. Most of these species rely on or are capable of periods of burst locomotion. Previously a link between burst locomotion and high whole muscle non-bicarbonate buffering capacity has been established in several marine and terrestrial mammals, including the rabbit (Castellini & Somero, 1981). Gradual increases in β_{Mb} occur in the order Cetartiodactyla with independent increases occurring in ruminants, pygmy hippo, *Choeropsis liberiensis*, and cetaceans. The increases in the β_{Mb} of these animals are harder to

explain, as the ruminants observed in this study and the pygmy hippo are not well known for burst locomotion. However other members of the ruminant family include the antelope and other sprinters that rely on burst locomotion to evade predators. In this context the 'aquatic escape' hypothesis (Meijaard et al., 2009) is interesting, which describes a behaviour thought to have led to the evolution of diving in cetacean ancestors. Several ruminants, when threatened, escape into water bodies and some of them dive into nearby rivers and remain submerged for considerable durations until the danger has passed or shelter is found (Meijaard et al., 2009). This behaviour is still observed today in the modern day mouse-deer, *Hyemoschus aquaticus* and *Tragulus napu* and a similar behaviour is seen in the pygmy hippo (Meijaard et al., 2009). Assuming this hypothesis is correct then increases in β_{Mb} could have facilitated the evolution of early cetaceans offering true 'Darwinian fitness', but may also have facilitated a similar role in early ruminant species. Thus increased β_{Mb} may have arisen once in an ancient common ancestor of ruminants and cetaceans to aid aquatic escape behaviour and the trait has then been retained in modern ruminants. This is supported in **Figure 2.3B**, which shows that 11 out of 12-14 His residues are conserved between cetartiodactylan species. It would therefore be of worth to sequence the Mb of mouse deer and other early ruminants, which could then be compared to early artiodactyl lineages such as, camelids, to date the origin of elevated β_{Mb}.

High β_{Mb} values of 3.2 (mol H^+ mol Mb^{-1} pH^{-1}) or above have evolved nine times within the mammalian species observed here. Five of these evolutions have occurred on branches where diving behaviour is present, however this does not prove to be an evolutionary event that is significantly concentrated in diving taxa, using the concentrated changes test. It therefore cannot be refuted that these occurrences in divers are more than chance evolutions. However, as has been shown in **Chapter 2**, there is a significant increase in the contribution of Mb towards whole muscle non-bicarbonate buffering (β_{muscle}) and

varied contribution from other buffering components such as, His-related dipeptides (**Fig 2.7**). This is a result of increases in total Mb buffering, which is the [Mb] multiplied by the Mb specific buffer value (β_{Mb}), rather than increases in β_{Mb} alone, i.e. Mb His content. Since High β_{Mb} occurs in terrestrial species that are capable of burst locomotion as well as in diving species, it is possible that an increase in β_{Mb} was a necessary adaptation in early evolution of species that had a greater demand for anaerobic metabolism due to the method of locomotion. The buffering potential of the Mb protein was then greatly increased in divers as [Mb] increased. In other words, the increase in Mb buffer groups likely preceded the increase in [Mb], at least in diving Cetartiodactyla.

A similar trend is observed within pinnipeds, where increases in β_{Mb} occur in both phocid and otariid seals. The evolution of β_{Mb} is achieved through different mechanisms in these two groups highlighted by independent evolutions of His residues (**Fig 2.3A; Fig A2**) and by the fact that β_{Mb} values rise above 3.2 only in phocids (**Fig 4.2**). Within diving insectivorans and mustelids, β_{Mb} does not increase above ancestral levels. Skeletal muscle [Mb] within diving insectivorans only increase modestly compared to their terrestrial relatives and given that they have relatively low muscle [Mb] then increases in β_{Mb} would be futile in an attempt to increase total Mb buffering. This may also be true of mustelids but so far [Mb] data for these species is not known. This study would predict low skeletal muscle concentrations.

Evolution of Mb net charge

Chapter 3 of this thesis showed that there is a significant correlation between [Mb] and Mb net charge and that there is a significant relationship between Mb net charge and diving behaviour. Here the evolution of Mb net charge will be discussed. The ancestral state is to have a low Mb net charge, something that has been retained in some modern day

species, hedgehog, tree shrew, Ehrenberg's mole-rat, plains viscacha, casiragua, and the guinea pig, despite numerous amino acid changes during their evolution, for example there are sixteen amino acid substitutions on the route towards the evolution of the rodent lineages and none of these involve charged residues (**Fig A2**). This would suggest that these species have low [Mb] and indeed the concentrations observed in Ehrenberg's mole-rat and Guinea pig of 0.4 and 0.7 (g $100g^{-1}$) (Ar et al., 1977; Leniger-Follert & Lubbers 1973) respectively, are low compared to the concentrations observed in diving species, but they are consistent with other terrestrial mammals (**Table A3**).

Independent increases in Mb net charge are seen in all diving species (**Fig 4.3**) this may be seen as an example of convergent evolution, where remarkably all species display similarly high charge that has been achieved through independent amino acid substitutions (**Fig A2**). Early in the evolution of the orders Carnivora, Chiroptera and Insectivora, an increase of one positive charge is observed. In the former two orders it is the result of an increase in a strongly positively charged residue at position 132 whereas in the latter order it results from a loss of a strongly negatively charged residue at position 83. A similar increase in Mb net charge can be seen in the early evolution in the order Cetartiodactyla although early charge increase in this order is the result of addition of His residues (**Fig A2**). After the Hippopotamidae-Cetacea divergence at approximately 55 MYA (McGowen et al., 2009) a large increase in Mb net charge is observed (**Fig 4.3**) with further small increases in Mb net charge observed in the toothed whales, culminating in the highest Mb net charges seen in the beaked whales (**Fig 4.3**) this has been shown, in this study, to correlate to high [Mb] (**Fig 3.5**).

Increases in Mb net charge above the basal Carnivora charge of 1.27 are observed in all diving carnivoran species, apart from the American mink, due to independent evolutions of strongly positively charged residues. Even further independent evolutions of high Mb net

charge are seen in phocid and otariid seals (**Fig A2**). Independent evolutions of high Mb net charge are also seen in the terminal branches of diving rodents and diving insectivores due to increases in His and strongly positively charged residues in diving rodents and due to further losses of strongly negatively charged residues in diving Insectivora species. Increases in Mb net charge are also observed in both diving and terrestrial species of Monotremata, and this is reflected by [Mb] that is higher than in terrestrial species of other orders (**Table A3**). The high Mb net charge and [Mb] in the echidna, *Tachyglossus aculeatus*, may be a remnant of an aquatic ancestry (Phillips et al., 2009). Similarly an increase in Mb net charge from 0.40 to 2.12 is observed in the elephants (**Fig 4.3**), which may be a reflection of their aquatic ancestry and something that has been retained even after they returned to a terrestrial existence even though [Mb] is low in the African elephant, *Loxodonta africana*, (**Table A3**). There are also increases in Mb net charge seen in some terrestrial species at the terminal branches of the red kangaroo, red deer and the badger. This suggests that these species have a high [Mb], although unfortunately measurements of [Mb] are not available.

As a burrowing species, the badger may be subjected to hypoxic environments and therefore may have evolved high [Mb] to enable continued muscle function in low oxygen, similar to diving mammals (Butler & Jones, 1997). There is a secondary reduction in Mb net charge in the pygmy hippo, which suggests that the pygmy hippo has a low [Mb], which unfortunately is unknown at this time. It may be that hippopotamus species evolved a large body size early in their evolution and therefore had a lower mass specific O_2 consumption (Butler & Jones, 1982) which negated the need for high [Mb] to enabled aquatic escape behaviour, and therefore Mb is not as highly expressed in these species as it is in other divers.

The evolution of a high Mb net charge has been observed eleven times (**Fig 4.3**). Of these eleven evolutions, eight occur on branches where diving behaviour is also present. Concentrated changes tests indicate that the evolution of high Mb net charge in diving species

is in fact not due to random evolutionary events and that it is significantly linked to the evolutionary history of diving species ($P < 0.01$). The significant concentration of incidences of high Mb net charge in diving species maybe an indication that structural changes in the Mb protein occur before high [Mb] can be established. As high Mb net charge may increase the solubility of apoMb and holoMb (see **Chapter 3** for discussion), this may therefore allow increased skeletal muscle [Mb] and more oxygen to be stored for use during long dives.

Conclusion

Chapters 2 and **3** of this thesis have shown that several diving mammals have a larger contribution of β_{Mb} towards increased whole muscle non-bicarbonate buffering compared to terrestrial species and that an increased Mb net charge that correlates strongly with increased [Mb] is a significant feature of diving mammals. This chapter has shown that high β_{Mb} is seen in some divers and terrestrial species that show burst locomotion. It suggests that evolutionary increases in β_{Mb} are not significantly more frequently found in diving than in terrestrial species, but rather a step in the evolution of modes of locomotion that are more heavily reliant upon anaerobic metabolism. Thus increased contributions of total Mb buffering towards β_{muscle} in diving species were likely first achieved by an increases in β_{Mb}, followed by increased Mb concentrations. Interestingly the increases in β_{Mb} have been achieved through increases in His residues. Unlike other good buffering groups with a p*K*a value close to physiological pH such as cysteine (Cys), His residues already convey a slightly higher net positive charge to Mb. While a free Cys residue would aid buffering, it would convey less towards net positive charge. Therefore increases in His to aid buffering may have laid the foundation for increased Mb net charge and thereby Mb solubility.

In contrast to β_{Mb}, the present work confirms that the evolution of high Mb net charge is significantly concentrated in mammalian lineages where diving behaviour occurs. Increases

from ancestral Mb net charge are observed in all divers, with most diving species showing a high Mb net charge which has either increased very recently within the terminal branches (**Fig 4.3**) or has increased throughout the lineage such as in the cetaceans and pinnipeds. Since Mb net charge is strongly correlated with [Mb], the structural changes in the Mb protein leading to an increased net charge very likely evolved throughout all lineages of diving mammals as a mechanism to provide O_2 to under-perfused locomotory muscles during a dive.

Chapter 5 – Myoglobin and maximal dive duration: Correlations and predictions

Introduction

For aquatic mammals, the maximal duration of a dive has long been believed to depend upon the total amount of usable oxygen (O_2) stored within the body and the overall rate at which O_2 is consumed (Scholander, 1940; Butler & Jones, 1997). Three main O_2 reservoirs can be utilised during a dive: Lung, blood and muscle O_2 stores. Using these finite O_2 stores with maximum efficiency, remarkable feats of breath-hold endurance have been observed among mammalian divers. Thus some species routinely dive for approximately an hour or longer, such as the Northern elephant seal, *Mirounga angustirostris* (Le Boeuf, 2000), Northern bottlenose whale, *Hyperoodon ampullatus* (Hooker & Baird, 1999), bowhead whale, *Balaena mysticetus* (Krutikowsky & Mate, 2000) and sperm whale, *Physeter catodon* (Watkins et al., 1993). It is accepted that during most dives undertaken by mammals and birds, metabolism remains aerobic in nature. Kooyman et al. (1980) identified that ~97% of adult Weddell seal, *Leptonychotes weddellii*, dives used predominately aerobic metabolism. This conclusion was drawn from post-dive lactate concentrations being approximately equal to pre-dive levels. They noted that the Weddell seal could dive for approximately twenty minutes before blood lactate levels increased above resting levels. This was termed as the aerobic dive limit (ADL), defined as the duration of a dive after which post-dive blood lactate concentrations increased above pre-dive levels (Kooyman et al., 1980). Later researchers have proposed to rename this term as the diving lactate threshold (DLT) to prevent confusion between ADL, and the time it takes to consume all usable O_2 termed the calculated aerobic dive limit (cADL), for discussion see Butler & Jones (1997). Thus DLT will be used during the remainder of this chapter.

Aquatic mammals have employed certain physiological adaptations, which assist them to sustain aerobic metabolism during a dive. Reduction in heart rate frequently occurs

during the beginning of a dive. The Baikal seal, *Phoca sibirica*, reduces its heart rate from between 120-160 beats per minute (bpm) immediately before a dive to less than 50 bpm during the dive (Ponganis et al., 1997) and several other seal species will reduce their heart rate to 10 bpm (Odden et al., 1999; Thornton & Hochachka, 2004). Reduced heart rate is often coupled with peripheral vasoconstriction, which is a reduction in the blood flowing to the extremities and non-essential organs, (Kooyman & Ponganis, 1989). Blood flow is also reduced to skeletal muscle tissues, leading researchers to believe that they eventually work anaerobically. This is indicated by an increase in blood lactate concentration only after a dive, when the tissues have been re-perfused (Scholander, 1940). Because of reduced heart rate and localised vasoconstriction, an animal's metabolism is also reduced. The body of diving animals is also tolerant to reductions in body temperature that accompany reduced metabolism. Diving ducks have even demonstrated rapid brain cooling, as a means of protection against ischemia (Caputa et al., 1998). This has also been noted in seals, where a decrease of up to 3°C has been observed in the hooded seal (Odden et al., 1999). All of the above mechanisms, termed the dive response, aid a diver to maintain aerobic metabolism for prolonged periods during a dive, although muscle tissues can become eventually anaerobic during a dive (Williams et al., 2011).

DLT has only been measured in Weddell seals (Kooyman et al., 1980), Baikal seals (Ponganis et al., 1997) and emperor penguins, *Aptenodytes forsteri*, (Ponganis et al., 1997). Estimates of DLT have been calculated based on the amount of oxygen stored in the lungs, blood and muscles of divers, and the rate of consumption during a dive. These calculated aerobic dive limits (cADL) have given an idea of the aerobic capacities of many seals and cetaceans. One of the biggest flaws in these calculations is the assumption that all usable oxygen is depleted before lactic acid starts being produced; see Butler (2004) for discussion. Recent studies have suggested that the main O_2 stores used in cADL, maybe treated as

independent. Dives may be limited by the depletion of one or more of these stores (Davis & Kanatous, 1999; Ponganis et al., 2010) and some of the isolated tissue stores, i.e. muscle, may de-saturate during a dive therefore relying on a certain amount of anaerobic metabolism, while other stores remain oxygenated (Williams et al., 2011). While knowing the aerobic limits of diving mammals and understanding usual dive habits is very important, it is noted that these calculated limits are exceeded in nearly every mammal. The Weddell seal has a DLT of 20-26 min yet its maximum dive time is 82 min (Castellini et al., 1992), the emperor penguin has a DLT of 5.6 min (Ponganis et al., 1997), yet a maximum dive time of 22 min (Robertson, 1995; as quoted in Ponganis, 1997). Similarly, cADLs are reported to be exceeded in harbour seal, *Phoca vitulina*, (Burns et al., 2005), ringed seal, *Phoca hispida*, (Lydersen et al., 1992) and the Australian sea lion, *Neophoca cinerea*, (Fowler et al., 2007). Therefore aerobic limits cannot convey the true diving capacity of any mammalian species.

O_2 stores tend to increase in direct proportion to body mass, i.e. isometric scaling with an exponent of 1 (Hudson & Jones, 1986), however O_2 consumption tends to increase allometrically with body mass, i.e. with an exponent less than 1, namely 0.65-0.75 (Butler & Jones, 1982; Halsey et al., 2006). This means that maximum dive duration increases allometrically with an exponent of 1-0.65 to 1-0.75, namely 0.25-0.35. Prediction of maximum dive duration is usually attempted using allometry (Boyd & Croxall, 1996; Schreer & Kovacs, 1997; Halsey et al., 2006). The allometric equations used seem to vary depending on what species are used during the study. It should be noted that these comprehensive studies using numerous divers from various orders have shown that a single allometric exponent cannot be used for predicting max dive durations. For example using only pinniped seals, one study claims that the allometric exponent is 0.28 (Boyd & Croxall, 1996) whereas a second study claims an allometric exponent of 0.48 is a better estimate of maximum dive duration (Schreer & Kovacs, 1997). It is noteworthy that the allometric equations used in

certain studies may not be as reliable as stated, because of inclusion of data without recognizing the interrelatedness of individuals due to common ancestry. It seems that order specific exponents produce more accurate predictions of max dive duration (Halsey et al., 2006).

Another method of predicting maximum dive duration was used by Noren & Williams (2000), they found that maximum Mb content ($[Mb_{max}]$) significantly correlates with maximum dive duration in toothed whales. **Chapter 3** of this study has identified that $[Mb_{max}]$ significantly correlates with Mb net charge (**Fig 3.5**) and so Mb net charge can be used as a proxy for $[Mb_{max}]$. In the study by Noren & Williams (2000) a simple correlation between maximum dive duration and body mass was analysed, finding that body mass is a better predictor of dive duration, than $[Mb_{max}]$ in cetacean species. A similar type body mass correlation has been repeated in this study, using thirty four mammalian divers, from five phylogenetic orders (**Fig 5.1**). The species used in this correlation vary in body mass from a 15 g water shrew to a 79 ton bowhead whale. This study finds that there is a significant positive correlation between body mass and maximum dive duration, however the r^2 value in this study is reduced, 0.49 (**Fig 5.1**) compared to the r^2 values of 0.72 observed for cetaceans and 0.98 when only considering toothed whales in the previous study (Noren & Williams, 2000). The findings here highlight that body mass is a good predictor of dive duration only when observing a group of closely related species.

Many diving mammals however are bigger than their terrestrial relatives and this should prolong dive time because of allometry of basal metabolic rate (BMR) (Butler & Jones, 1982) and the isometric scaling of O_2 stores (Hudson & Jones, 1986). An increase in body size has also been documented in the evolutionary transition of early whales from terrestrial ancestors, which suggests that increases in size were linked to diving behaviour. However, stores can also be increased independently of body mass by elevated Mb, larger

lungs or larger blood O2 stores. The relative contribution of these two ways for increasing diving capacity appears not to have been investigated before.

It is the aim of this study to test whether considering the three main oxygen stores separately yields better predictions of maximum dive duration than cADL or body mass and to develop a simple method to predict maximum dive duration for multiple orders of diving mammals.

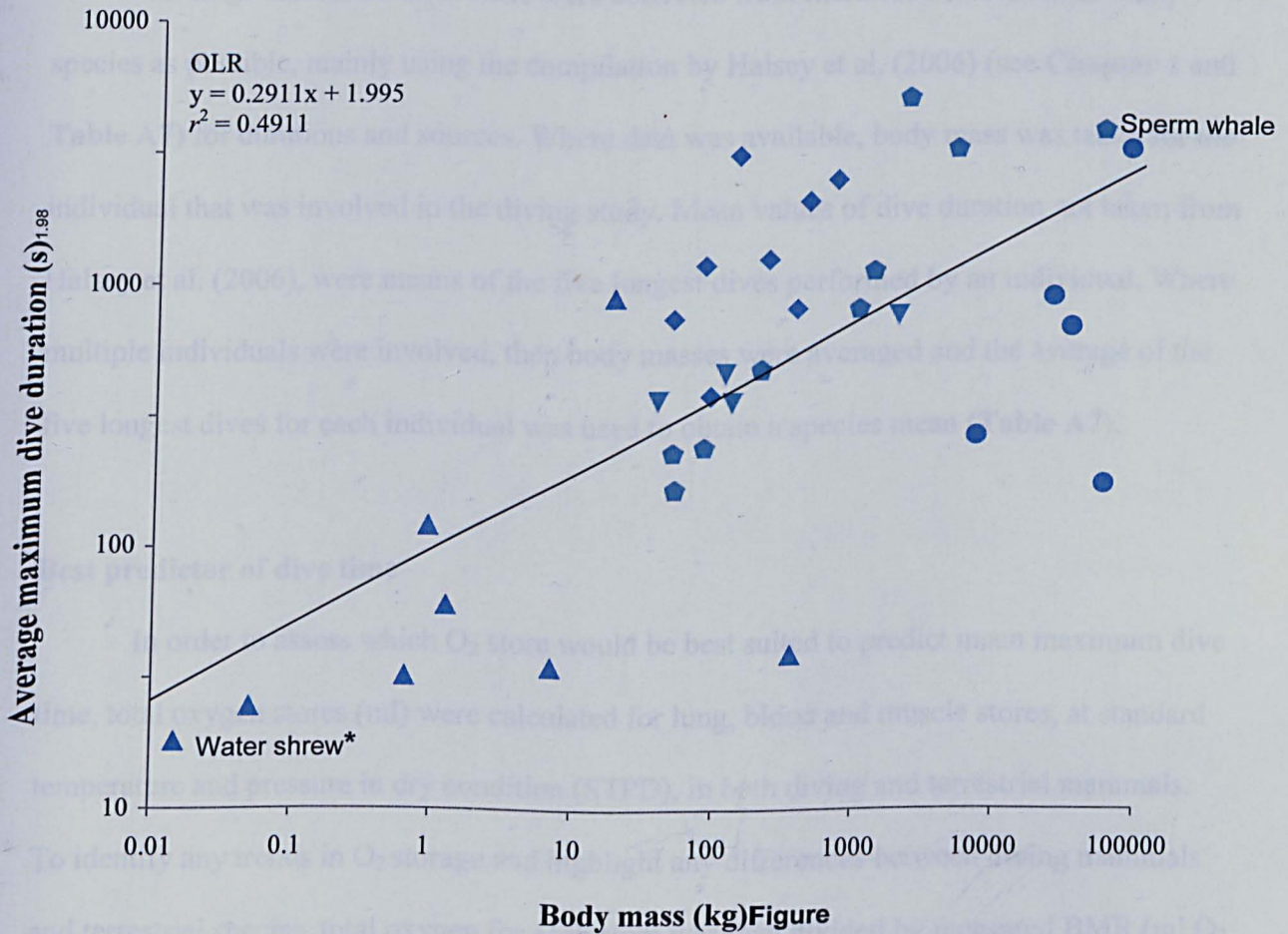


Figure 5.1 Correlation between average maximum dive duration and body mass for thirty four species of diving mammals with known Mb amino acid sequence. Data span five phylogenetic orders and are plotted on double logarithmic axes. A weak yet significant ($P < 0.01$) positive correlation is found with ordinary linear regression (OLR) analysis. Triangles = semi-aquatic species, diamonds = phocid seals, inverted triangles = otariid seals, circles = baleen whales, pentagon = toothed whales.

Methods

Maximum dive durations

Average maximum dive times were collected from literature sources for as many species as possible, mainly using the compilation by Halsey et al. (2006) (see **Chapter 1** and **Table A7**) for durations and sources. Where data was available, body mass was taken for the individual that was involved in the diving study. Mean values of dive duration not taken from Halsey et al. (2006), were means of the five longest dives performed by an individual. Where multiple individuals were involved, then body masses were averaged and the average of the five longest dives for each individual was used to obtain a species mean (**Table A7**).

Best predictor of dive time

In order to assess which O_2 store would be best suited to predict mean maximum dive time, total oxygen stores (ml) were calculated for lung, blood and muscle stores, at standard temperature and pressure in dry condition (STPD), in both diving and terrestrial mammals. To identify any trends in O_2 storage and highlight any differences between diving mammals and terrestrial species, total oxygen for each store was then divided by measured BMR (ml O_2 s^{-1}) for as many species as possible and the $\log_{10}$ of the resulting value then correlated with $\log_{10}$ body mass (kg). Measured BMR data was used to try to avoid the problems associated with using allometry defined BMR (White et al., 2009). However, BMR, which is defined as energy expenditure of a postabsorptive, non-reproducing animal, whilst at rest and measured under thermoneutrality, is impossible to measure for certain large species, e.g. cetaceans, ruminants that may never be post-absorptive, and for some smaller species that may never rest, such as shrews (White et al 2009). Thus allometric based BMR data have been used to allow inclusion of certain key species highlighted with blue text (**Table A5-7**).

Calculating oxygen stores:

Lung oxygen store

Total lung oxygen store, $\dot{V}O_{2Lung}$ (ml), was calculated according **Equation 5.1,** where mass specific lung volume (V_{Lung}) in ml kg^{-1} was taken from literature sources and converted to absolute volumes in ml using an average body mass M for that species in kg (**Table A5**). If unavailable, V_{Lung} was calculated using **Equation 5.2** (Stahl, 1967). Lung O_2 content was taken as 15% of V_{Lung} (Dejours, 1981). Body mass was taken from the same literature sources that reported BMR measurements, whenever possible (**Table A5**).

$\dot{V}O_{2Lung}$ = V_{Lung} X O_2 content X Body mass **Equation 5.1**

$V_{Lung} = 53.5\ M^{1.06}$ **Equation 5.2**

Blood oxygen store

Total blood oxygen, $\dot{V}O_{2Blood}$ (ml) was calculated using **Equations 5.3**, and arterial and venous O_2 was calculated using **Equations 5.4 and 5.5,** respectively. Haemoglobin concentrations [Hb] (g 100 ml^{-1}) were taken from literature sources (**Table A6**). The oxygen-binding capacity of Hb is 1.34 in ml O_2 g^{-1} Hb (Hlastala & Berger, 2001). The O_2 saturation of arterial blood is assumed to be 95% and the arterial volume was assumed to be 33% of total blood volume (V_{Blood}). The O_2 saturation of venous blood is assumed to be 75% at the onset of a dive and the venous volume was assumed 66% of total V_{Blood}. These V_{Blood} assumptions were taken following the methods previously published for calculating cADL (Kooyman et al., 1983; Davis & Kanatous, 1999; Fowler et al., 2007). Measured V_{Blood} (ml kg^{-1}) was taken from literature sources for animals ranging in mass from a 14 g water shrew to a 3 ton killer whale, this data was then multiplied by body mass to give the absolute V_{Blood} (ml). Measured volumes were taken mainly for diving species, as V_{Blood} scales well with

allometry for terrestrial species (Stahl, 1967). If measured V_{Blood} was not available then V_{Blood} was calculated (ml) using **Equation 5.6** where M is body mass in kg (Stahl, 1967).

$V\dot{O}_{2Blood}$ = Arterial O_2 + Venous O_2 **Equation 5.3**

Arterial O_2 = ([Hb]/100) X 1.34 X 0.95 X Arterial vol X V_{Blood} **Equation 5.4**

Venous O_2 = ([Hb]/100) X 1.34 X 0.75 X Venous vol X V_{Blood} **Equation 5.5**

$V_{Blood} = 5.6M^{1.02}$ **Equation 5.6**

Muscle oxygen store

Total muscle O_2 stores, $V\dot{O}_{2Muscle}$ (ml), were calculated according to **Equation 5.7** by multiplying the amount of muscle by the amount of O_2 carried by the muscle. The body mass used in calculations was the body mass of the individual for which measured BMR data was obtained. Where body mass from BMR measurements was not available, average mass for that species was taken from other literature sources (**Table A7**). Muscle mass was assumed to be 33.66% as an average of measured muscle masses in twenty species, ranging from 23% in the sloth to 41.5% as an average of eight bovid species, with an even mix of diving and terrestrial species (**Table A9**). Skeletal [Mb] (g 100 g^{-1}) was taken from literature sources, where available (**Table A7**) and 1.30 was taken as the O_2 binding capacity of Mb, O_{2capMb} (ml O_2 g^{-1}Mb).

$V\dot{O}_{2Muscle}$ = (Body mass X Muscle % / 100) X (Skeletal [Mb] X O_{2capMb}) **Equation 5.7**

The O_{2capMb} of 1.30 used here was determined by modifying the formula used to calculate Hb O_2 binding capacity (Hlastala & Berger, 2001) given as **Equation 5.8**. The molecular weight of Mb was taken as an average of values given in the ExPASy proteomics server for twenty seven species, based on amino acid sequences (**Table A8**).

$$O_{2cap}Mb = 1\ X \frac{mole\ O_2}{mole\ Mb}\ X\ 22{,}400\ \frac{ml\ O_2}{mole\ O_2}\ X\ 1\ \frac{mole\ Mb}{17{,}173\ g\ Mb} = 1.304\ \frac{ml\ O_2}{g\ Mb}$$ **Equation 5.8**

Analysis of O_2 stores

The volume of each O_2 store was divided by BMR to give a rate of O_2 consumption, which was then correlated with body mass for diving and terrestrial species. Ordinary least squares regression analysis (OLR) was conducted on the data for terrestrial and diving species separately, then an analysis of covariance (ANCOVA) was performed (StatsDirect UK) to identify any differences between the regression analysis of divers and terrestrial for each of the major O_2 stores.

Mb sequence and charge calculation

Mb sequences were obtained and Mb net charge was calculated as described in **Chapter 3**. Briefly Mb sequences for as many species as possible were collected from literature sources and online databases or determined by PCR from cDNA. Mb net charge was calculated from the amino acid sequence and the p*K*a values of ionisable groups by totalling the estimated charge contribution of each ionisable residue. The charge of each residue at every pH value ranging from 4 - 11 with increments of 0.1 was calculated using a simple hyperbolic H^+ binding curve according to **Equation 3.1** in **Chapter 3** with the necessary p*K*a values obtained from the literature or determined by comparing titration curves of purified Mbs with known differences in the number of ionisable groups. It was identified in **Chapter 3** that [Mb_{max}] significantly correlates with estimated Mb net charge (**Fig 3.8**). This allows the use of Mb net charge as a proxy for [Mb_{max}] in later analyses.

Evolutionary reconstruction of myoglobin net charge

Estimated Mb net charge was reconstructed on an evolutionary species trees described in **Chapter 4**. Briefly, a composite phylogenetic tree was constructed from literature sources for all mammalian species with available Mb sequence data (**Fig. 4.1**). Mb amino acid substitutions for each position in the sequences were then mapped onto branches, using the 'trace character' function in MacClade version 4.0 (Maddison & Maddison, 2000). For this study maximum parsimony reconstruction was used. Maximum parsimony traces the characters, amino acids, in a way to minimise the number of evolutionary changes. Comparisons have shown that when there are few changes involved parsimony gives similar result to maximum likelihood (Kuhner & Felsenstein, 1994). All amino acid changes were then highlighted on the composite tree and analysed for charged residue substitutions (**Fig 4.3**). Since the charge of each ionisable residue has been calculated at pH 6.5 (**Table 2.2**) and Mb net charge is known at the terminal branch, with the extant species, (**Table A2**),working backwards through the most parsimonious charged amino acid substitutions, it is possible to reconstruct an estimate of Mb net charge at each node.

Modelling dive time using body mass and myoglobin net charge

Body mass data was collected from literature sources (**Table A7**) and combined with Mb net charge data (**Table A7**), used as a proxy for $[Mb_{max}]$, for thirty four diving mammals from five different orders. Maximum dive duration was modelled, using the formula:

$$\log t_{max} = (a \text{ X } \log m) + (b \text{ X } z) \qquad \textbf{Equation 5.9}$$

where t_{max} is the average maximum dive duration in seconds, m is body mass in kg and z is the Mb net charge at pH 6.5. Values for a and $b \pm$ standard error, are 0.1751 ± 0.0363 and

0.5856 ± 0.0255, respectively, and were calculated using a non-linear iterative curve fit algorithm (SigmaPlot version 11.0, Systat software, USA), that would produce the best match to measured average maximum dive durations (**Table A7**).

Taking this further, Mb net charge reconstruction at ancestral nodes was then compiled along with body mass estimates to predict average maximum dive durations for extinct species close to the split of the ancestral cetacean lineage and its closest artiodactylan relatives, such as *Pakicetus* and *Indohyus,* respectively. Fossil evidence suggests that *Indohyus* species existed approximately 60 million years ago (MYA), and that they were semi-aquatic artiodactylans positioned close to the origin of the cetacean lineage, diverging just after the two groups diverged from the branch leading to the Hippopotamidae (Thewissen et al., 2007) (**Fig 5.9**). Fossil evidence suggests that *Packicetus* species were alive approximately 45-55 MYA (Thewissen et al., 2001), and that they are the closest relatives to the ancestral whales, diverging from the lineage to modern whales soon after the divergence of Cetacea from *Indohyus* (**Fig 5.9**). Estimates of dive duration for these species are shown in **Table 5.1**.

Results

Figure 5.2 shows a double logarithmic plot of how long lung O_2 stores can theoretically sustain BMR ($\dot{V}O_{2,lung}$ BMR^{-1}) against body mass in a range of terrestrial mammals from a 2.2 g Etruscan shrew, *Suncus etruscus*, (Nagel, 1980) to a 3.7 ton African elephant, *Loxodonta africana*, (Kleiber, 1947). An ordinary least squares linear regression was fitted to the data with a high r^2 value of 0.93. The same data for diving species was then overlaid, with r^2 value of 0.85, to identify any differences between divers (blue symbols) and terrestrial mammals (red symbols) (**Fig 5.2**). In the minke whale, *Balaenoptera acutorostrata*, the California sea lion, *Zalophus californianus*, the star-nosed mole and Eurasian beaver $\dot{V}O_{2Lung}$ BMR^{-1} is no different to terrestrial species of similar size, whereas in all other diving species $\dot{V}O_{2Lung}$ BMR^{-1} is reduced (**Fig 5.2**). ANCOVA shows that there is no significant difference between the regression slopes of divers and terrestrial species $P = 0.542$. However there is a significant difference between the y-intercepts between divers and terrestrial species $P < 0.005$. Meaning that the duration that $\dot{V}O_{2Lung}$ can sustain BMR is reduced in diving species compared to terrestrial mammals (**Fig 5.2**). The red open squares indicate terrestrial species that routinely burrow or occupy high altitudes have been added as a comparison, but have not been included in the regression analysis.

The same analyses were made for both blood and muscle O_2 stores (**Fig 5.3** and **Fig 5.4,** respectively). **Fig 5.3** suggests that $\dot{V}O_{2Blood}$ sustains metabolism at BMR equally long for terrestrial and diving mammals of similar body mass. This is supported by ANCOVA, finding no significant difference between the regression slopes of divers and terrestrial species $P = 0.059$ and no significant difference between the y-intercepts either $P = 0.928$. There are three species; the Weddell seal, minke whale and echidna, *Tachyglossus aculeatus*, where $\dot{V}O_{2Blood}$ can sustain BMR longer than in terrestrial species of similar size (**Fig 5.3**). This can be explained by either an unusually low BMR or an unusually high $\dot{V}O_{2Blood}$. The

former is true for the echidna, which has a measured BMR similar to an animal that is half its body mass, the platypus, *Ornithorhynchus anatinus* (McNab, 1984). In contrast, the minke whale has a high V_{Blood}. The Weddell seal has a higher measured V_{Blood} compared to its allometrically calculated volume (**Table A6**).

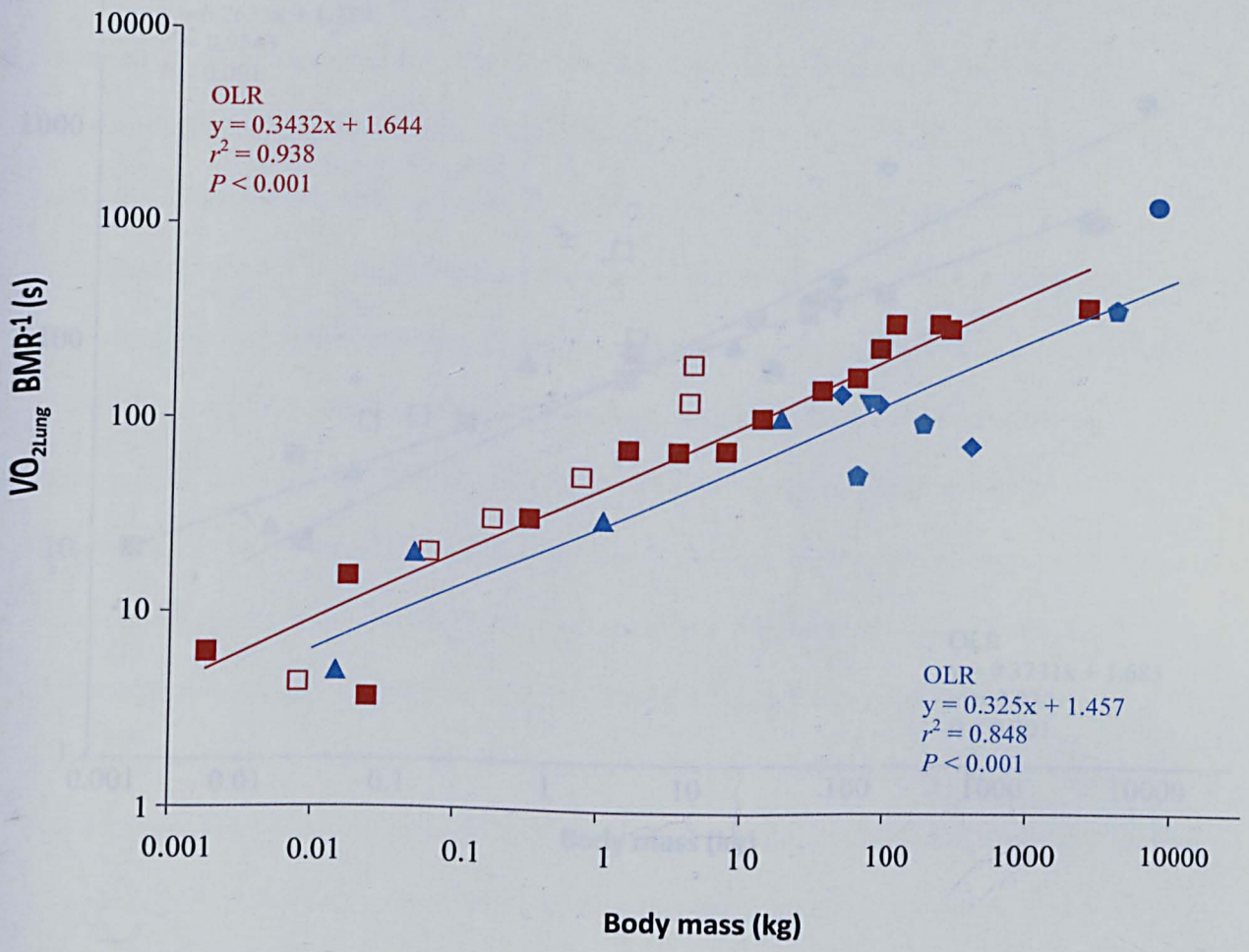


Figure 5.2 Duration VO_{2Lung} can sustain BMR, as a function of body mass on a double logarithmic plot. A significant positive relationship is found with ordinary least squares regression (OLR) (solid red line) for terrestrial and diving mammals (solid red and blue lines respectively). Data for diving mammal is overlaid to allow comparison with terrestrial species. Red filled squares = terrestrial species, Red open squares = altitude or burrowing species, blue triangles = semi-aquatic species, blue diamonds = phocid seals, blue inverted triangles = otariid seals, blue circles = baleen whales, blue pentagons = toothed whales.

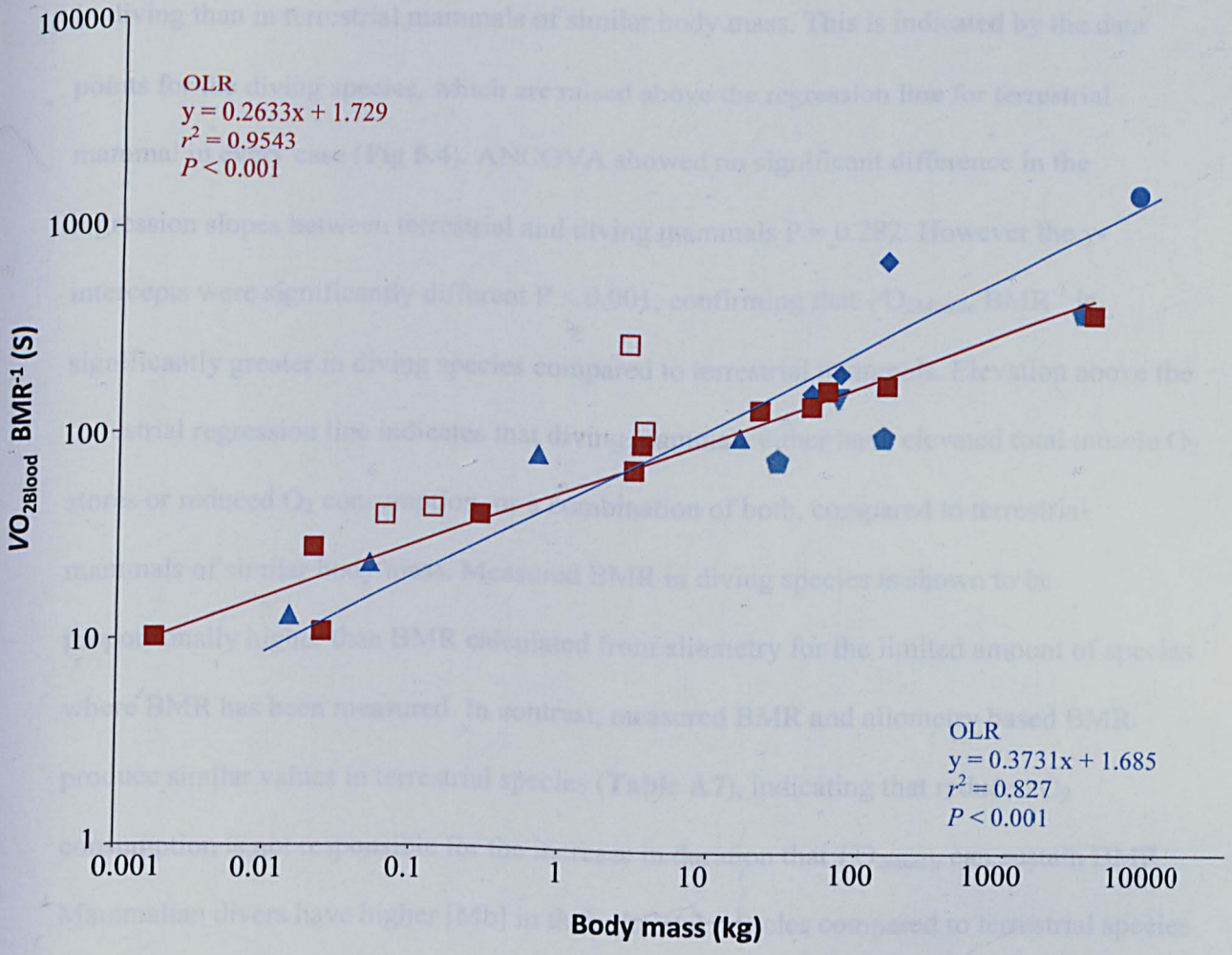


Figure 5.3 Duration VO_{2Blood} can sustain BMR as a function of body mass on a double logarithmic plot. A significant positive relationship is found with ordinary least squares regression (OLR) (solid red line) for terrestrial and diving mammals (solid red and blue lines respectively). Data for diving mammal blood O_2 store and consumption is overlaid to allow comparison with terrestrial species. Red filled squares = terrestrial species, Red open squares = altitude or burrowing species, blue triangles = semi-aquatic species, blue diamonds = phocid seals, blue inverted triangles = otariid seals, blue circles = baleen whales, blue pentagons = toothed whales.

The muscle O_2 store is the only O_2 reservoir that consistently can support BMR longer in diving than in terrestrial mammals of similar body mass. This is indicated by the data points for the diving species, which are raised above the regression line for terrestrial mammal in every case (**Fig 5.4**). ANCOVA showed no significant difference in the regression slopes between terrestrial and diving mammals $P = 0.282$. However the y-intercepts were significantly different $P < 0.001$, confirming that $\dot{V}O_{2Muscle}$ BMR^{-1} is significantly greater in diving species compared to terrestrial mammals. Elevation above the terrestrial regression line indicates that diving mammals either have elevated total muscle O_2 stores or reduced O_2 consumption, or a combination of both, compared to terrestrial mammals of similar body mass. Measured BMR in diving species is shown to be proportionally higher than BMR calculated from allometry for the limited amount of species where BMR has been measured. In contrast, measured BMR and allometry based BMR produce similar values in terrestrial species (**Table A7**), indicating that reduced O_2 consumption is not responsible for the increase in duration that $\dot{V}O_{2muscle}$ can sustain BMR. Mammalian divers have higher [Mb] in their skeletal muscles compared to terrestrial species (**Table A7**) and since Mb is the store of O_2 then this can explain the elevation above terrestrial levels in $\dot{V}O_{2muscle}$ BMR^{-1}.

To ascertain the predictive power of $\dot{V}O_{2muscle}$ BMR^{-1}, for diving capacity, this parameter was compared with average maximum dive duration on a double logarithmic plot in as many mammalian divers as possible. A strong positive correlation was observed, where OLR produces an r^2 value of 0.788, and a slope that is significantly different from zero ($P < 0.001$), indicating that $\dot{V}O_{2muscle}$ BMR^{-1} alone, can explain approximately 78% of the variation in average maximum dive duration (**Fig 5.5**). The regression between average maximum dive duration and body mass on a double logarithmic plot, **Fig. 5.1** had an r^2 value of 0.49 when considering diving species from multiple phylogenetic orders. This suggests

that the time for which $\dot{V}O_{2muscle}$ can fuel BMR is a better predictor of maximum dive duration in a diving mammal than body mass alone.

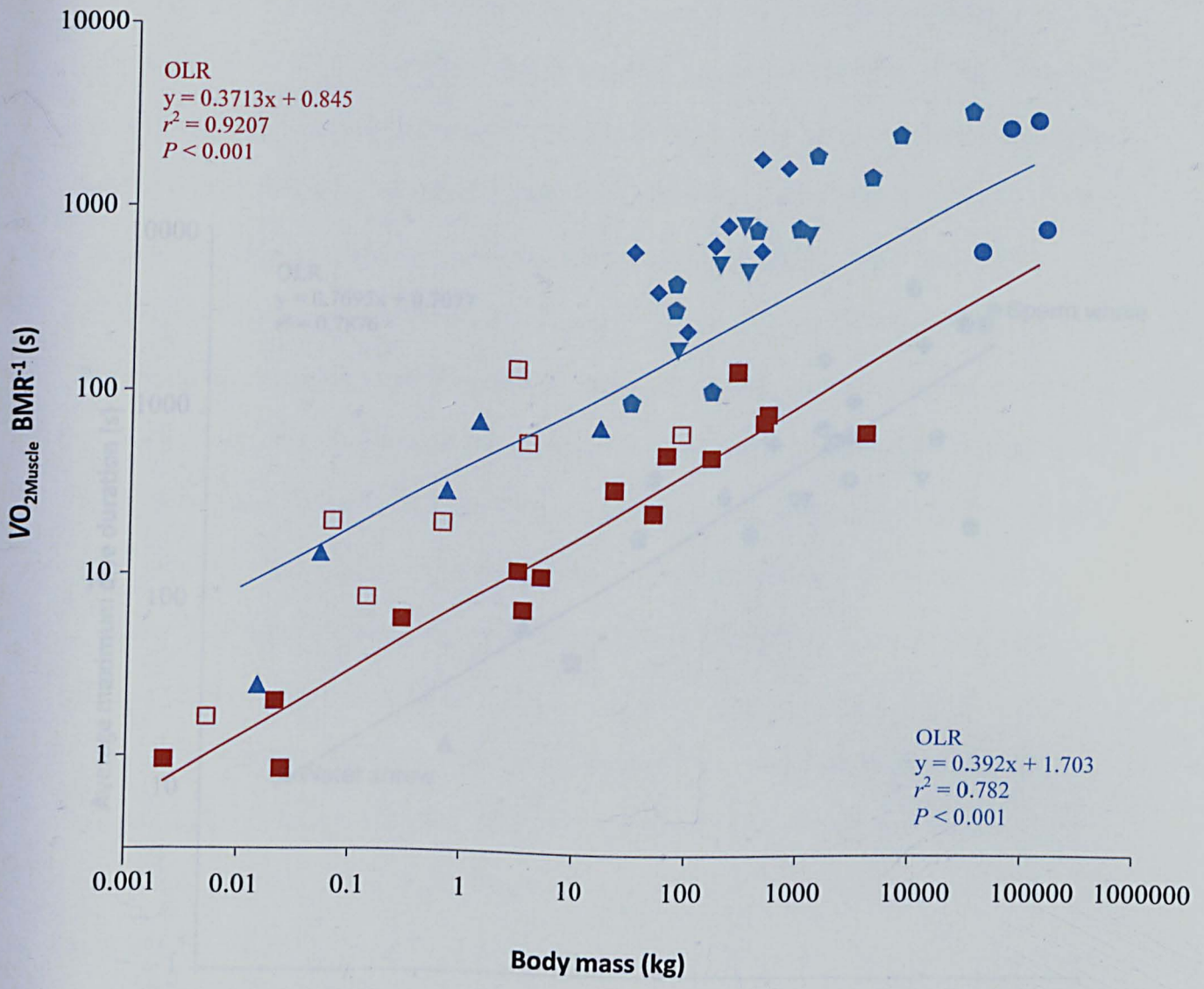


Figure 5.4 Duration $VO_{2Muscle}$ can sustain BMR as a function of body mass on a double logarithmic plot. A significant positive relationship is found with ordinary least squares regression (OLR) (solid red line) for terrestrial and diving mammals (solid red and blue lines respectively). Data for diving mammal muscle O_2 store and consumption is overlaid to allow comparison with terrestrial species. Red filled squares = terrestrial species, Red open squares = altitude or burrowing species, blue triangles = semi-aquatic species, blue diamonds = phocid seals, blue inverted triangles = otariid seals, blue circles = baleen whales, blue pentagons = toothed whales.

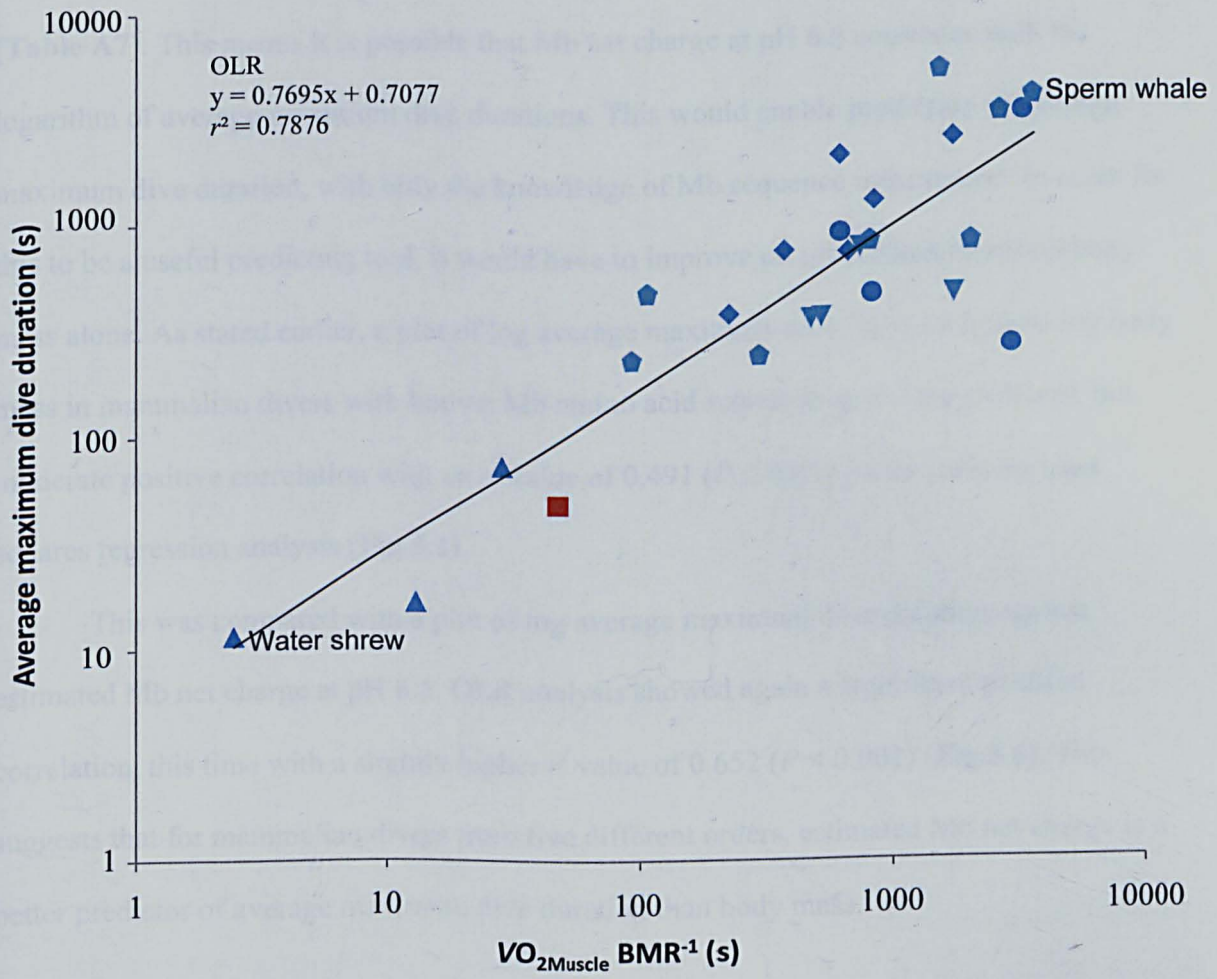


Figure 5.5 Double logarithmic plot of average maximum dive duration against the duration $VO_{2Muscle}$ can sustain BMR. A significant, but moderate positive correlation is observed for all diving mammals where both dive duration and [Mb] has been measured ($P < 0.05$). Red squares = human, blue triangles = semi-aquatic species, blue diamonds = phocid seals, blue inverted triangles = otariid seals, blue circles = baleen whales, blue pentagons = toothed whales)

Chapter 3 identified that log [Mb_{max}] was significantly correlated with estimations of Mb net charge at physiological pH (**Fig 3.8**). $\dot{V}O_{2muscle}$ BMR^{-1} strongly correlates with average maximum dive durations (**Fig 5.5**) and $\dot{V}O_{2muscle}$ is influenced by [Mb_{max}] more so than BMR, as indicated by increased [Mb_{max}] in diving species compared to terrestrial species and by a BMR that is higher than expected from allometry in divers but not terrestrial species (**Table A7**). This means it is possible that Mb net charge at pH 6.5 correlates with the logarithm of average maximum dive durations. This would enable prediction of average maximum dive duration, with only the knowledge of Mb sequence information. In order for this to be a useful predicting tool, it would have to improve on predictions based on body mass alone. As stated earlier, a plot of log average maximum dive duration against log body mass in mammalian divers with known Mb amino acid sequence shows a significant, but moderate positive correlation with an r^2 value of 0.491 ($P < 0.001$) with ordinary least squares regression analysis (**Fig 5.1**).

This was compared with a plot of log average maximum dive duration against estimated Mb net charge at pH 6.5. OLR analysis showed again a significant positive correlation, this time with a slightly higher r^2 value of 0.652 ($P < 0.001$) (**Fig 5.6**). This suggests that for mammalian divers from five different orders, estimated Mb net charge is a better predictor of average maximum dive duration than body mass.

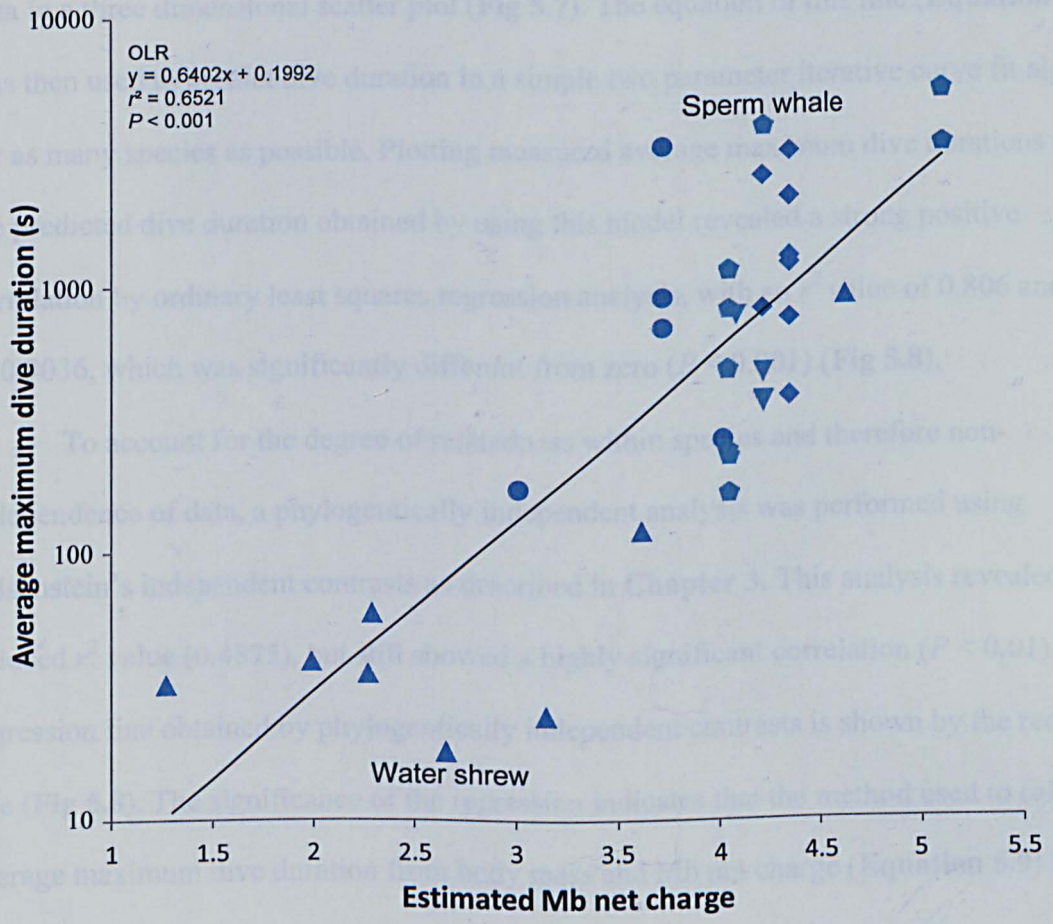


Figure 5.6 Average maximum dive duration against estimated Mb net charge at pH 6.5 on a semi-logarithmic plot, for thirty four species of diving mammals from five different orders. Blue triangles = semi-aquatic species, blue diamonds = phocid seals, blue inverted triangles = otariid seals, blue circles = baleen whales, blue pentagons = toothed whales

To assess if an improvement in correlation with dive durations could be made, estimated Mb net charge was combined with body mass and average maximum dive duration data in a three dimensional scatter plot (**Fig 5.7**). The equation of this line (**Equation 5.9**) was then used to predict dive duration in a simple two parameter iterative curve fit algorithm for as many species as possible. Plotting measured average maximum dive durations against the predicted dive duration obtained by using this model revealed a strong positive correlation by ordinary least squares regression analysis, with an r^2 value of 0.806 and a slope of 0.9036, which was significantly different from zero ($P < 0.001$) (**Fig 5.8**).

To account for the degree of relatedness within species and therefore non-independence of data, a phylogentically independent analysis was performed using Felsenstein's independent contrasts as described in **Chapter 3**. This analysis revealed a reduced r^2 value (0.4875), but still showed a highly significant correlation ($P < 0.01$). The regression line obtained by phylogentically independent contrasts is shown by the red dotted line (**Fig 5.8**). The significance of the regression indicates that the method used to calculate average maximum dive duration from body mass and Mb net charge (**Equation 5.9**) has good predictive power.

As a proof of concept, the average maximum dive duration was calculated for a 31,000 kg humpback whale, *Megaptera novaeangliae*, which has a Mb net charge at pH 6.5 of 3.72 (**Table A2**). Average maximum dive duration was calculated as 922 s (15.37 min), which is within 4% of the measured average maximum dive duration of 884 s (14.73 min; Goldbogen et al., 2008). The formula can also be used to evaluate the relative influence of body mass and Mb net charge (or log [Mb_{max}]) on maximum dive duration. Repeating the calculation for the humpback whale, but increasing body mass by 10%, whilst maintaining the Mb net charge, leads to a 16 s (1.7%) increase in maximum dive duration. Similarly a 10% increase in Mb net charge from 3.72 to 4.09, whilst maintaining body mass of 31,000

kg, leads to an estimated dive duration of 1523 s, showing an increase of 601 s (10 min) or a 65% increase in maximum dive duration. However, because Mb net charge correlates with the logarithm of [Mb_{max}] (**Chapter 3**), it is better to recalculate the increase in dive duration due to a 10% increase in [Mb]. Using the same 31,000 kg humpback whale; a Mb net charge of 3.72 (**Table A1**), using the regression in **Figure 3.5**, gives a [Mb_{max}] of 2.95 g 100 g^{-1} a 10% increase gives a concentration of 3.24 g $100g^{-1}$, which equates to a Mb net charge of 3.90. Using **Equation 5.9** this new charge gives an average maximum dive duration of 1176 s (20 min). Therefore for a 10% increase in [Mb] a 28% increase in maximum dive duration is observed, compared to the 1.7% increase due to a 10% increase in body mass. Thus increases in [Mb] influence dive duration to a greater degree than similar changes in body mass.

Taking this further, the Mb net charge reconstruction for the extinct species suggests an estimated Mb net charge at pH 6.5 ranging between 1.12 and 3.76 for both *Indohyus* and *Pakicetus* species and other basal whales. Using the body mass estimates from **Table 5.1**, the simple model presented in this study, predicts a range of maximum dive durations for the artiodactylan *Indohyus* between 6.4 - 223 s, and a range of maximum dive durations between 8.8 – 309 s for the larger *Pakicetus* species, *P. attocki*.

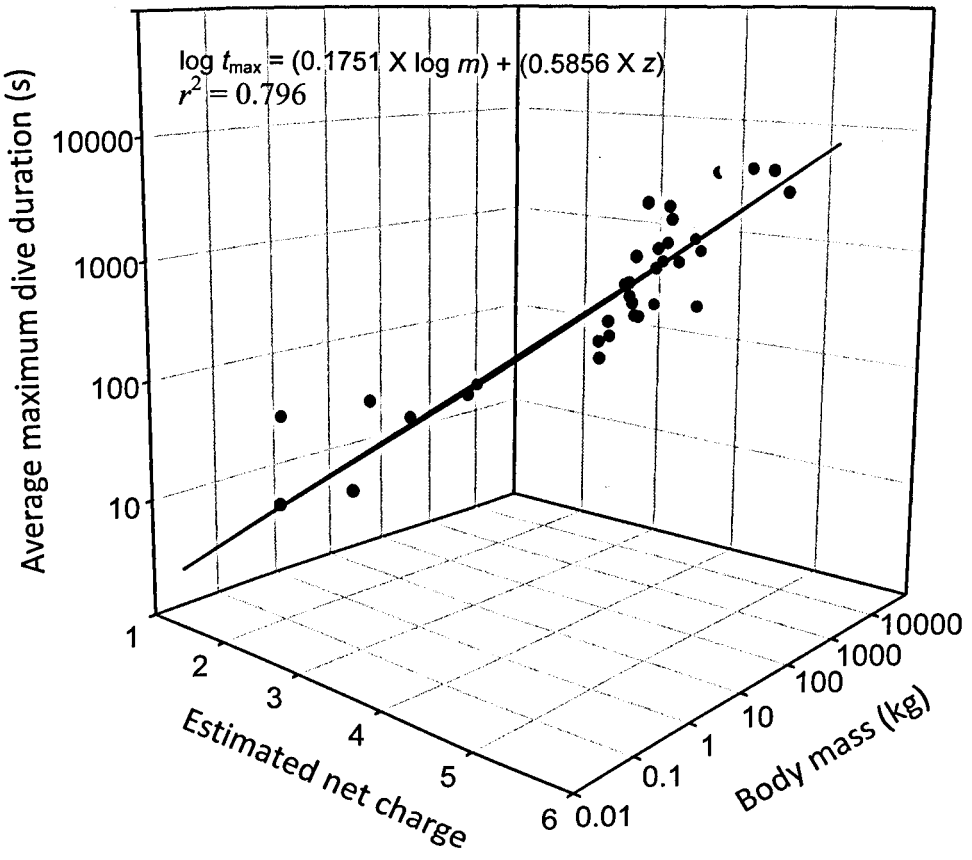


Figure 5.7 Three dimensional scatterplot showing the relationship between estimated net charge at pH 6.5, body mass and measured average maximum dive duration. Plotted is the equation that best fits through all data points, log t_{max} = (0.1751 X log m) + (0.5856 X z), where log t_{max} is average maximum dive duration (s), m is body mass (kg) and z is estimated net charge at pH 6.5.

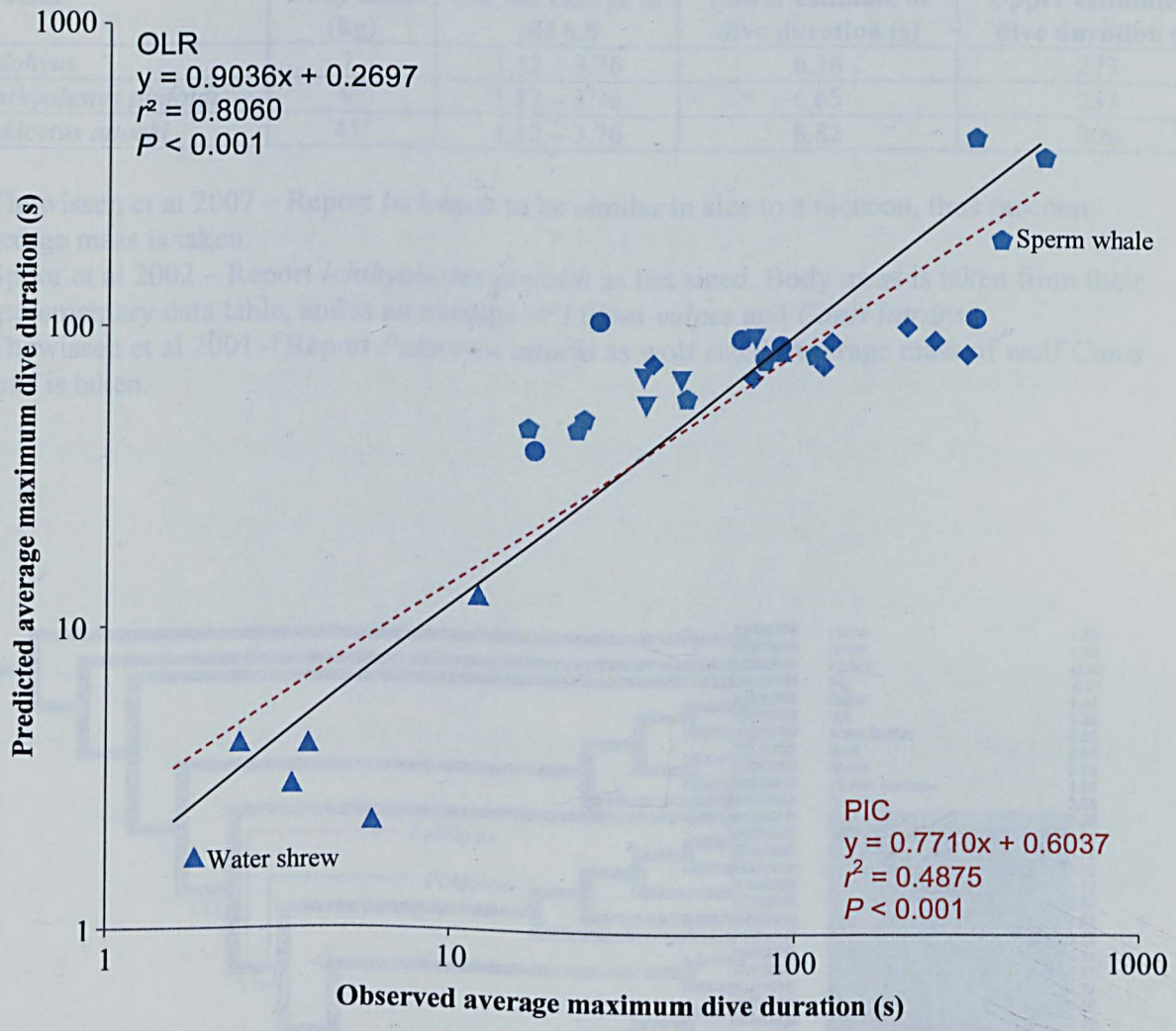


Figure 5.8 A significant and strong positive correlation between measured and calculated average maximum dive duration on a double logarithmic plot for thirty four species of diving mammal across five orders. Blue triangles = semi-aquatic species, blue diamonds = phocid seals, blue inverted triangles = otariid seals, blue circles = baleen whales, blue pentagons = toothed whales. Using ordinary least squares regression analysis (solid black line) and phylogenetically independent contrast regression analysis (dotted red line).

Table 5.1 Dive duration estimates for two extinct basal whale species and their closest artiodactylan relative, based on estimates of body mass from the literature and Mb net charge at pH 6.5 from phylogenetic reconstruction. Equation 5.9 was used to determine both lower and upper estimates of dive duration.

Species	Body mass (kg)	Mb net charge at pH 6.5	Lower estimate of dive duration (s)	Upper estimate of dive duration (s)
Indohyus	7[1]	1.12 – 3.76	6.36	223
Ichthyolestes pinfoldi	9[2]	1.12 – 3.76	6.65	233
Pakicetus attocki	45[3]	1.12 – 3.76	8.82	309

[1] Thewissen et al 2007 – Report *Indohyus* to be similar in size to a raccoon, thus raccoon average mass is taken.
[2] Spoor et al 2002 – Report *Ichthyolestes pinfoldi* as fox sized. Body mass is taken from their supplementary data table, and is an average of *Vulpes vulpes* and *Canis latrans*.
[3] Thewissen et al 2001 – Report *Pakicetus attocki* as wolf sized. Average mass of wolf *Canis lupus* is taken.

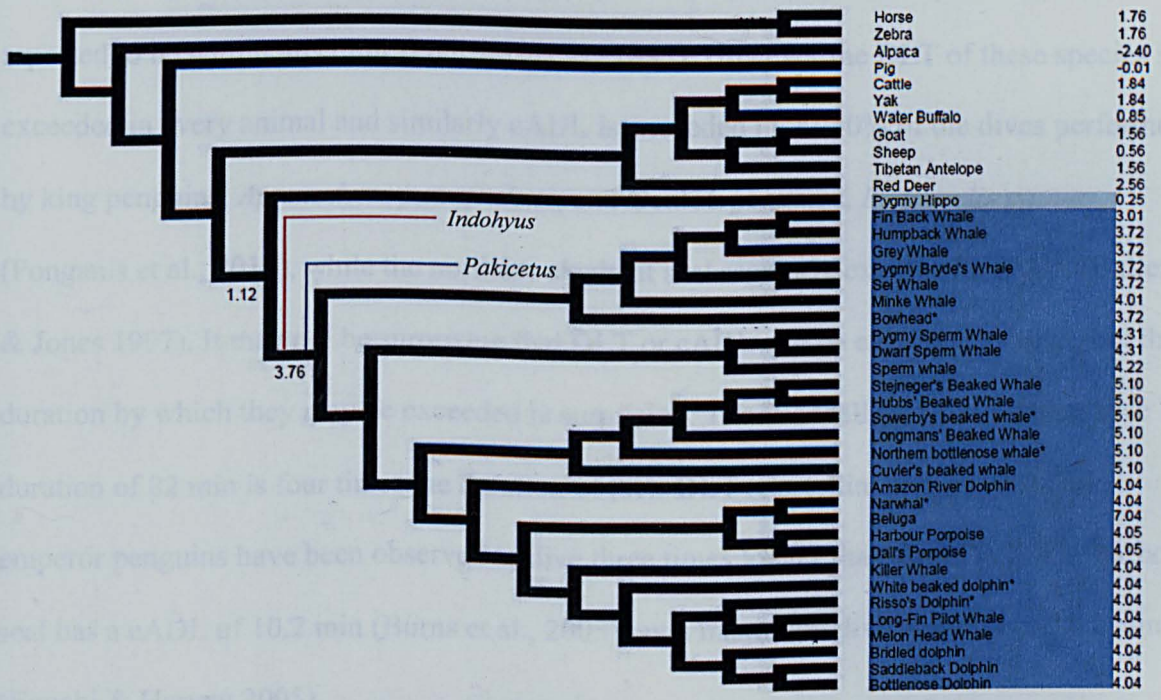


Figure 5.9 Reconstruction of Mb net charge evolution in Cetartiodactyla. Diving species are highlighted in blue. Mb net charge (at pH 6.5) is higher in diving mammals compared to terrestrial mammals. Red and blue branches indicate the position where *Indohyus* and *Pakicetus* species have evolved. The numbers correspond to the Mb net charge for those nodes, according to amino acid substitutions reconstructed by maximum parsimony.

Discussion

This study has developed a new method for estimating diving capacity that is valid across several orders of aquatic and semi-aquatic mammals ranging from the smallest to the largest known divers. Before a detailed discussion of the new method, limitations of earlier methods for estimating mammalian diving capacity are discussed.

Limitations of DLT/cADL for estimating diving performance

Diving lactate threshold (DLT) and calculated aerobic dive limits (cADL) inform us about the aerobic capacity of a species. They provide a good measure of the normal dive habits of an animal. For example, 94% of dives carried out by the Weddell seal are within the 20 min DLT (Kooyman et al., 1980). This has also been noted in diving birds with the emperor penguin reaching its DLT after 5.6 min of diving and almost 80% of dives are reported to be within this limit (Ponganis et al., 1997). However the DLT of these species is exceeded in every animal and similarly cADL is exceeded in 20-40% of the dives performed by king penguins, *Aptenodytes patagonicus*, and Gentoo penguins, *Pygoscelis papua* (Ponganis et al., 2010), while the northern elephant seal regularly exceeds its cADL (Butler & Jones 1997). It may not be surprising that DLT or cADL can be exceeded as such, but the duration by which they may be exceeded is surprising. The Weddell seal's maximum dive duration of 82 min is four times the duration of their DLT (Castellini et al., 1992) and emperor penguins have been observed to dive three times longer than their DLT. The harbour seal has a cADL of 10.2 min (Burns et al., 2005) but a maximum dive duration of 35.2 min (Eguchi & Harvey 2005).

Recent reviews suggest that previous cADL measurements are inaccurate due to incorrect estimates of total body O_2 stores, with data for the emperor penguin showing that usable O_2 in the lung store has been under estimated (Ponganis et al., 2010); and more

importantly, values that define diving metabolic rate (DMR) are not known in most cases. Therefore use of other metabolism estimates such as field metabolic rate (FMR) or various multiples of resting metabolic rate (RMR) e.g. 2 xRMR or 3 x RMR, are problematic (Butler 2006; Ponganis et al., 2010), Ponganis et al. (2010) show that a cADL of 23 min is possible for the emperor penguin if a DMR of approximately half RMR is assumed. The reviews of Butler (2006) and Ponganis et al. (2010) highlight that dives exceeding cADL are most likely the result of physiological responses that reduce metabolism and maximise aerobic capacity beyond what can be modelled. Such responses include the onset and the extent of bradycardia (Ponganis et al., 2010) and localised core hypothermia that ensue resulting from reduced blood flow (Caputa et al., 1998; Odden et al., 1999). The behavioural reduction in metabolic cost by using sinking and gliding locomotion during a dive is another method of reducing metabolism (Williams et al., 2000; Williams et al., 2008). In addition, blood and lung O_2 stores have been shown to still carry O_2 at the end of a dive, and it has therefore been implicated that depleted muscle oxygen stores alone are the likely explanation for observed increases in post dive plasma lactate concentrations (Ponganis et al., 2010).

Limitations of using allometry to predict diving performance

The only other method to predict dive time relies on allometry based equations. While these are capable of estimating maximum dive durations, they are reliant upon calculations based on a multiple of body mass to an allometric exponent. While body mass has been shown to correlate with dive time (Noren & Williams, 2000), there have been numerous studies that give different allometric exponents for maximum dive duration in diving mammals (Boyd & Croxall, 1996; Schreer & Kovacs, 1997; Hochachka & Mottishaw, 1998 and Noren & Williams, 2000). Even using a small number of species from the same

phylogenetic group reveals vastly different allometric exponents (Boyd & Croxall, 1996; Schreer & Kovacs, 1997).

Many studies failed to account for species relatedness in their studies, see Halsey et al. (2006) for discussion. This may mean that their findings were highly significant through the inclusion of data that should have not been so heavily weighted, because data from very closely related species are more likely to be similar because of the inheritance of characteristics that may influence dive duration within these species from a recent common ancestor. When phylogenetic relatedness has been accounted for in the allometry of maximum dive duration in mammals; an allometric exponent of 0.326 is found which is again different to previous work, suggesting an alternate scaling relationship (Halsey et al., 2006).

One issue that has been raised with allometric relationships that do include phylogenetic analyses comes from allometric estimates of BMR. It has been found that the accuracy of the equations is improved when a separate exponent is used for mammalian orders with smaller body sizes such as shrews within the order Soricidae as they may never be inactive or postabsorptive; the latter argument has also been made for ruminants in the order Cetartiodactyla (White et al., 2009). This may equally occur in groups of diving mammals, which cover an even larger range of body mass than terrestrial mammals, from a 14 g water shrew to a 100 ton blue whale (**Table A7**), compared to a range of body mass for terrestrial species from a 2 g Etruscan shrew to a 3.6 ton African elephant (**Table A5**).

It is for this reason that this study has set out to find an alternative method to estimate maximum dive duration.

Identifying the best predictor of diving performance

There is no indication of an increase in $\dot{V}O_{2lung}$ in diving mammals that would increase the duration that $\dot{V}O_{2Lung}$ can sustain BMR relative to terrestrial mammals of similar body mass (**Fig 5.2**). In fact evidence shown here indicates that $\dot{V}O_{2Lung}$ BMR^{-1} is actually significantly reduced in divers compared to terrestrial mammals (**Fig 5.2**). This is to be expected considering some of the larger diving mammals, sperm whale and other deep diving species, reduce V_{lung} in order to minimise the risk of embolism injuries (Berta et al., 2006). Although cetaceans tend to dive after inhalation, their lungs are capable of complete collapse which aids in the prevention of decompression sickness and nitrogen narcosis. Phocid seals will have been shown to exhale before diving and therefore lung oxygen stores play a little role in supplying O_2 during a dive in these species (Falke et al., 1985; Schmidt-Nielsen, 1991).

The duration that $\dot{V}O_{2Blood}$ can sustain BMR is statistically identical in terrestrial and diving mammals of equal body mass (**Fig 5.3**). There are three exceptions to this where species can sustain BMR above terrestrial levels and these occur in the echidna, Weddell seal and the minke whale. The echidna is a burrowing species and therefore may be subjected to periods of anoxia, similar to divers, therefore may have similar adaptations. The echidna has a low measured BMR which is almost equal to an animal half its body mass; it also hase a higher V_{Blood} than other species of the same size (McNab, 1984). A reduced BMR has also been reported by Dhinsda et al. (1971), who also noted a reduced body temperature and a high blood O_2 affinity in this species. Taken together, this can explain the increased duration that $\dot{V}O_{2Blood}$ can sustain BMR relative to other terrestrial species of a similar size. Interestingly, there is also evidence that the echidna had an aquatic ancestry (Phillips et al., 2009), and therefore high V_{Blood} and or high Hb concentrations may be a residual characteristic remaining from an aquatic way of life.

In the Weddell seal VO_{2Blood} can also sustain BMR for a greater duration than in other species of a similar size. This is clearly explained by the high V_{Blood} measured in this species (**Table A6**), which may be an adaptation to compensate for a reduction in V_{lung} and exhalation upon initiation of a dive and thereby reduced lung oxygen stores.

For the minke whale, an explanation is harder to ascertain. The measured Hb concentration and V_{Blood} do not appear to be different to any other animal of similar body mass, this is not surprising because V_{Blood} was calculated from allometry. This suggests that departure away from the terrestrial regression line (**Fig 5.3**) must be, reduced BMR. However BMR for this species had to be obtained by allometry (White et al., 2009). Previous research has shown that whales have a reduced body temperature compared to terrestrial mammals of approximately 35°C (Sharp & Marsh, 1953) and therefore use of allometry to calculate BMR based on terrestrial mammals with a body temperature of 37°C would in fact overestimated whale BMR. Similarly a large percentage of a whales mass is presumably poorly perfused blubber (Sharp & Marsh, 1953), which would increase mass but not V_{Blood}, then in fact calculations of V_{Blood} based on allometry for a terrestrial body mass would also be an over estimate. Taking this in to account, it seems likely that although both BMR and V_{Blood} are over estimated by allometry, but V_{Blood} has been over estimated by a larger factor.

Of all the three main O_2 reservoirs, only muscle O_2 stores show a clear increase from terrestrial levels to those observed in diving mammals of similar body mass. They last significantly longer, when consumed at BMR, in all diving species (**Fig 5.4**). All diving species show an elevated concentration of Mb compared to non-burrowing, non-high-altitude mammals (**Table A7**). This may well be coupled with a reduction in BMR which can account for the differences observed among different lineages of divers. The semi-aquatic star-nosed mole, *Condylura cristata*, has a BMR one third that of the terrestrial guinea pig, *Cavia porcellus*, an animal of similar body mass (**Table A7**). The reason behind consistently

elevated concentrations of Mb in diving mammals is linked to the occurrence and severity of the bradycardia that is part of the dive response. During the initial phase of a dive, blood flow is re-directed away from the non-essential muscle tissues, due to localised vaso-constriction (Scholander, 1940). Although most species have some behavioural adaptation to reduce the cost of locomotion (Williams et al., 2000), ischemia during a dive means that locomotion is powered by muscles that use stored O_2 only.

By measuring the O_2 desaturation of muscle tissue during a dive, Williams et al. (2011) have recently proven that skeletal muscles switch to anaerobic metabolism when O_2 stores are depleted They found two approaches to diving taken by emperor penguins. During type A dives Mb O_2 is de-saturated at a constant rate until the DLT of 5.6 min is reached, then the rest of the dive relies heavily on anaerobic metabolism in muscle tissue. In contrast, during type B dives Mb is only half de-saturated at the 5.6 min DLT. The penguins used in the study demonstrated an alteration in stroke rate during type B dives, therefore a behavioural adjustment in swimming action is also responsible for reducing metabolism (Williams et al., 2000; Williams et al., 2007). In contrast to type A dives, Williams et al., (2011) also suggest that muscle beds are not completely isolated during type B dives. Thus, allowing mild blood flow, and hence muscle O_2 is not fully consumed until later in the dive, which is similar to what as has been shown in elephant seals during sleep apnoea (Ponganis et al., 2008).

Since it has been recently shown that blood and lung stores do not fully de-saturate during emperor penguin dives (Ponganis et al., 2010), and since underwater movement is powered by the skeletal muscles which have to rely on the O_2 store within the tissues, then muscle O_2 stores appear to be the best indicator of maximum dive duration. Estimates in this study of the BMR specific muscle O_2 store ($\dot{V}O_{2muscle}BMR^{-1}$) in diving mammals show a strong positive correlation with maximum dive duration (**Fig 5.4**) and on their own can

explain nearly 80% of the variation in maximum dive duration across all investigated diving mammals.

One of the main criticisms of using the above method would be that during a dive metabolic rate would not be at a basal rate. In calculating dive time researchers have used FMR, DMR and other variations of RMR that were often obtained from forced submersion studies, and have been criticised as inaccurate (Butler, 2004; Ponganis et al., 2010). However, recently Williams et al. (2011) have produced the only measurements of muscle O_2 consumption during a dive of emperor penguins. Combining their work with measurements of blood and lung O_2 store depletion, they find that during a dive total body O_2 metabolism is close to the resting metabolic rate of emperor penguins as measured when they are floating in a flume. It could be criticised that to use this formula for every species is not practical as it has been reported that some divers may never meet the criterion of BMR, as they are never post-absorptive, or they are never completely at rest, such as the water shrew and some cetaceans as never rest (White et al., 2009).

As BMR is not available for many species and technically never reached in others, this study tried to simplify the method for estimating average maximum dive duration. Mb-bound O_2 is the major oxygen reservoir of muscle tissue and [Mb_{max}] has been found to correlate with maximum dive durations in toothed whales, but not in baleen whales, (Noren & Williams, 2000). Baleen whales have lower [Mb] compared to other cetaceans (**Table A7**) with reasonably high dive durations. Noren & Williams (2000) have suggested that the lack of correlation in baleen whales is due to a small sample. However, baleen whales may employ other methods of extending dive duration, such as more energy efficient locomotion or increase in body mass. Thus, Noren & Williams (2000) found that maximum dive durations for mysticete whales correlated better with body mass than with muscle [Mb] implying that size is responsible for increasing dive duration, presumably due to lower mass

specific O_2 consumption (Butler & Jones, 1981). The present study shows that body mass of diving mammals from five orders is weakly positively correlated with average maximum dive time (**Fig 5.5**) and so body mass correlations may only be strong across several orders of diving mammals.

This study looked to improve the correlation between [Mb_{max}] and maximum dive time, by increasing the data set. **Chapter 3** has demonstrated that the logarithm of [Mb_{max}] correlates strongly with Mb net charge (**Fig 3.5**). Since Mb sequence data is available for many more species than [Mb_{max}] data, it is plausible to use Mb net charge at pH 6.5 as a proxy for [Mb_{max}]. Regression analysis between Mb net charge at pH 6.5 and the logarithm of maximum dive duration gives a significant, strong positive correlation (**Fig 5.6**) that can account for 65% of the variation of maximum dive duration in five different lineages of mammalian divers. This shows that Mb net charge at physiological pH is a good estimator of maximum dive duration.

In a previous study that was restricted to cetaceans, multiple linear regression techniques have been applied to show that combining [Mb_{max}] with body mass produced better correlations with maximum dive time, than either did alone (Noren & Williams, 2000). Working on a method to combine Mb net charge, body mass and maximum dive duration, **Figure 5.7** led to the development of **Equation 5.9,** which can be used to directly predict maximum dive duration based solely on two parameters. Maximum dive durations estimated with this method significantly and very strongly correlate with measured maximum dive durations (**Fig 5.8**): Using ordinary least squares regression analysis yields an r^2 value of 0.81 and a slope of 0.90, which is significantly different from zero ($P < 0.001$). When accounting for the relatedness of species, using PIC analysis; the regression still gives a significant correlation between calculated and measured values for average maximum dive duration, however, with a slightly reduced slope and r^2 value of 0.77

and 0.49, respectively ($P < 0.01$) (**Fig 5.8**). These results show that **Equation 5.9** may be the best tool yet to estimate maximum dive durations, although it requires only two parameters, body mass and Mb protein sequence. The first is readily available for most species, and knowledge of the second has been significantly increased with the present study.

Equation 5.9 was tested by predicting the maximum dive time for a 31,000 kg humpback whale, a species whose measured maximum dive time became known to the author only after the construction of the formula. The calculated estimate of average maximum dive duration is within 4% of the measured value for this species. The formula has also been used to evaluate the relative influence of body mass and Mb net charge (or log [Mb_{max}]) on maximum dive duration. Using the humpback whale as an example a 10% increase in body mass, whilst maintain [Mb] lead to a 1.7% increase in maximum dive duration. Similarly a 10% increase in [Mb], whilst maintaining body mass, lead to a 28% increase in maximum dive duration. This shows that increases in [Mb] influence dive duration to a greater degree than similar changes in body mass. Previous research has disagreed with this suggesting that body mass has a greater effect on dive duration (Butler & Jones, 1981; Noren & Williams, 2000; Williams et al., 2001), this may be due to problems in obtaining accurate [Mb_{max}]. When using the standard spectrophotometric method of determining [Mb] (Reynafarje, 1963) there are other light absorbing components that can never be fully removed such as, Hb, mitochondria and other cell organelles, which can influence the concentration determination of Mb, especially if baseline fluctuations are not accounted for (Masuda et al., 2008). Furthermore, Mb leaches out of the muscle cells upon death (Miura et al., 2011) and so if tissue samples are not fresh i.e. a result of stranding events then [Mb] determination may lead to values that are lower than the actual in vivo concentration.

Chapter 4 of this thesis demonstrated how Mb net charge at pH 6.5 can be reconstructed using a mammalian phylogeny (**Fig 4.3**). Using **Equation 5.9**, the combination of Mb net charge with body mass data would allow the prediction of maximum dive duration of the early ancestors of any diving mammal. This study has looked at the dive durations for the early ancestors of cetaceans and their closest non-cetacean relative, the pakicetids *I. pinifoldi* and *P. attocki* and *Indohyus*, respectively, (**Fig. 5.9**). Fossil evidence suggests that *Indohyus* had a body mass of approximately 7 kg (Thewissen et al., 2007). Mb net charge reconstruction (**Fig 5.9**), gives a range of estimated dive durations between 6 – 223 s (**Tab. 5.1**). This supports fossil evidence that suggests that these species were aquatic waders and not divers (Thewissen et al., 2007), and that they may have used water as a means to evade predators in much the same way that the modern pygmy hippo and mouse deer do today (Meijaard et al., 2010). *Packicetus* species also have an estimated Mb net charge ranging between 1.12 and 3.76 (**Fig 5.9**). Fossil evidence suggests body mass for the smaller *I. pinfoldi* of 9 kg (Spoor et al., 2002) and 45 kg for the larger *P. attocki* (Thewissen et al., 2001; **Tab. 5.1**). Therefore these species show a trend of increasing body mass that is also observed in many species that evolved diving, with a six fold increase in body mass being observed from *Indohyus* to *P. attocki* (**Table 5.1**). **Table 5.1** shows that if Mb net charge remains the same then the six fold increase in body mass leads to a 1.4 fold increase in maximum dive duration. However, if the cetacean ancestors increased Mb net charge early on during their evolution, then the three fold increase in Mb net charge would lead to a 36 fold increase in dive duration in *I. pinfoldi* and a 49 fold increase in dive duration in *P. attocki.* This indicates that as diving evolved Mb had a bigger impact on increasing dive duration than increases in body mass. In fact, time-calibrated molecular phylogenetic analysis indicates that body size niches were attributed early in the evolution of cetaceans and furthermore that

shifts in cetacean body size coincide with changes in foraging strategies and food sources (Slater et al., 2010).

Conclusion

Previous estimates of dive duration were mainly based on calculated aerobic dive limits of a species and have therefore ignored those rarer occasions where some of the most remarkable dives occur. Estimates of maximum dive duration based on body mass and allometry leave a large part of the observed variation unexplained and result in a need to calculate dive durations for major orders of mammals separately. This study presents a simple model based on only two parameters that allows estimation of average maximum dive duration over the full size range of diving mammals from water shrews to whales. The model can be used along with phylogenetic reconstruction to estimate dive durations in the ancestors of diving species, which may aid our understanding of the evolution of diving behaviour. This study shows that molecular characteristics of Mb can be used to help explain the physiology and dive behaviour of mammals, and suggests that during the evolution of diving behaviour increases in Mb concentration offer greater increases in dive duration than increases in body mass.

Chapter 6 – Conclusion

Diving behaviour has evolved independently at least ten times in mammals. Each time the problems associated with an aquatic life for an air breathing mammal have been over come through a series of adaptations, with the degree of adaptation reflecting the amount of time a species spends immersed in water (Berta et al., 2006). These adaptations include the reduction of heat loss by alterations to the skin (Tarasoff, 1974; Caldwell & Cadwell, 1985), changes to limbs to aid movement in water (Bert et al., 2006), alterations to the sensory organs to aid vision and hearing under water (Sivak, 1980; Pardue et al., 1993; Pilleri & Wanderler, 1964; Berta et al., 2006).

Most dives undertaken by mammals are aerobic in nature (Kooyman et al., 1981; Ponganis et al., 1997). Aerobic metabolism is maintained during a dive through the dive response, which incorporates bradycardia and peripheral vaso-constriction (Scholander, 1940) and is observed in both diving mammals and birds. Bradycardia is a reduction in heart rate that frequently occurs during the beginning of a dive. Reductions in heart rate of approximately 70% have been noted in the Baikal seal (Ponganis et al., 1997) with larger decreases seen in other seals and sea lions (Odden et al., 1999; Thornton & Hochachka, 2004).

A reduction in heart rate is often coupled with peripheral vaso-constriction, which is the tightening of arterial and venous wall to restrict the flow of blood to certain areas of the body including; extremities, non-essential organs and skeletal muscle tissues including the locomotory muscles (Kooyman & Ponganis, 1989). Whilst this mechanism preserves essential oxygen (O_2) for heart and brain function, it also means some parts of the body such as the locomotory muscles become isolated and have to rely on O_2 stored within the tissues to power aerobic metabolism during a dive (Butler and Jones, 1997). The protein responsible for storing molecular O_2 is myoglobin (Mb), a small 17 kDa monomeric globular haemoprotein

consisting of 153 amino acids, that can bind one O_2 molecule (Kendrew, 1962). The primary function of Mb is the reversible binding of O_2 and the facilitated diffusion of O_2 across the sarcoplasm to the mitochondria (Wittenberg & Wittenberg, 2003) in skeletal and cardiac tissue. It is the classic O_2 storage ability of Mb, which makes it one of the most important proteins in diving mammals (Dolar et al., 1999). The content of Mb ([Mb]) in mammalian divers is far greater than that found in terrestrial mammals (**Table A3**), with divers exhibiting concentrations up to thirty times those seen in non-diving species (Castellini & Somero, 1981; Noren and Williams, 2000). These large increases in [Mb] in diving species provide enough O_2 to maintain aerobic metabolism in most dives undertaken by mammals (Kooyman et al., 1981; Ponganis et al., 1997).

Recent work on diving birds has shown that O_2 stores in the blood and the lungs never fully de-saturate during a dive in the emperor penguin, *Aptenodytes forsteri*, (Ponganis et al., 2010). If this is also true for mammalian divers, then during the small percentage of dives that exceed the diving lactate threshold (DLT) the exercising muscles are responsible for producing the lactic acid observed post-dive (Kooyman et al., 1981; Ponganis et al., 1997). Even though only a small percentage of dives exceed the aerobic limits of an animal, what is truly remarkable is the duration which anaerobic metabolism can be sustained. The DLT of the Weddell seal, *Leptonychotes weddellii*, is 20 min (Kooyman et al., 1981) yet its maximum dive duration is 82 min (Castellini et al., 1992). Thus the Weddell seal has an anaerobic capacity four times greater than its aerobic limit. During these anaerobic dives, a mammalian diver must be capable of buffering acidic end products in order to maintain normal muscular function (Surenkok et al., 2006). Proton (H^+) buffering is achieved by two mechanisms bicarbonate and non-bicarbonate buffers, the magnitude of the former depends on the tissue bicarbonate concentration, which is similar in all mammals (Fernandez et al., 1989). Non-

bicarbonate buffering is achieved by free histidine (His), His residues in proteins and His-related dipeptides (Abe, 2000).

This study has determined 24 novel mammalian Mb amino acid sequences, five of which; short-tailed shrew, star-nosed mole, coast mole, American mink and grey squirrel are deemed synthetic constructs due to the first twenty eight nucleotides being influenced by the primer involved in PCR. However due to the conserved nature of the corresponding first eight amino acids, this is not believed to influence the results in this study. Analysis of mammalian Mb amino acid sequences show a general trend towards increasing the Mb His content to a high level (10 or more His) in 7 out of 11 lineages of diving species. This suggests an increased potential towards proton buffering in the Mb of mammalian divers. Acid base titration curves of purified Mbs from 10 mammalian species, together with their primary sequence information and known p*K*a values for ionisable groups in Mb, have been used to develop a model that can accurately predict the specific Mb buffer value (β_{Mb}) of any Mb from its primary sequence. Analysing the evolution of β_{Mb}, revealed that high β_{Mb} (values over 3.2 mol H^+ mol Mb^{-1} pH^{-1}) did not significantly evolve more frequently in lineages of diving mammals. This is due to high β_{Mb} in some terrestrial species. High β_{Mb} generally occurs in terrestrial animals that are capable of burst locomotion, such as the rabbit, *Oryctolagus cuniculus*, red kangaroo, *Macropus rufus*, Horse, *Equus caballus*, Zebra, *Equus burchellii*, and all of the canine species observed in this study. High β_{Mb} values are also noted in ruminant artiodactyls, which may be due to an ancestral aquatic escape strategy (Meijaard et al., 2009), which the species observed here have subsequently lost, but is still retained in more basal species such as the water chevrotain, *Hyemoschus aquaticus* and greater mouse deer, *Tragulus napu* (Meijaard et al., 2009).

Combining β_{Mb} values with data on muscle [Mb] has allowed for the first time, quantification of the contribution of Mb towards whole muscle non-bicarbonate buffering

capacity ($\beta_{muscleNB}$) in diving and terrestrial mammals. This study found that the contribution of Mb to $\beta_{muscleNB}$ ($\beta_{muscleMb}$) was significantly higher in divers compared to terrestrial species (**Fig 2.6**) due to the large increase in [Mb] observed in divers (**Table A3**). This study was also able to compare $\beta_{muscleMb}$ with the contribution from specific His-related dipeptides that have previously been shown to be elevated in divers (Crush et al., 1970; Abe, 2000). Data here suggests, contrary to previous findings (Castellini & Somero, 1981), that the contribution to $\beta_{muscleNB}$ from Mb in some species can be a substantial proportion of the increased muscle non-bicarbonate buffer value generally seen in diving species. The increase in β_{Mb} due to three His residues in phocid seals, together with the increase in [Mb] observed in these species, can explain almost half (45%) of the total increase in $\beta_{muscleNB}$ above the $\beta_{muscleNB}$ values of their close terrestrial relatives. Thus, increased $\beta_{muscleMb}$ is significant in divers when increased β_{Mb} is coupled with large increases in [Mb].

High protein concentrations are known to lead to protein aggregations (Rumen & Appella, 1962; Fink, 1998). **Chapter 3** of this study highlights that both holoMb and apoMb are susceptible to aggregation even at physiological conditions. Previous research has shown that the Mbs of cetaceans are generally more stable (Scott et al., 2000) and have greater solubility (Deyoung et al., 1993; Regis et al., 2005; Chow et al., 2006) compared to some terrestrial species. Alterations responsible for improved protein solubility are charge changes in the amino acid sequence, that lead to an increase the overall net charge of the protein. These changes include increases in His residues and strongly positively charged residues or decreases in the number of strongly negatively charged residues.

This study shows a remarkable trend in all diving species to significantly increase the net charge of the Mb protein (**Fig 3.2A-D**; **Table A2**) compared to their close terrestrial relatives. This has been achieved via two mechanisms in mammalian divers. Increases in the number of often different His residues and often different strongly positively charged Mb

amino acid residues has evolved independently in seven lineages of divers, including members from the orders Cetartiodactyla, Carnivora and Rodentia. In addition, two independent evolutions of a decrease in the quantity of strongly negatively charged residues have been observed in the diving insectivores (**Table 3.1, Fig A2**). These mechanisms to increase overall Mb net charge would therefore convey an increased solubility in the Mbs of diving species. Research has shown that Mb has an aggregation prone region in the G-helix, (Fandrich et al., 2003; Trovato et al., 2007). The present study has shown that diving species generally have more His in this region compared to their close terrestrial relatives (**Fig 3.2A-D**). Thus, this study proposes that a His rich G- helix, or close surrounding residues, are important in preventing Mb aggregation in mammalian divers.

Chapter 4 of this study has shown that all mammalian divers have increased Mb net charge from the ancestral low Mb net charge state. The number and phylogenetic position of diving species with known Mb sequences within pinnipeds and cetaceans has allowed observing a gradual increase of Mb net charge over evolutionary time in these groups. This can currently not be verified for the diving members in the orders Monotremata, Insectivora and Rodentia where most diving species have evolved a high Mb net charge along the terminal branches of the phylogenies (**Fig 4.3**). The evolution of high Mb net charge is significantly concentrated on branches of the mammalian phylogeny where diving occurs. This is therefore a significant evolutionary event in the Mb protein of mammalian divers. The present study has also shown that Mb net charge is significantly correlated with the logarithm of maximal muscle [Mb] (**Fig 3.5**). Thus any increase in Mb net charge is associated with an exponential increase in maximal [Mb] in skeletal muscles. One can then hypothesise that changes in the structure of the Mb protein to accommodate high Mb concentrations have evolved throughout all lineages of diving mammals as a mechanism to provide increased amounts of muscle bound O_2 to increase dive durations.

The ability of certain species to dive for long durations and to great depths is one of the most fascinating aspects of mammalian physiology. By calculating the duration that each of the three main O_2 stores of the body could theoretically support basal metabolic rate, this study found muscle O_2 stores to be the best indicator of observed maximum dive durations over a large range of diving mammals (**Fig 5.2-5.4**). This makes sense if lung and blood O_2 stores are reserved for brain and heart functioning as has been identified in the emperor penguin, mentioned previously (Ponganis et al., 2010).

Previous estimates of dive duration have focused mainly on the aerobic capacity of a species and have therefore ignored those rarer occasions where some of the most remarkable dives occur. Estimates of maximum dive duration based on body mass and allometry leave a large part of the observed variation unexplained and result in a need to calculate dive durations for major orders of mammals separately. This study presents a simple model (**Equation 5.9**) based only on two parameters that allows estimation of average maximum dive duration in a full range of mammalian divers. Furthermore this study shows how the model can be used in conjunction with phylogenetic reconstruction of Mb net charge and fossil estimations of body mass to estimate dive durations of ancestors to mammalian divers, which may aid in understanding the evolution of diving behaviour.

Mammalian divers have greatly increased in body mass during their evolution, with the blue whale, *Balaenoptera musculus*, being the most extreme example of this. Due to the allometry of BMR (Butler & Jones, 1982), meaning that larger animals have a lower mass specific O_2 consumption than smaller animals (Brody, 1945; White & Seymour, 2003), and based on the isometric scaling of O_2 stores (Hudson & Jones, 1986), researchers have concluded that increases in body mass have been an important evolutionary step in diving mammals. Contrary to previous findings (Castellini & Somero, 1981), the present study, using the simple model mentioned earlier, finds that increases in [Mb] convey greater

increases in dive duration than increases in body mass. Evidence has recently suggested that body mass of cetaceans is linked to the prey items an animal forages for and has shown a secondary reduction in body mass in dolphin species due to changes in diet and foraging strategy (Slater et al., 2010). Therefore this suggests that size is not as important to diving ability as Mb.

This study provides novel insights into how cumulative substitutions on the molecular surface of Mb, away from the active site of the haem group, can have a profound adaptive effects on the physiological properties conveyed to the whole animal. Amino acid changes leading to increased Mb protein charge have permitted an increase in Mb protein concentration, likely due to an increase in solubility by electrostatic repulsion. Increases in Mb His-content of diving species have increased $\beta_{muscleNB}$ and therefore led to an increased capacity for buffering acidic anaerobic end products in the muscles of mammalian divers. This study has further shown that the molecular characteristics of Mb can be used to help explain the physiology and evolution of dive behaviour in mammals. Evidence within this study supports the hypothesis that mammalian Mb protein has undergone previously unrecognised, parallel and adaptive evolution in several lineages of mammalian divers that has profoundly increased their maximal physiological diving capacity. In summary, this work shows that adaptations to diving in mammals involve not only quantitative increases in Mb O_2 stores, but also qualitative changes to the protein to allow higher [Mb] and increased buffering.

Future perspectives for this work include experimentally verifying the proposed increase in apoMb solubility in mammalian divers identified in **Chapter 3**. Confirming that calculations of [Mb] based on amino acid sequences are accurate, by directly measuring the concentration in some of the species that have been identified here, such as the polar bear, *Ursus maritimus* and the pygmy hippo, *Choeropsis liberiensis*. Exploring the reasons for high

Mb net charge in elephant species by looking at the Mb of sirenians the dugong and manatee. Finally in the future it may be possible to confirm the Mb reconstruction within ancient whales, if techniques in ancient DNA extraction and sequencing improve to include fossil samples, then the Mb gene of *Indohyus* and/or *Pakicetus* could possibly be sequenced.

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Table A1 Myoglobin amino acid sequence for 124 mammalian species observed in this study. Accession numbers are given for those species that have been obtained from molecular databases in black text, those obtained from NCBI genome projects in green text and those obtained from TGI genome projects in blue text. Residues given in red text have been influenced by the PCR primer. Species marked with an asterisk have been sequenced for the first time in this study. Diving mammals are indicated by blue boxes at the common species name. Taxonomic orders are marked in bold and are given above the common species name. Helix names are marked with red boxes and helix positions are given above the sequences, following Scott et al. (2000). Black boxes above the sequence indicate residues that are conserved among all mammals. White boxes above the sequences indicate residues that are conserved in all except for one or two species.

Helix: N A | A-Helix 1–16 | AB 1 | B-Helix 1–16 | C-Helix 1–6 | CD 1–8 | D-Helix 1–5 | DE 1–2 | E-Helix 1–20 | EF 1–8 | F-Helix 1–9 | FG 1–5 | G-Helix 1–19 | GH 1–6 | H-Helix 1–24 | HC 1–5

Residue positions 1–153

Species	Species scientific name	Accession No	Sequence (1–153)
Monotremata			
Echidna	*Tachyglossus aculeatus*	P02195	GLSDGEWQLVLKVWGKVETDITGHGQDVLIRLFKTHPETLEKFDKFKHLKTEDEMKASADLKKHGGVVLTALGSILKKKGQHEAELKPLAQSHATKHKISIKFLEFISEAIIHVLQSKHSADFGADAQAAMGKALELFRNDMATKYKEFGFQG
Platypus	*Ornithorhynchus anatinus*	P02196	GLSDGEWQLVLKVWGKVEGDLPGHGQEVLIRLFKTHPETLEKFDKFKGLKTEDEMKASADLKKHGGTVLTALGNILKKKGQHEAELKPLAQSHATKHKISIKFLEYISEAIIHVLQSKHSADFGADAQAAMGKALELFRNDMAAKYKEFGFQG
Marsupialia			
Red kangaroo	*Macropus rufus*	P02194	GLSDGEWQLVLNIWGKVETDEGGHGKDVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGITVLTALGNILKKKGHHEAELKPLAQSHATKHKIPVQFLEFISDAIIQVIQSKHAGNFGADAQAAMKKALELFRHDMAAKYKEFGFQG
Wallaby	*Macropus eugenii*	gn1/ti/1607551647/1357517402	-------------------------------LFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGITVLTALGNILKKKGHHEAELKPLAQSHATKHKIPVQFLEFISDAIIQVIQSKHAGDFGADAQAAMKKALELFRHDMAAKYKEFGFQG
Opossum	*Didelphis virginiana*	P02193	GLSDGEWQLVLNAWGKVEADIPGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGATVLTALGNILKKKGNHEAELKPLAQSHATKHKISVQFLEFISEAIIQVIQSKHPGDFGGDAQAAMGKALELFRNDMAAKYKELGFQG
Afrotheria			
Aardvark	*Orycteropus afer*	P02164	GLSDAEWQLVLNVWGKVEADIPGHGQDVLIRLFKGHPETLEKFDRFKHLKTEDEMKASEDLKKHGTTVLTALGGILKKKGQHEAEIQPLAQSHATKHKIPVKYLEFISEAIIQVIQSKHSGDFGADAQGAMSKALELFRNDIAAKYKELGFQG
Lesser hedgehog tenrec	*Echinops telfairi*	gn1/ti/670539989	-------------------------------LFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGTTVLTALGGILKKKGQHEAELKPLAQSHATKHKIPVKYLEFISEAIIQVLKSKHGGDFGADAQGAMGKALELFRNDIAAKYKELGFQG
Cape hyrax	*Procavia capensis*	gn1/ti/1202104509/1305421800/1203686178	GLSDGEWQLVLNAWGKVEADIPGHGQEVLIRLFQGHPETLEKFDKFKHLKTADEMKASEDLKKHGGVVLTALGGILKKKGDHQAEIQPLAQSHATKHKIPIKYLEFISEAIIHVLQSKHAADFGADAQGAMRKALELFRNDIAAKYKELGFQG
African elephant	*Loxodonta africana*	Campbell unpublished	GLSDGEWELVLKTWGKVEADIPGHGLEVFVRLFTGHPETLEKFDKFKHLKTEGEMKASEDLKKQGVTVLTALGGILKKKGHHQAEIQPLAQSHATKHKIPIKYLEFISDAIIHVLQSKHPAEFGADAQAAMKKALELFRNDIAAKYKELGFQG
Asian elephant	*Elephas maximus*	Campbell unpublished	GLSDGEWELVLKTWGKVEADIPGHGLEVFVRLFTGHPETLEKFDKFKHLKTEGEMKASEDLKKQGVTVLTALGGILKKKGHHQAEIQPLAQSHATKHKIPIKYLEFISDAIIHVLQSKHPAEFGADAQGAMKKALELFRNDIAAKYKELGFQG
Xenarthra			
Armadillo	*Dasypus novemcinctus*	gn1/ti/562681578/566969190/1864760437	GLSDGEWQSVLNIWGKVEADIPGHGQEILIRLFKGHPETLEKFDKFKHLKSEDEMKACEDLKKHGATVLNALGGILKKKGQHEAEIKPLAQSHATKHKIPVKYLEFISEAILQVLQSKHPGDFGADAQGAMSKALELFRNDIAAKYKELGFQG
Insectivora			
Water shrew *	*Sorex palustris*		GLSDGEWQLVLNTWGKVEADIPGYGQEVLIRLFQGHPETLEKFEKFKNLKSEGDMKASEDLKKHGTTVLTALGGILKKKGQHQAELAPLAQSHANKHKIPIKYLEFISEAIIQVLQAKQGGNFGADAQSAMKKALQLFRNDIAAKYKELGFQG
Short-tailed shrew *	*Blarina brevicauda*		GLSDGEWQQVLNIWGKVEADIPSHGQAVLIRLFQGHPETLEKFDKFKNLKSEDEMKGSEDLKKHGTTVLTALGGILKKKGNHQAELQPLAQSHANKHKIPIKYLEFISEAIIDVLKAKHGGDFGADAQGAMSKALELFRNDIAAKYKELGFQG
Hedgehog	*Erinaceus europaeus*	P02156	GLSDGEWQLVLNVWGKVEADIPGHGQEVLIRLFKDHPETLEKFDKFKHLKSEDEMKSSEDLKKHGTTVLTALGGILKKKGHHEAQLAPLAQSHANKHKIPVKYLEFISEAIIQVLKSKHAGDFGADAQGAMSKALELFRNDIAAKYKELGFQG
Star-nosed mole *	*Condylura cristata*		GLSDGEWQLVLNIWGKVEADIPGHGQEVLIRLFQSHPETLEKFDKFKNLKSEGDMKASEDLKKHGTTVLTALGGILKKKGQHQAELQPLAQSHANKHKIPVKYLEFISEAIIQVLQTKHGGNFGADAQSAMKKALQLFRNDIAAKYKELGFQG
Coast mole *	*Scapanus orarius*		GLSDGEWQLVLNVWGKVEADISGHGQEVLISLFKSHPETLEKFDKFKNLKSEGEMKASEDLKKHGATVLTALGGILKKKGQHGAEVQPLAQSHANKHKIPVKYLEFISEVIIQVLQSKHPGDFGADAQGAMRKALELFRNDMAAKYKELGFQG
Chiroptera			
Little brown bat	*Myotis lucifugus*	gn1/ti/974010787/997923600/970851479	GLSDAEWQLVLNAWGKVEADIPGHGQAVLISLFKGHPETQDKFEKLKNLKSEDEMKASEDLKKQGATVLTALGGVLKKKGQHEAELKPLAESHATKHKVPVEYLEFISDSIIEVLKSKLGGDFGDDAKGAMKKALELFRNDMAAKYKELGFQG
Egyptian rousette	*Rousettus aegyptiacus*	P02163	GLSDGEWQLVLNVWGKVEADIPGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGATVLTALGGILKKKGQHEAQLKPLAQSHATKHKIPVKYLEFISEVIIQVLQSKHPGDFGADAQGAMGKALELFRNDIAAKYKELGFQG
Large flying fox	*Pteropus vampyrus*	gn1/ti/1379095081	-------------------------------LFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGATVLTALGGILKKKGQHEAELKPLAQSHATKHKIPVKYLEFISEVIIQVLQSKHPGDFGADAQGAMKKALELFRNDIAAKYKELGFQG
Carnivora			
Grey seal	*Halichoerus grypus*	P68081	GLSDGEWHLVLNVWGKVETDLAGHGQEVLIRLFKSHPETLEKFDKFKHLKSEDDMRRSEDLRKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSKHPAEFGADAQAAMKKALELFRNDIAAKYKELGFHG
Baikal seal	*Phoca sibirica*	P30562	GLSDGEWHLVLNVWGKWETDLAGHGQEVLIRLFKSHPETLEKFDKFKHLKSEDDMRRSFDLRKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSKHPAEFGADAQAAMKKALELFRNDIAAKIKELGFHG
Harbour seal	*Phoca vitulina*	P68080	GLSDGEWHLVLNVWGKVETDLAGHGQEVLIRLFKSHPETLEKFDKFKHLKSEDDMRRSEDLRKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSKHPAEFGADAQAAMKKALELFRNDIAAKYKELGFHG
Ringed seal *	*Phoca hispida*		GLSDGEWHLVLNVWGKVETDLAGHGQEVLIRLFKSHPETLEKFDKFKHLKSEDDMRRSEDLRKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSKHPAEFGADAQAAMKKALELFRNDIAAKYKELGFHG
Harp seal *	*Pagophilus groenlandicus*		GLSDGEWHLVLNVWGKVETDLAGHGQEVLIRLFKSHPETLEKFDKFKHLKSEDDMRRSEDLRKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSKHPAEFGADAQAAMKKALELFRNDIAAKYKELGFHG
Hooded seal *	*Cystophora cristata*		GLSDGEWHLVLNVWGKVETDLAGHGQEVLIRLFKSHPETLEKFDKFKHLKSEDDMRRSEDLRKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSKHPAEFGADAQAAMKKALELFRNDIAAKYKELGFHG
Bearded seal *	*Erignathus barbatus*		GLSDGEWHLVLNVWGKVETDLAGHGQEVLIRLFKSHPETLEKFDKFKHLKSEDDMRRSEDLRKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSKHPAEFGADAQAAMKKALELFRNDIAAKYKELGFHG
Weddell seal *	*Leptonychotes weddellii*		GLSDGEWHLVLNIWGKVETDLAGHGQEVLIRLFKSHPETLEKFDKFKHLKSEDDMRRSEDLRKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIQVLHSKHPGEFGADTQAAMKKALELFRNDIAAKYKELGFHG
Elephant seal *	*Mirounga angustirostris*		GLSDGEWHLVLNIWGKVETDLAGHGQEVLIRLFKSHPETLEKFDKFKHLKSEDDMRRSEDLRKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSKHPAEFGADTQAAMKKALELFRNDIAAKYKELGFHG
California sea lion *	*Zalophus californianus*	P02161	GLSDGEWHLVLNIWGKVETDLAGHGQEVLIRLFRSHPETLEKFDKFKHLKSEDDMRRSEDLRKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIQVLHNKHPGEFGADTQAAMKKALELFRNDIATKYKELGFHG
Steller sea lion *	*Eumetopias jubatus*		GLSDGEWQLVLNIWGKVEADLVGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKRSEDLKKHGKTVLTALGGILKKKGHHDAELKPLAQSHATKHKIPIKYLEFISEAIIHVLQSKHPGDFGADTHAAMKKALELFRNDIAAKYRELGFQG
Australian sea lion *	*Neophoca cinerea*		GLSDGEWQLVLNIWGKVEADLVGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKRSEDLKKHGKTVLTALGGILKKKGHHDAELKPLAQSHATKHKIPIKYLEFISEAIIHVLQSKHPGDFGADTHAAMKKALELFRNDIAAKYRELGFQG
Northern fur seal *	*Callorhinus ursinus*		GLSDGEWQLVLNIWGKVEADLVGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKRSEDLKKHGKTVLTALGGILKKKGHHDAELKPLAQSHATKHKIPIKYLEFISEAIIHVLQSKHPGDFGADTHAAMKKALELFRNDIAAKYRELGFQG
Walrus *	*Odobenus rosmarus*		GLSDGEWQLVLNIWGKVEADLVGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKRSEDLKKHGKTVLTALGGILKKKGHHDAELKPLAQSHATKHKIPIKYLEFISEAIIHVLQSKHPGDFGADTHAAMKKALELFRNDIAAKYRELGFQG
Raccoon *	*Procyon lotor*		GLSDGEWQLVLNVWGKVEADLAGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKRSEDLKKHGKTVLTALGGIFKKKGHHDAELKPLAQSHATKHKIPIKYLEFISEAIIQVLQSKHPGDFGADTHAAMKKALELFRNDIAAKYRELGFQG
Badger	*Meles meles*	P02157	GLSDGEWQLVLNVWGKVEADLAGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGNTVLTALGGILKKKGQHEAELKPLAQSHATKHKIPVKYLEFISDAIIQVLQSKHPGDFGADAQAAMKKALELFRNDIAAKYKELGFQG
American mink *	*Neovison vison*		GLSDGEWQLVLNVWGKVETDLAGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKGSEDLKKHGNTVLTALGGILKKKGHQEAELKPLAQSHATKHKIPVKYLEFISDAIAQVLQSKHPGNFAAEAQAAMKKALELFRNDIAAKYKELGFQG
River otter	*Lutra lutra*	P11343	GLSDGEWQLVLNVWGKVEADLAGHGQEVLIRLFK[illegible]
Black bear	*Ursus americanus*	TC5202 TC212 TC1035 TC1537 TC2057	GLSDGEWQLVLNIWGKVEADLAGHGQEVLISLFKNHPETLEKFDKFKHLKSEDEMKGSEDLKKHGNTVLTALGGILKKKGQHEAELKPLAQSHATKHKIPVKYLEFISEAIIQVLQRKHSGDFGADAQAAMKKALELFRNDIAAKYKELGFQG
Polar bear *	*Ursus maritimus*		GLSDGEWQLVLNVWGKVEADLAGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKGSEDLKKHGNTVLTALGGILKKKGKHEAELKPLAQSHATKHKIPIKYLEFISEAIIQVLQSKHPGDFGADAQGAMKRALELFRNDIAAKYKELGFQG
Giant panda	*Ailuropoda melanoleuca*	XP002925619	GLSDGEWQLVLNVWGKVEADLAGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKGSEDLKKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIQVLQSKHPGDFGADAQGAMRKALELFRNDIAAKYKELGFQG
Cape fox	*Vulpes chama*	P02160	GLSDGEWQLVLNVWGKVEADLAGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKGSEDLKKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIQVLQSKHPGDFGADAQGAMRKALELFRNDIAAKYKELGFQG
Bat-eared fox	*Otocyon megalotis*	P63114	GLSDGEWQLVLNVWGKVEADLAGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKGSEDLKKHGNTVFTALGGILKKKGQHEAELKPLAQSHATKHKIPVKYLEFISEAIIQVLQSKHPGDFGADAQAAMRKALELFRNDIAAKYKELGFQG
Dog	*Canis lupus familiaris*	P63113	GLSDGEWQLVLNVWGKVEADLAGHGQEVLIRLFK[illegible]
African hunting dog	*Lycaon pictus*	P02159	GLSDGEWQLVLNIWGKVETDLAGHGQEVLIRLFKNHPETLDKFDKFKHLKTEDEMKGSEDLKKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPVKYLEFISDAIIQVLQSKHSGDFHADTEAAMKKALELFRNDIAAKYKELGFQG
Cat	*Felis catus*	gb/ACBE01246425/GI:220426232	GLSDGEWQIVLNIWGKVETDLAGHGQEVLIRLFKNHPETLDKFDKFKHLKTEDEMKGSEDLKKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPVKYLEFISDAIIQVLQSKHSGDFHADTEAAMKKALELFRNDIAAKYKELGFQG
Perrisodactyla			
Horse	*Equus caballus*	P68082	GLSDGEWQQVLNVWGKVEADIAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKASEDLKKHGTVVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSKHPGDFGADAQGAMTKALELFRNDIAAKYKELGFQG
Zebra	*Equus burchellii*	P68083	GLSDGEWQQVLNVWGKVEADIAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKASEDLKKHGTVVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSKHPGDFGADAQGAMTKALELFRNDIAAKYKELGFQG
Cetartiodactyla			
Alpaca	*Lama pacos*	gn1/ti/1988085346	--QFISDAIIHVLQSKHPGDFGADAQAAMNKALELFRNDMAAKYKELGFQG
Pig	*Sus scrofa*	NP999401	GLSDGEWQLVLNVWGKVEADVAGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGNTVLTALGGILKKKGHHEAELTPLAQSHATKHKIPVKYLEFISEAIIQVLQSKHPGDFGADAQGAMSKALELFRNDMAAKYKELGFQG
Cattle	*Bos taurus*	NP776306	GLSDGEWQLVLNAWGKVEADVAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAEVKHLAESHANKHKIPVKYLEFISDAIIHVLHAKHPSDFGADAQAAMSKALELFRNDMAAQYKVLGFHG
Yak	*Bos grunniens*	Q2MJN4	GLSDGEWQLVLNAWGKVEADVAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAEVKHLAESHANKHKIPVKYLEFISDAIIHVLHAKHPSDFGADAQAAMSKALELFRNDMAAQYKVLGFHG
Water buffalo	*Bubalus bubalis*	P84997	GLSDGEWQLVLNAWGKVETDVAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAEVKHLAESHANKHKIPVKYLEFISDAIIHVLHDKHPSDFGADAQAAMSKALELFRNEMAAQYKVLGFHG
Goat	*Capra hircus*	ACJ68031	GLSDGEWTLVLNAWGKVEADVAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAEVKHLAESHANKHKIPVKYLEFISDAIIHVLHAKHPSDFGADAQGAMSKALELFRNDMAAQYKVLGFQG
Sheep	*Ovis aries*	NP001072126	GLSDGEWQLVLNAWGKVEADVAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILEKKGHHEAEVKHLAESHANKHKIPVKYLEFISDAIIHVLHAKHPSDFGADAQGAMSKALELFRNDMAAQYKVLGFQG
Tibetan antelope	*Pantholops hodgsonii*	ABR24111	GLSDGEWQLVLNAWGKVEAGVAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAEVKHLAESHANKHKIPVKYLEFISDAIIHVLHAKHPSDFGADAQGAMSKALELFRNDMAAQYKVLGFQG
Red deer	*Cervus elaphus*	P02191	GLSDGEWQLVLNAWGKVEADVAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAEVKHLAESHANKHKIPVKYLEFISDAIIHVLHAKHPSNFGADAQGAMSKALELFRNDMAAQYKVLGFQG
Pygmy hippo *	*Choeropsis liberiensis*		GLSDGEWQLVLNAWGKVEADVAGHGQEVLIRLFTGHPETLEKFEKFKNLKTEDEMRACENLKKHGNTVLTALGGILKKKGHHEEELKPLAHSHATKHKIPIKYLEFISEAIIHVLQSKHPGDFGADAQGAMNKALELFRNDIAAKYKELGFQG
Fin-back whale	*Balaenoptera physalus*	P02180	GLSDGEWQLVLNVWGKVEADVAGHGQEVLIRLFT[illegible]
Humpback whale	*Megaptera novaeangliae*	P02178	VLTDAEWHLVLNIWAKVEADVAGHGQDILISLFKGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSRHPADFGADAQAAMNKALELFRKDIAAKYKELGFQG
Grey whale	*Eschrichtius robustus*	P02177	VLSDAEWQLVLNIWAKVEADVAGHGQDILIRLFKGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSRHPADFGADAQAAMNKALELFRKDIAAKYKELGFQG
Pygmy Bryde's whale	*Balaenoptera edeni*	Q0KIY2	VLSDAEWQLVLNIWAKVEADVAGHGQDILIRLFKGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSRHPGDFGADAQAAMNKALELFRKDIAAKYKELGFQG
Sei whale	*Balaenoptera borealis*	Q0KIY1	VLSDAEWQLVLNIWAKVEADVAGHGQDILIRLFKGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSRHPGDFGADAQAAMNKALELFRKDIAAKYKELGFQG
Minke whale	*Balaenoptera acutorostrata*	P02179	VLSDAEWQLVLNIWAKVEADVAGHGQDILIRLFKGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSRHPGDFGADAQAAMNKALELFRKDIAAKYKELGFQG
Bowhead *	*Balaena mysticetus*		VLSDAEWQLVLNIWAKVEADVAGHGQDILIRLFKGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSRHPAEFGADAQAAMNKALELFRKDIAAKYKELGFQG
Pygmy sperm whale	*Kogia breviceps*	Q0KIY5	VLSDAEWHLVLNIWAKVEADVAGHGQDILIRLFKGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSRHPGDFGADAQGAMNKALELFRKDIAAKYKELGFQG
Dwarf sperm whale	*Kogia simus*	P02184	VLSDGEWQLVLNIWAKVEADVAGHGQDVLIRLFKGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHPADFGADAQGAMSKALELFRKDIAAKYKELGYQG
Sperm whale	*Physeter catodon*	P02185	VLSEGEWQLVLHVWAKVEADIAGHGQDILIRLFKHHPETLEKFDRFKHLKSEAEMKASEDLKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHPADFGADAQGAMSKALELFRKDIAAKYKELGYQG
Stejneger's beaked whale	*Mesoplodon stejnegeri*	Q0KIY0	VLSEGEWQLVLHVWAKVEADIAGHGQDILIRLFKHHPETLEKFDRFKHLKSEAEMKASEDLKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHPGDFGADAQGAMNKALELFRKDIAAKYKELGYQG
Hubbs' beaked whale	*Mesoplodon carlhubbsi*	P02183	VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFDKFKHLKTEAEMKASEDLKKHGHTVLTALGGILKKKGHHEAELKPLAQSHATKH[illegible]
Sowerby's beaked whale *	*Mesoplodon bidens*		VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFK[illegible]
Longmans' beaked whale	*Indopacetus pacificus*	Q0KIY9	GLSEAEWQLVLHVWAKVEADLSGHGQEILIRLFKGHPETLEKFDKFKHLKSEAEMKASEDLKKHGHTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSKHPSDFGADAQGAMTKALELFRKDIAAKYKELGFHG
Northern bottlenose whale *	*Hyperoodon ampullatus*		GLSEAEWQLVLHVWAKVEADLSGHGQEILIRLFKGHPETLEKFDKFKHLKSEAEMKASEDLKKHGHTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSKHPSDFGADAQGAMTKALELFRKDIAAKYKELGFHG
Cuvier's beaked whale	*Ziphius cavirostris*	P02182	GLSEAEWQLVLHVWAKVEADLSGHGQEILIRLFKGHPETLEKFDKFKHLKSEAEMKASEDLKKHGHTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSKHPSDFGADAQGAMTKALELFRKDIAAKYKELGFHG
Amazon river dolphin	*Inia geoffrensis*	P02181	GLSEAEWQLVLHVWAKVEADLSGHGQEILIRLFKGHPETLEKFEKFKHLKSEAEMKASEDLKKHGHTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSKHPSDFGADAQAAMTKALELFRKDIAAKYKELGFHG
Narwhal *	*Monodon monoceros*		GLSEAEWQLVLHVWAKVEADLSGHGQEILIRLFKGHPETLEKFDKFKHLKSEAEMKASEDLKKHGHTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSKHPSDFGADAQGAMTKALELFRKDIAAKYKELGFHG
Beluga	*Delphinapterus leucas*	P83682	GLSEAEWQLVLHVWAKVEADLSGHGQEILIRLFK[illegible]KIPIKYLEFISDAIIHVLHSRHPSDFGADAQAAMTKALELFRKDIAAKYKELGFHG
Harbour porpoise	*Phocoena phocoena*	P68278	GLSDGEWQLVLNIWGKVEADLAGHGQDVLIRLFKGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHPGDFGADAQAAMNKALELFRKDIAAKYKELGFHG
Dall's porpoise	*Phocoenoides dalli dalli*	P68277	GLSEGEWQLVLNVWGKVEADLAGHGQDVLIRLFK[illegible]KIPIKYLEFISEAIIHVLHSRHPGDFGADAQGAMNKALELFRKDIAAKYKELGFHG
Killer whale	*Orcinus orca*	P02173	GLSEGEWQLVLNVWGKVEADLAGHGQDVLIRLFKGHPETLEKFDKFRHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHDAELKPLAQSHATKH[illegible]
White beaked dolphin *	*Lagenorhynchus albirostris*		GLSEGEWQLVL----KVEADLAGHGQDVLIRLFKGHPETLEKFDKFKHLKT---MKASEDLKKHGNTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYL----EAIIHVLHSRHPAEFGADAQGAMNKALELFRKDIATKYKELGFHG
Risso's dolphin *	*Grampus griseus*		GLSEGEWQLVLNVWGKVEADLAGHGQDVLIRLFKGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVLTALGGILKKKGHHDAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHPAEFGADAQGAMNKALELFRKDIATKYKELGFHG
Long-fin pilot whale	*Globicephala melas*	P02174	GLSEGEWQLVLNVWGKVEADLAGHGQDVLIRLFKGHPETLEKFDKFKHLKTEADMKASEDLKKHGNTVLTALGGILKKKGHHDAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHPAEFGADAQGAMNKALELFRKDIAAKYKELGFHG
Melon-head whale	*Peponocephala electra*	Q0KIY3	GLSDGEWQLVLNVWGKVEADLAGHGQDILIRLFK[illegible]
Bridled dolphin	*Stenella attenuata*	Q0KIY7	GLSDGEWQLVLNVWGKVEADLAGHGQDVLIRLFK[illegible]
Saddleback dolphin	*Delphinus delphis*	P68276	GLSDGEWQLVLNVWGKVEADLAGHGQDVLIRLFKGHPETLEKFDKFKHLKTEADMKASEDLKKHGNTVLTALGAILKKKGHHDAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHPAEFGADAQGAMNKALELFRKDIAAKYKELGFHG
Bottlenose dolphin	*Tursiops truncatus*	P68279	GLSDGEWQLVLNVWGKVEADLAGHGQDILIRLFKGHPETLEKFDKFKHLKTEADMKASEDLKKHGNTVLTALGAILKKKGHHDAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHPAEFGADAQGAMNKALELFRKDIAAKYKELGFHG
Lagomorpha			
Rabbit	*Oryctolagus cuniculus*	P02170	GLSDAEWQLVLNVWGKVEADLAGHGQEVLIRLFHTHPETLEKFDKFKHLKSEDEMKASEDLKKHGNTVLTALGAILKKKGHHEAEIKPLAQSHATKHKIPVKYLEFISEAIIHVLHSKHPGDFGADAQAAMSKALELFRNDIAAQYKELGFQG
Black-lipped pika	*Ochotona curzoniae*	Q6PL31	GLSDGEWQLVLTVWGKVEADLAGHGQEVLIRLFKNHPETLEKFDKFKNLKSEDEMKGSDDLKKHGNTVLSALGGILKKKGQHEAELKPLAQSHATKHKIPVKYLEFISEAIIQVLQSKHPGDFGADAQGAMSKALELFRNDMAAKYKELGFHG
American pika	*Ochotona princeps*	P02171	GLSDGEWQLVLNVWGKVEADLAGHGQEVLIRLFKNHPETLEKFDKFKNLKSEDEMKGSDDLKKHGNTVLSALGGILKKKGQHEAELKPLAQSHATKHKIPVKYLEFISEAIIQVLQSKHPGDFGADAQGAMSKALELFRNDMAAKYKELGFQG
Rodentia			
House mouse	*Mus musculus*	NP038621	GLSDGEWQLVLNVWGKVEADLAGHGQEVLIGLFKTHPETLDKFDKFKNLKSEEDMKGSEDLKKHGCTVLTALGTILKKKGQHAAEIQPLAQSHATKHKIPVKYLEFISEIIIEVLKKRHSGDFGADAQGAMSKALELFRNDIAAKYKELGFQG
Norway rat	*Rattus norvegicus*	NP067599	GLSDGEWQMVLNIWGKVEGDLAGHGQEVLISLFKAHPETLEKFDKFKNLKSEEEMKSSEDLKKHGCTVLTALGTILKKKGQHAAEIQPLAQSHATKHKIPVKYLEFISEVIIQVLKKRYSGDFGADAQGAMSKALELFRNDIAAKYKELGFQG
Muskrat	*Ondatra zibethicus*	P32428	GLSDGEWQLVLHVWGKVEADLAGHGQDVLIRLFKAHPETLEKFDKFKHIKSEDEMKGSEDLKKHGNTVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPIKYLEFISEAIIHVLQSKHPSNFGADVQGAMKRALELFRNDIAAKYKELGFQG
Mole rat	*Spalax carmeli*	CAL91965	GLSDGEWQLVLKVWGKVESDLAGHGQEVLIKLFKNHPETLEKFDKFKNLKSEDEMKGSEDLKKHGNTVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPIKYLEFISEAIIQVLQSKHPGDFGADAQGAMSKALELFRNDIAAKYKELGFQG
Ehrenberg's mole-rat	*Nannospalax ehrenbergi*	P04248	GLSDGEWQLVLNVWGKVEGDLAGHGQEVLIKLFKNHPETLEKFDKFKHLKSEDEMKGSEDLKKHGNTVLTALGGILKKKGQHAAEIQPLAQSHATKHKIPIKYLEFISEAIIQVLQSKHPGDFGADAQGAMSKALELFRNDIAAKYKELGFQG
Kangaroo rat	*Dipodomys ordii*	gn1/ti/1526078076/1583645431/1570078438	GLSDGEWQLVLNVWGKVEADLAGNGQEVLIRLFKNHSETLEKFDKFKNLKSEDEMKASEDLKKHGTTVLTALGGILKKKGQHEAEIKPLAQSHATKHKIPMAYLEFISEAIIEVLKCKYPGDFGADAQGAMSKALELFRNDIAAKYKELGFQG
Eurasian beaver	*Castor fiber*	P14396	GLSDGEWQLVLHVWGKVEADLAGHGQEVLIRLFKGHPETLEKFNKFKHIKSEDEMKASEDLKKHGVTVLTALGGVLKKKGHHEAEIKPLAQSHATKHKIPIKYLEFISEAIIHVLQSKHPGNFGADANGAMNKALELFRKDIAAKYKELGFQG
Plains viscacha	*Lagostomus maximus*	P04250	GLSDGEWQLVLNVWGKVEADLGGHGQEVLIRLFKGHPETLEKFDKFKHLKAEDEMRASEDLKKHGTTVLTALGGILKKRGQHAAELAPLAQSHATKHKIPVKYLEFISEAIIQVLQSKHPGDFGADAQAAMSKALELFRNDIAAKYKELGFQG
Casiragua	*Proechimys guairae*	P04249	GLSDGEWQLVLNVWGKVEGDLSGHGQEVLIRLFKGHPETLEKFDKFKHLKAEDEMRASEELKKHGTTVLTALGGILKKKGQHAAELAPLAQSHATKHKIPVKYLEFISEAIIQVLQSKHPGDFGADAQGAMSKALELFRNDIAAKYKELGFQG
Guinea pig	*Cavia porcellus*	gn1/ti/1666012444/743544164/1664737761	GLSDGEWQLVLNVWGKVEGDLGGHGQEVLIRLFKAHPETLEKFDKFKHLKGEDEMRASEDLKKHGNTVLTALGGILKKKGQHAAELAPLAQSHATKHKIPVKYLEFISEAIIQVLQSKHPGDFGADAQGAMSKALELFRNDIAAKYKELGFQG
Northern gundi	*Ctenodactylus gundi*	P20856	GLSDGEWQLVLNAWGKVETDIGGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGTTVLTALGNILKKKGQHEAELAPLAQSHATKHKIPVKYLEFISEAIIQVLESKHPGDFGADAQGAMSKALELFRNDIAAKYKELGFQG
Grey squirrel *	*Sciurus carolinensis*		GLSDGEWQLVLSVWGKVEADLAGHGQEVLIRLFKDHPETLEKFDKFKNLKSEEEMKASEELKKHGSTVLGALGGILKKKGQHEAEIKPLAQSHATKHKIPVKYLEFISEAIIQVLKGKHSGDFGDAAQGAMSKALELFRNDIAAKYKELGFQG
Ground squirrel	*Spermophilus tridecemlineatus*	gn1/ti/999164977/1024530269	GLSDAECQLVLNIWGKVETDLAGHGQQVLISLFKGHPETLDKFDKFKHLKSEDEMKASEDLKKHGTTVLTALGGILKKKGQHEAEIKPLAQSHATKHKIPVKYLE--
Primates			
Tree shrew	*Tupaia glis*	P02165	GLSDGEWQLVLNVWGKVEADVAGHGQEVLIRLFKGHPETLEKFDKFKHLKTEDEMKASEDLKKHGNTVLSALGGILKKKGQHEAEIKPLAQSHATKHKIPVKYLEFISEAIIQVLQSKHPGDFGADAQAAMSKALELFRNDIAAKYKELGFQG
Thick-tailed bush baby	*Otolemur crassicaudatus*	P02168	GLSDGEWQLVLKIWGKVEADLAGHGQDVLIRLFTAHPETLEKFDKFKNLKTADEMKASEDLKKHGVTVLTALGGILKKKGQHEAEIKPLAQSHATKHKIPVKYLEFISEAIIHVLQNKHSGDFGTDVQGAMSKALELFRNDIAAKYKELGFQG
Potto	*Perodicticus potto edwarsi*	P02166	GLSDGEWQSVLNVWGKVEADLAGHGQEILIRLFTAHPETLEKFDKFKNLKTPDEMKASEDLKKHGVTVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLEFISEAIIHVLQSKHPGDFGADAQGAMNKALELFRNDIAAKYKELGFQG
Slow loris	*Nycticebus coucang*	P02167	GLSDGEWQSVLNVWGKVEADLAGHGQEILIRLFTAHPETLEKFDKFKNLKTPDEMKASEDLKKHGVTVLTALGGILKKKGQHEAEIKPLAQSHATKHKIPVKYLEFISGAIIHVLQSKHPGDFGADAQGAMSKALELFRNDIAAKYKELGFQG
Weasel lemur	*Lepilemur mustelinus*	P02169	GLSDGEWQLVLNVWGKVEADVGGHGQEVLIRLFTGHPETLEKFDKFKHLKTADEMKASEDLKKHGTTVLTALGGILKKKGQHEAELKPLAQSHATKHKIPIKYLEFISDAIVHVLHSKHPAEFGADAQAAMKKALELFRNDIAAKYKELGFQG
Grey mouse lemur	*Microcebus murinus*	gn1/ti/1555755140/1560357904/1558262175	GLSDGEWQLVLNVWGKVEADVSGHGQEVLIRLFTGHPETLEKFDKFKHLKTADEMKASEDLKKHGTTVLTALGAILKKKGQHEAELKPLAQSHATKHKIPVKYLEFISDAIIHVLQSKHPADFGADAQGAMKKALELFRNDIAAKYKELGFQG
Common woolly monkey	*Lagothrix lagotricha*	P02154	GLSDGEWQLVLNIWGKVEADIPSHGQEVLISLFKGHPETLEKFDKFKHLKSEDEMKASEELKKHGVTVLTALGGILKKKGQHEAELKPLAQSHATKHKIPVKYLEFISDAIIHALQKKHPGDFGADAQGAMKKALELFRNDMAAKYKELGFQG
Douroucouli	*Aotus trivirgatus*	P02151	GLSDGEWQLVLNVWGKVEADVPSHGQEVLISLFKGHPETLEKFDKFKHLKSEDEMKASEELKKHGVTVLTALGGILKKKGHHEAELKPLAQSHATKHKIPVKYLEFISDAIVHVLQKKHPGDFGADAQGAMKKALELFRNDMAAKYKELGFQG
Squirrel monkey	*Saimiri sciureus*	P02155	GLSDGEWQLVLNIWGKVEADIPSHGQEVLISLFKGHPETLEKFDKFKHLKSEDEMKASEELKKHGTTVLTALGGILKKKGQHEAELKPLAQSHATKHKIPVKYLELISDAIVHVLQKKHPGDFGADAQGAMKKALELFRNDMAAKYKELGFQG
Brown capuchin	*Cebus apella*	P02153	GLSDGEWQLVLNVWGKVEADIPSHGQEVLISLFKGHPETLEKFDKFKHLKSEDEMKASEELKKHGATVLTALGGILKKKGQHEAELKPLAQSHATKHKIPVKYLEFISDAIVHVLQKKHPGDFGADAQGAMKKALELFRNDMAAKYKELGFQG
White tuft-ear marmoset	*Callithrix jacchus*	P02152	GLSDGEWQLVLNVWGKVEADIPSHGQEVLISLFKGHPETLEKFDKFKHLKSEDEMKASEELKKHGVTVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLEFISDAIVHVLQKKHPGDFGADAQGAMKKALELFRNDMAAKYKELGFQG
Rhesus monkey	*Macaca mulatta*	gn1/ti/665763401/513343268/496218168	GLSDGEWQLVLNVWGKVEADIPSHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGVTVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLELISESIIQVLQSKHPGDFGADAQGAMNKALELFRNDMAAKYKELGFQG
Crab-eating macaque	*Macaca fascicularis*	P02150	GLSDGEWQLVLNVWGKVEADIPSHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGVTVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLELISESIIQVLQSKHPGDFGADAQGAMNKALELFRNDMAAKYKELGFQG
Olive baboon	*Papio anubis*	P68084	GLSDGEWQLVLNVWGKVEADIPSHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGATVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLELISESIIQVLQSKHPGDFGADAQGAMNKALELFRNDMAAKYKELGFQG
Red guenon	*Erythrocebus patas*	P68086	GLSDGEWQLVLNVWGKVEADIPSHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGATVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLELISESIIQVLQSKHPGDFGADAQGAMNKALELFRNDMAAKYKELGFQG
Hanuman langur	*Semnopithecus entellus*	P68085	GLSDGEWQLVLNVWGKVEADIPSHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGATVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLELISESIIQVLQSKHPGDFGADAQGAMNKALELFRNDMAAKYKELGFQG
Agile gibbon	*Hylobates agilis*	P62735	GLSDGEWQLVLNVWGKVEADIPSHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGATVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLEFISECIIQVLQSKHPGDFGADAQGAMNKALELFRKDMASNYKELGFQG
Siamang	*Symphalangus syndactylus*	P62735	GLSDGEWQLVLNVWGKVEADIPSHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGATVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLEFISECIIQVLQSKHPGDFGADAQGAMNKALELFRKDMASNYKELGFQG
Orangutan	*Pongo pygmaeus*	P02148	GLSDGEWQLVLNVWGKVEADIPSHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGATVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLEFISESIIQVLQSKHPGDFGADAQGAMNKALELFRKDMASNYKELGFQG
Mountain gorilla	*Gorilla gorilla beringei*	P02147	GLSDGEWQLVLNVWGKVEADIPSHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGATVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLEFISECIIQVLQSKHPGDFGADAQGAMNKALELFRKDMASNYKELGFQG
Chimpanzee	*Pan troglodytes*	P02145	GLSDGEWQLVLNVWGKVEADISGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGATVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLEFISECIIQVLHSKHPGDFGADAQGAMNKALELFRKDMASNYKELGFQG
Human	*Homo sapiens*	NP976311	GLSDGEWQLVLNVWGKVEADIPGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGATVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLEFISECIIQVLQSKHPGDFGADAQGAMNKALELFRKDMASNYKELGFQG

Table A2 Data for the quantity of charged residues in the myoglobin (Mb) of mammalian species. The Mb net charge and specific buffer values for three pHs of physiological relevance, calculated from amino acid sequence. Species highlighted in blue boxes are diving species. Species in red text have incomplete Mb amino acid sequence.

Species	Quantity of strongly charged residues					Mb net charge			Mb specific buffer value		
	His	Glu	Asp	Lys	Arg	pH 6.0	pH 6.5	pH 7.0	pH 6.0	pH 6.5	pH 7.0
Monotremata											
Echidna	9	12	9	21	2	3.65	2.40	1.41	2.81	2.23	1.73
Platypus	8	13	8	21	2	3.39	2.29	1.38	2.38	2.02	1.65
Marsupialia											
Red kangaroo	10	13	9	21	2	4.03	2.54	1.10	2.93	3.01	2.67
Wallaby	9	10	7	19	1						
Opossum	8	14	8	19	2	0.35	-0.73	-1.63	2.37	1.98	1.63
Afrotheria											
Aardvark	8	13	9	18	3	0.34	-0.73	-1.63	2.34	1.98	1.63
Lesser hedgehog tenrec	7	11	6	20	1						
Cape hyrax	9	12	9	18	3	1.65	0.40	-0.59	2.81	2.23	1.73
African elephant	9	14	7	20	2	3.57	2.12	0.86	3.09	2.71	2.30
Asian elephant	9	14	7	20	2	3.57	2.12	0.86	3.09	2.71	2.30
Xenarthra											
Armadillo	8	14	8	20	2	1.22	-0.06	-1.24	2.64	2.50	2.18
Insectivora											
Water shrew	5	12	6	19	2	3.28	2.65	2.09	1.39	1.16	1.13
Short-tailed shrew	7	12	9	19	2	1.04	0.15	-0.67	1.84	1.74	1.54
Hedgehog	8	13	9	20	2	1.34	0.27	-0.63	2.34	1.98	1.63
Star-nosed mole	7	11	7	19	2	4.02	3.15	2.33	1.79	1.72	1.54
Coast mole	7	13	7	19	2	2.05	1.16	0.33	1.86	1.75	1.54
Chiroptera											
Little brown bat	5	15	10	22	1	-1.66	-2.33	-2.90	1.53	1.20	1.14
Eygptian rousette	8	13	8	20	2	2.34	1.27	0.37	2.33	1.97	1.63
Large flying fox	7	11	6	20	1						
Carnivora											
Grey seal	13	14	8	19	5	6.55	4.34	2.45	4.53	4.19	3.30
Baikal seal	13	13	8	19	5	7.53	5.34	3.45	4.49	4.18	3.29
Harbour seal	13	14	8	19	5	6.55	4.34	2.45	4.53	4.19	3.30
Ringed seal	13	14	8	19	5	6.55	4.34	2.45	4.53	4.19	3.30
Harp seal	13	14	8	19	5	6.55	4.34	2.45	4.53	4.19	3.30
Hooded seal	13	14	8	19	5	6.55	4.34	2.45	4.53	4.19	3.30
Bearded seal	12	14	8	19	5	6.22	4.21	2.41	4.02	3.93	3.20
Weddell seal	13	14	8	19	5	6.55	4.34	2.45	4.53	4.19	3.30
Elephant seal	12	14	8	18	6	6.22	4.21	2.41	4.02	3.93	3.20
California sea lion	11	13	9	21	4	5.81	4.22	2.89	3.51	2.91	2.38
Steller sea lion	11	13	9	21	4	5.81	4.22	2.89	3.51	2.91	2.38
Australian sea lion	11	13	9	21	4	5.81	4.22	2.89	3.51	2.91	2.38
Northern fur seal	11	13	9	21	4	5.81	4.22	2.89	3.51	2.91	2.38
Walrus	10	13	9	21	4	5.48	4.08	2.85	3.00	2.64	2.27
Raccoon	8	13	9	21	2	2.34	1.27	0.37	2.34	1.98	1.63
Badger	8	14	7	21	2	3.44	2.43	1.53	2.22	1.88	1.73
American mink	8	14	8	21	2	2.35	1.27	0.37	2.37	1.99	1.63
River otter	8	14	8	21	3	3.35	2.27	1.37	2.37	1.99	1.63
Black bear	9	14	8	20	3	3.24	1.99	0.82	2.59	2.45	2.20
Polar bear	9	14	8	20	3	3.24	1.99	0.82	2.59	2.45	2.20
Giant panda	8	14	8	20	3	2.35	1.27	0.37	2.37	1.98	1.63
Cape fox	10	13	10	21	2	3.03	1.54	0.10	2.94	3.01	2.67
Bat-eared fox	10	13	10	21	2	3.03	1.54	0.10	2.94	3.01	2.67
Dog	10	13	10	21	2	3.03	1.54	0.10	2.94	3.01	2.67
African hunting dog	10	13	10	21	2	3.03	1.54	0.10	2.94	3.01	2.67
Cat	10	15	7	20	3	3.49	1.97	0.70	3.27	2.83	2.21
Perrisodactyla											
Horse	11	13	8	19	2	3.41	1.76	0.22	3.36	3.24	2.83
Zebra	11	13	8	19	2	3.41	1.76	0.22	3.36	3.24	2.83
Cetartiodactyla											
Alpaca	2	2	4	4	1						
Pig	9	14	8	19	2	1.24	-0.01	-1.18	2.59	2.45	2.20
Cow	13	13	8	18	2	3.89	1.84	-0.11	4.09	4.07	3.63
Yak	13	13	8	18	2	3.89	1.84	-0.11	4.09	4.07	3.63
Water buffalo	13	14	8	18	2	2.90	0.85	-1.11	4.12	4.08	3.63
Goat	12	13	8	18	2	3.33	1.56	-0.22	3.52	3.60	3.40
Sheep	12	14	7	17	2	2.34	0.56	-1.22	3.55	3.61	3.40
Tibetan antelope	12	13	8	18	2	3.33	1.56	-0.22	3.52	3.60	3.40
Red deer	12	13	7	18	2	4.33	2.56	0.78	3.51	3.60	3.40

Species	Quantity of strongly charged residues					Mb net charge			Mb specific buffer value		
	His	Glu	Asp	Lys	Arg	pH 6.0	pH 6.5	pH 7.0	pH 6.0	pH 6.5	pH 7.0
Cetartiodactyla (continued)											
Pygmy hippo	10	16	6	18	3	1.98	0.25	-1.50	3.33	3.59	3.24
Fin-back whale	12	12	9	20	2	4.95	3.01	1.24	3.93	3.79	3.21
Humpback whale	11	12	9	20	3	5.38	3.72	2.12	3.36	3.31	2.97
Grey whale	11	12	9	20	3	5.38	3.72	2.12	3.36	3.31	2.97
Pygmy Bryde's whale	11	12	9	20	3	5.38	3.72	2.12	3.36	3.31	2.97
Sei whale	11	12	9	20	3	5.38	3.72	2.12	3.36	3.31	2.97
Minke whale	12	13	8	20	3	5.96	4.01	2.24	3.96	3.79	3.21
Bowhead	11	12	9	20	3	5.38	3.72	2.12	3.36	3.31	2.97
Pygmy sperm whale	13	14	7	19	4	6.42	4.31	2.39	4.28	4.10	3.46
Dwarf sperm whale	13	14	7	19	4	6.42	4.31	2.39	4.28	4.10	3.46
Sperm whale	12	14	7	19	4	6.17	4.22	2.36	3.85	3.90	3.39
Stejneger's beaked whale	14	14	7	21	2	7.53	5.10	2.86	4.75	4.86	3.95
Hubbs' beaked whale	14	14	7	21	2	7.53	5.10	2.86	4.75	4.86	3.95
Sowerby's beaked whale	14	14	7	21	2	7.53	5.10	2.86	4.75	4.86	3.95
Longmans' beaked whale	14	14	7	21	2	7.53	5.10	2.86	4.75	4.86	3.95
Northern bottlenose whale	14	15	6	21	2	7.54	5.10	2.86	4.78	4.87	3.95
Cuvier's beaked whale	14	14	7	20	3	7.53	5.10	2.86	4.75	4.86	3.95
Amazon river dolphin	12	13	8	20	3	5.96	4.04	2.33	3.93	3.70	3.06
Narwhal	12	13	8	19	4	5.96	4.04	2.33	3.93	3.70	3.06
Beluga	12	12	6	20	3						
Harbour porpoise	12	14	7	20	3	5.97	4.05	2.34	3.96	3.71	3.06
Dall's porpoise	12	14	7	20	3	5.97	4.05	2.34	3.96	3.71	3.06
Killer whale	12	12	9	20	3	5.95	4.04	2.33	3.90	3.70	3.06
White beaked dolphin	12	12	9	20	3	5.95	4.04	2.33	3.90	3.70	3.06
Risso's dolphin	12	12	9	20	3	5.95	4.04	2.33	3.90	3.70	3.06
Long-fin pilot whale	12	13	8	20	3	5.96	4.04	2.33	3.93	3.70	3.06
Melon-head whale	12	12	9	20	3	5.95	4.04	2.33	3.90	3.70	3.06
Bridled dolphin	12	12	9	20	3	5.95	4.04	2.33	3.90	3.70	3.06
Saddleback dolphin	12	12	9	20	3	5.95	4.04	2.33	3.90	3.70	3.06
Bottlenose dolphin	12	12	9	20	3	5.95	4.04	2.33	3.90	3.70	3.06
Lagomorpha											
Rabbit	12	14	8	18	2	2.22	0.32	-1.49	3.77	3.81	3.30
Black-lipped pika	8	13	9	20	2	1.62	0.44	-0.56	2.44	2.22	1.77
American pika	7	13	9	20	2	1.06	0.16	-0.67	1.88	1.75	1.55
Rodentia											
House mouse	7	13	9	20	2	0.92	-0.18	-1.28	2.15	2.26	2.09
Norway rat	6	14	7	20	2	1.18	0.33	-0.52	1.74	1.69	1.68
Muskrat	11	13	8	20	3	5.31	3.61	2.10	3.49	3.28	2.72
Mole rat	7	13	8	21	1	2.06	1.16	0.33	1.87	1.75	1.55
Ehrenberg's mole-rat	8	13	8	20	1	1.34	0.27	-0.63	2.33	1.97	1.63
Kangaroo rat	5	15	8	20	2	-0.80	-1.66	-2.52	1.79	1.71	1.68
Eurasian beaver	11	14	6	21	2	6.32	4.62	3.10	3.51	3.29	2.72
Plains viscacha	8	13	8	17	4	1.34	0.27	-0.63	2.33	1.97	1.63
Casiragua	8	14	7	18	3	1.35	0.27	-0.63	2.36	1.98	1.63
Guinea pig	8	13	8	18	3	1.34	0.27	-0.63	2.33	1.97	1.63
Northern gundi	8	15	8	19	2	-0.63	-1.72	-2.63	2.40	2.00	1.64
Grey squirrel	7	16	7	21	2	1.10	0.17	-0.67	1.97	1.78	1.56
Ground squirrel	7	9	6	16	0						
Primates											
Tree shrew	8	14	8	20	2	1.35	0.27	-0.63	2.37	1.98	1.63
Thick-tailed bush baby	8	12	9	20	2	2.37	1.29	0.38	2.35	2.01	1.65
Potto	9	13	8	19	2	2.28	1.01	-0.17	2.60	2.48	2.22
Slow loris	8	12	8	19	2	2.37	1.29	0.38	2.34	2.00	1.64
Weasel lemur	10	13	8	20	2	3.51	2.04	0.77	3.14	2.77	2.26
Grey mouse lemur	9	12	9	20	2	2.65	1.40	0.41	2.81	2.23	1.73
Common woolly monkey	9	14	8	22	1	2.68	1.40	0.41	2.88	2.25	1.74
Douroucouli	10	14	8	22	1	3.57	2.12	0.86	3.10	2.72	2.31
Squirrel monkey	9	14	8	22	1	2.68	1.40	0.41	2.88	2.25	1.74
Brown capuchin	9	14	8	22	1	2.68	1.40	0.41	2.88	2.25	1.74
White tuft-ear marmoset	10	14	8	22	1	3.57	2.12	0.86	3.10	2.72	2.31
Rhesus monkey	9	14	8	20	2	2.24	0.99	-0.18	2.59	2.45	2.20
Crab-eating macaque	9	14	8	20	2	2.24	0.99	-0.18	2.59	2.45	2.20
Olive baboon	9	14	8	20	2	2.24	0.99	-0.18	2.59	2.45	2.20
Red guenon	9	14	8	20	2	2.24	0.99	-0.18	2.59	2.45	2.20
Hanuman langur	9	14	8	20	2	2.24	0.99	-0.18	2.59	2.45	2.20
Agile gibbon	9	14	8	20	2	2.11	0.66	-0.80	2.86	2.96	2.75
Siamang	9	14	8	20	2	2.11	0.66	-0.80	2.86	2.96	2.75
Orangutan	9	14	8	20	2	2.24	0.99	-0.18	2.59	2.45	2.20
Mountain gorilla	9	14	8	20	2	2.11	0.66	-0.80	2.86	2.96	2.75
Chimpanzee	10	14	8	20	2	2.96	1.30	-0.44	3.16	3.49	3.28
Human	9	14	8	20	2	2.11	0.66	-0.80	2.86	2.96	2.75

Table A3 Maximum muscle myoglobin (Mb) content, maximal dive duration and associated body mass, and whole muscle non- bicarbonate buffering capacity ($\beta_{muscleNB}$, between pH 6.0 -7.0) for mammalian species considered in this study. Species in blue boxes are diving species. Reference 19 refers to data determined in this study.

Species	Max Mb content (g 100 g^{-1} w.wt)	Muscle	Ref	Body Mass (kg)	Average maximum dive duration (s)	Ref	$\beta_{muscleNB}$ (µmol g^{-1} and pH (6-7))	Muscle	Ref
Echidna	1.35	Triceps	1						
Platypus	1.60	Triceps	2	1.25	60.0	35			
African elephant	0.460	Psoas	3						
Armadillo	0.845	Thigh	4						
Water shrew	1.10	Cardiac	5	0.0148	18.2	36	38.2	Skeletal	36
Short-tailed shrew	0.877	Cardiac	5				24.9	Skeletal	36
Star-nosed mole	1.44	Forelimb	6	0.0502	24.6	6	48.0	Hindlimb	6
Coast mole	1.21	Forelimb	6				38.9	Hindlimb	6
Little brown bat	0.519	Pectoralis	7						
Grey seal	5.40	Longissimus dorsi	8	217	1265	37	67.7	Longissimus Dorsi	19
Baikal seal	6.70	Posterior spinal muscles	9						
Harbour seal	4.97	Hind extremities	10	86.5	381	37	76.2	Longissimus Dorsi	20
Ringed seal	7.18	Longissimus dorsi	11	46.4	745	37			
Harp seal	9.70	Longissimus dorsi	12	76.5	1200	38	85.0	Longissimus Dorsi	44
Hooded seal	10.4	Longissimus dorsi	12	129	3120	39	81.5	Longissimus Dorsi	44
Bearded seal				350	812	37			
Weddell seal	7.24	Longissimus dorsi	13	415	2093	37	72.1	Longissimus Dorsi	20
Elephant seal	7.90	Longissimus dorsi	14	655	2519	37			
California sea lion	4.90	Dorsal triceps complex	15	111	463	37	61.2	Longissimus Dorsi	20
Steller sea lion	4.90	Pectoralis	16						
Australian sea lion	2.70	Locomotor muscle complex	17	125	360	37			
Northern fur seal	3.48	unknown	18	37.0	366	37			
Walrus	2.96	unknown	18	1900	762	37			
Raccoon	0.290	Forelimb	19				35.8	Forelimb	19
American mink				0.648	32.0	40			
River otter				7.00	35.0	37			
Polar bear				350	39.0	41			
Dog	0.610	Longissimus dorsi	20				50.2	Longissimus Dorsi	20
Cat	0.301	Left ventricle	7						
Horse	0.705	Psoas	21						
Alpaca	0.510	Sartorius	22						
Pig	0.450	Psoas	21				51.9	Adductor	20
Cow	0.600	Psoas	3				49.7	Temporalis	20
Water buffalo	0.393	Longissimus dorsi	23						
Sheep	0.350	Psoas	3						
Fin-back whale	3.73		24	56300	174	37			
Grey whale				31800	688	37			
Sei whale	0.910		25	23600	900	37			
Minke whale				7000	266	37	111	Skeletal	45
Bowhead	3.54	Longissimus dorsi	26	79400	3300	37	75.0	Longissimus Dorsi	46
Pygmy sperm whale	4.33	Longissimus dorsi	26				87.4	Longissimus Dorsi	46
Sperm whale	7.00	Longissimus dorsi	27	51700	3918	37			
Northern bottlenose whale	6.34	unknown	24	4750	3300	37			
Cuvier's beaked whale	4.32	Longissimus dorsi	26	2113	5220	37	94.5	Longissimus Dorsi	46
Narwhal	7.87		26	989	812	37			
Beluga	3.44	Longissimus dorsi	26	1600	820	37	74.2	Longissimus Dorsi	46
Harbour porpoise	4.26	Dorsal muscle	28	48.0	231	37	80.1	Longissimus Dorsi	46
Dall's porpoise				50.0	167	37			
White beaked dolphin	3.05	Longissimus dorsi	19				73.4	Longissimus Dosri	19
Risso's dolphin	2.61	Longissimus dorsi	19						
Long-fin pilot whale				1250	1140	37			
Bridled dolphin	2.54	Skeletal muscle	20	80.0	242	37	84.1	Longissimus Dors	46
Saddleback dolphin	3.55	Longissimus dorsi	26				89.6	Longissimus Dors	46
Bottlenose dolphin	2.66	Longissimus dorsi	26	200	480	37	69.1	Longissimus Dorsi	46
Rabbit	0.170	Cardiac	29						
House mouse	0.604	Cardiac	11						
Norway rat	0.546	Cardiac	11				30.2	Hindlimb	19
Muskrat	1.38	Skeletal muscle	30	0.910	120	42	50.9	Hindlimb	19
Ehrenberg's mole-rat	0.400	Masseter	31						
Eurasian beaver	1.30	Gastrocnemius	32	17.6	900	43			
Guinea pig	0.676	Right ventricle	33						
Human	0.645		34	70.0	48.0	37			

1 Hochachka et al., 1984
2 Evans et al., 1994
3 Lawrie, 1953
4 Seab and Burns, 1976
5 Campbell Unpublished
6 McIntyre et al., 2002
7 Schuder et al., 1979
8 Reed et al., 1994
9 Neshumova & Cherepanoa, 1985
10 Blessing & Hartschen-Niemeyer, 1969
11 O'Brien et al., 1992
12 Lestyk et al., 2009
13 Kanatous et al., 2008
14 Hassrick et al., 2010
15 Weise & Costa, 2007
16 Richmond et al., 2006
17 Fowler et al., 2007
18 Lenfant et al., 1970
19 This study
20 Castellini & Somero, 1981
21 Lawrie, 1950
22 Reynafarje et al., 1975
23 Dosi et al., 2006
24 Scholander, 1940
25 Tawara, 1950
26 Noren & Williams, 2000
27 Sharp & Marsh, 1953
28 Blessing, 1972
29 Bailey & Driedzic, 1992
30 MacArthur et al., 2001
31 Ar et al., 1977
32 McKean & Carlton, 1977
33 Leniger-Follert & Lubbers, 1973
34 Blessing, 1971
35 Kruuk, 1993
36 Gusztak Msc thesis, 2008
37 Halsey et al., 2006
38 Irving & Orr, 1935
39 Folkow et al., 2004
40 Schreer & Kovacs, 1997
41 Hays et al., 2007
42 Dyck & Romberg, 2007
43 MacArthur, 1992
44 Lestyk et al., 2009
45 Abe 1995
46 Noren, 2004

Table A4 Tissue sample information. Species marked with an asterisk were processed in the lab of Dr. Kevin Campbell, University of Maintoba

Species common name	Scientific name	Sex	Age	Muscle	Obtained from	Year Received	Location	Country	Sample provided by	Affiliation
Humpback whale	*Megaptera novaeangliae*	M	Juvenile	Longissimus dorsi	Stranding	2009	Greater London	England	Dr. Robert Deaville	Zoological Society of London
Risso's dolphin	*Grampus griseus*	M	Adult	Longissimus dorsi	Stranding	2009	Anglesey	England	Dr. Robert Deaville	Zoological Society of London
Northern-bottlenose whale	*Hyperoodon ampullatus*	F	Juvenile	Longissimus dorsi	Stranding	2009	Dorset	England	Dr. Robert Deaville	Zoological Society of London
White beaked dolphin	*Lagenorhynchus albirostris*	F	Adult	Longissimus dorsi	Stranding	2009	Cornwall	England	Dr. Robert Deaville	Zoological Society of London
Sowerby's beaked whale	*Mesoplodon bidens*	F	Juvenile	Longissimus dorsi	Stranding	2009	Silverdale Morecombe	England	Dr. Robert Deaville	Zoological Society of London
Pygmy hippo	*Choeropsis liberiensis*	?	Juvenile	Skeletal muscle	Post mortem	2010	Whipsnade zoo	England	Dr. Edmund Flach	Zoological Society of London
Common dolphin	*Delphinus delphis*	F	Juvenile	Longissimus dorsi	Stranding	2009	Cornwall	England	Dr. James Barnett & Dr. Nick Davisor	Veterinary Laboratories Agency
Harbour porpoise	*Phocoena phocoena*	M	Juvenile	Longissimus dorsi	Stranding	2009	Cornwall	England	Dr. James Barnett & Dr. Nick Davisor	Veterinary Laboratories agency
Bowhead *	*Balaena mysticetus*	?		Skeletal muscle	Biopsy	2010	Wakeham Bay	Canada	Dr. Kevin Campbell	University of Manitoba
Narwhal *	*Monodon monoceros*	?		Skeletal muscle	Biopsy	2010	Arctic Bay	Canada	Dr. Kevin Campbell	University of Manitoba
Minke whale	*Balaenoptera acutorostrata*	M	Juvenile	Skeletal muscle	Stranding	2009	Merseyside	England	Scott Mirceta	University of Liverpool
Cow	*Bos taurus*	F	Adult	Left ventricle	Slaughter house	2008	Merseyside	England	Lee Moore	University of Liverpool
Sheep	*Ovis aries Sus*	F	Juvenile	Left ventricle	Slaughter house	2008	Merseyside	England	Lee Moore	University of Liverpool
Pig	*scrofa Sorex*	?	Adult	Left ventricle	Slaughter house	2008	Merseyside	England	Lee Moore	University of Liverpool
Water shrew	*palustris*	?	Various	Fore/Hind limb	Trapping	2008	Manitoba	Canada	Dr. Kevin Campbell	University of Manitoba
Short-tailed shrew	*Blarina brevicauda*	?	Various	Fore/Hind limb	Trapping	2008	Manitoba	Canada	Dr. Kevin Campbell	University of Manitoba
Star-nosed mole	*Condylura cristata*	?	Various	Fore/Hind limb	Trapping	2008	Manitoba	Canada	Dr. Kevin Campbell	University of Manitoba
Coast mole	*Scapanus orarius*	?	Various	Fore/Hind limb	Trapping	2008	Manitoba	Canada	Dr. Kevin Campbell	University of Manitoba
Muskrat	*Ondatra zibethicus*	?	Various	Fore/Hind limb	Trapping	2008	Manitoba	Canada	Dr. Kevin Campbell	University of Manitoba
Grey squirrel	*Sciurus carolinensis*	F	Adult	Hind limb	Trapping	2008	Merseyside	England	Dr. Julian Chantrey	University of Liverpool
American mink	*Neovison vison*	?		Hind limb Fore	Trapping	2008	Aberdeen	Scotland	Prof. Xavier Lambin	University of Aberdeen
Raccoon	*Procyon lotor*	F	Adult	limb Masseter	Hunting	2010	Hövelriege	Germany	Heinz Pauleickhoff	Hunter
Polar bear	*Ursus maritimus*	M	Adult	Longissimus dorsi	Hunting	2009	Skagafjordur	Iceland	Dr. Einar Arnason	University of Iceland
Grey seal	*Halichoerus grypus*	M	Adult	Skeletal muscle	Stranding	2009	Isle Of May	Scotland	Dr. Dominic McCafferty	University of Glasgow
Ringed seal	*Phoca hispida*	?		Skeletal muscle	Biopsy	2010	Alaska	USA	Dr. Jennifer Burns	University of Alaska
Harp seal	*Pagophilus groenlandicus*	F	Adult	Skeletal muscle	Biopsy	2010	Gulf of St. Lawrence	Canada	Dr. Jennifer Burns	University of Alaska
Hooded seal	*Cystophora cristata*	F	Adult	Skeletal muscle	Biopsy	2010	Gulf of St. Lawrence	Canada	Dr. Jennifer Burns	University of Alaska
Bearded seal	*Erignathus barbatus*	?	Adult	Skeletal muscle	Biopsy	2010	Alaska	USA	Dr. Jennifer Burns	University of Alaska
Weddell seal	*Leptonychotes weddellii*	M	Pup	Skeletal muscle	Biopsy	2010	Big Razorback Island	Antarctica	Dr. Jennifer Burns	University of Alaska
Northern elephant seal	*Mirounga angustirostris*	?	Pup		Biopsy	2010	Alaska	USA	Dr. Jennifer Burns	University of Alaska
Californian sea lion	*Zalophus californianus*	?	Neonate	Skeletal muscle	Biopsy	2010	Alaska	USA	Dr. Jennifer Burns	University of Alaska
Steller sea lion	*Eumetopias jubatus*	?	Adult	Skeletal muscle	Biopsy	2010	Alaska	USA	Dr. Jennifer Burns	University of Alaska
Australian sea lion	*Neophoca cinerea*	F	Adult	Skeletal muscle	Biopsy	2010	Alaska	USA	Dr. Jennifer Burns	University of Alaska
Northern fur seal	*Callorhinus ursinus*	F	Adult	Skeletal muscle	Biopsy	2010	Alaska	USA	Dr. Jennifer Burns	University of Alaska
Walrus	*Odobenus rosmarus*	M	Adult	Skeletal muscle	Biopsy	2010	Alaska	USA	Dr. Jennifer Burns	University of Alaska

Table A5 Volume of oxygen (O_2) stored in the lung of mammalian species and the duration lung O_2 could support basal metabolic rate (BMR). Measured BMR was used where possible. If data was not available then BMR was calculated from the allometric equation $3.03M^{0.72}$ (White et al., 2009). In order to calculate lung O_2, measured lung volume was used if available, if not then lung volume was calculated by allometry $65.6M^{1.02}$ (Stahl 1967). Data obtained by allometry are highlighted in blue text. Diving species are indicated by blue boxes. Although myoglobin sequence was taken from the Eurasian beaver, lung O2 data was obtained for the closely related Canadian beaver, *Castor canadensis*.

Species	Body mass (kg)	Measured BMR (ml O_2 s^{-1})	Ref	Measured lung vol (ml kg^{-1})	Ref	Average Body mass (Kg)	Allometry BMR (ml O_2 s^{-1})	Lung volume (ml)	VO_{2Lung} (ml O2)	VO_{2Lung}/ BMR (s)
Echidna	2.73	0.120	1			4.22	0.343	246	23.8	199
African elephant	3670	118	2			2430	33.3	207000	47100	398
Armadillo	3.51	0.240	3			4.00	0.330	233	30.61	127
Etruscan shrew	0.00220	0.00194	4			0.00180	0.00129	0.0659	0.0121	6.24
Water shrew	0.0146	0.0194	5			0.0125	0.00520	0.516	0.096	4.97
Short-tailed shrew	0.0243	0.0319	6			0.0169	0.00645	0.709	0.116	3.65
Star-nosed mole	0.0509	0.0306	7			0.0455	0.0132	2.02	0.621	20.3
Coast mole	0.0641	0.0229	8			0.0612	0.0163	2.77	0.468	20.5
Little brown bat	0.0052	0.0072	9			0.0080	0.00375	0.319	0.0312	4.32
Harbour seal	86.8	8.37	10	81.3	21	107	3.51	7550	1060	127
Ringed seal	46.6	3.92	10	79.4	22	75.8	2.74	5260	555	141
Weddell seal	389	20.7	11	27.3	23	367	8.54	28000	1590	76.8
California sea lion	73.0	8.86	11	103	24	111	3.61	7880	11300	1270
Raccoon	4.82	0.607	12			7.32	0.510	441	43.6	71.9
Black bear	130	4.24	13			110	3.60	7840	1380	325
Polar bear	181	6.19	14			225	6.00	16600	2010	325
Dog	20.8	1.83	2			12.9	0.768	806	195	106
Cat	3.00	0.367	2			3.41	0.294	196	25.9	70.7
Pig	150	6.46	2			86.2	3.01	6020	1573	244
Cow	445	16.3	2			269	6.83	20100	5000	307
Sheep	46.6	3.12	2			33.8	1.53	2230	462	148
Minke whale						7240	73.1	660000	99000	1350
Harbour porpoise	28.5	5.38	15			62.0	2.37	4250	293	54.4
Killer whale	3220	108	11			3880	46.7	341000	42400	392
Bottlenose dolphin	149	16.1	11			180	5.11	13100	1630	101
Rabbit	3.37	0.385	2			1.52	0.165	83.6	27.7	72.0
House mouse	0.0210	0.0087	2			0.0174	0.00658	0.730	0.132	15.2
Norway rat	0.282	0.0678	2			0.320	0.0535	16.0	2.11	31.2
Muskrat	0.681	0.191	16	58.2	25	1.04	0.125	55.9	5.95	31.2
Ehrenberg's mole-rat	0.135	0.0319	17			0.176	0.0348	8.48	0.976	30.6
Eurasian beaver	15.3	1.29	18	60.5	26	17.6	0.961	1120	139	107
Guinea pig	0.629	0.0961	19			0.728	0.0968	38.2	4.95	51.5
Human	70.0	4.17	20			59.7	2.31	4080	718	172

1 McNab, 1984
2 Klieber, 1947
3 McNab, 1980
4 Frey, 1980
5 Gusztak Msc thesis, 2008
6 Hindle et al., 2003
7 McIntyre et al., 2002
8 Campbell unpublished
9 Geiser, 1988
10 Ochoa-Acuna et al., 2009
11 Williams et al., 2001
12 Mugaas et al., 1993
13 Watts & Cuyler, 1988
14 Watts et al., 1987
15 Kanwisher & Sundnes, 1965
16 Campbell et al., 1998
17 Haim & Izhaki, 1993
18 MacArthur, 1989
19 Arends & McNab, 2001
20 Blakemore & Jennett, 2001
21 Burns et al., 2005
22 Lydersen et al., 1992
23 Burns & Castellini, 1996
24 Kooyman & Sinnett,1982
25 MacArthur et al., 2001
26 McKean & Carlton, 1977

Table A6 Volume of oxygen (O_2) stored in the blood of mammalian species and the duration blood O_2 could support basal metabolic rate (BMR). Measured BMR was used where possible. If data was not available then BMR was calculated from the allometric equation $3.03M^{0.72}$ (White et al., 2009). In order to calculate blood O_2, measured blood volume was used if available, if not then blood volume was calculated by allometry $65.6M^{1.02}$ (Stahl 1967). Data obtained by allometry are highlighted in blue text. Diving species are indicated by blue boxes. Although myoglobin sequence was taken from the Eurasian beaver, lung O2 data was obtained for the closely related Canadian beaver, *Castor canadensis*.

Species	Body mass (kg)	Measured BMR (ml O_2 s^{-1})	Ref	Measured blood vol (ml kg^{-1})	Ref	Average Body mass (kg)	Allometry BMR (ml O_2 s^{-1})	Allometry blood volume (ml kg^{-1})	Hb (g 100 ml^{-1})	Ref	Arterial O_2 (ml kg^{-1})	Venous O_2 (ml kg^{-1})	VO_{2Blood} (ml O_2)	VO_{2Blood} / BMR (s)
Echidna	2.73	0.120	1			4.22	0.34	67.5	17.2	23	4.88	7.70	34.3	286
African elephant	3670	118	2			2420	33.27	76.7	15.3	24	4.93	7.78	46700	395
Armadillo	3.51	0.240	3			4.00	0.33	67.4	10.3	24	2.92	4.61	26.4	110
Etruscan shrew	0.00220	0.00194	4			0.00180	0.00	57.8	17.4	25	4.23	6.67	0.0196	10.1
Water shrew	0.0146	0.0194	5	76.0	16	0.0125	0.01	60.1	20.9	8	6.67	10.5	0.251	13.0
Short-tailed shrew	0.0243	0.0319	6	76.0	16	0.0169	0.01	60.5	17.3	8	5.52	8.72	0.345	10.8
Star-nosed mole	0.0509	0.0306	7	76.0	17	0.0455	0.01	61.7	17.2	17	5.49	8.67	0.721	23.5
Coast mole	0.0641	0.0229	8	76.0	17	0.0612	0.02	62.0	17.4	17	5.56	8.77	0.918	40.2
Harbour seal	86.8	8.37	9	132	18	107	3.51	72.0	21.2	18	11.7	18.5	1410	168
Ringed seal	46.6	3.92	9			75.8	2.74	71.5	24.9	26	7.48	11.8	2880	736
Weddell seal	389	20.7	10	210	19	367	8.54	73.8	26.0	19	22.9	36.2	43200	2080
California sea lion	73.0	8.86	10	96.0	19	111	3.61	110	18.0	27	7.26	11.5	2080	235
Dog	20.8	1.83	2			12.9	0.77	69.0	16.4	28	4.75	7.49	255	139
Cat	3.00	0.367	2			3.41	0.29	67.2	11.7	29	3.30	5.22	25.6	69.8
Pig	150	6.46	2			86.2	3.01	71.7	10.1	30	3.04	4.80	1180	182
Sheep	46.6	3.12	2			33.8	1.53	70.4	12.7	31	3.76	5.93	451	145
Minke whale						7240	73.10	78.4	18.3	32	6.01	9.49	112000	1530
Harbour porpoise	28.5	5.38	11			62.0	2.37	71.2	19.3	33	5.78	9.12	425	78.9
Killer whale	3220	108	10			3880	46.67	77.4	16.3	34	5.30	8.37	44000	407
Bottlenose dolphin	149	16.1	10	71.0	20	180	5.11	72.8	14.4	20	4.30	6.78	1650	102
Rabbit	3.37	0.385	2			1.52	0.16	66.2	14.8	35	4.11	6.49	35.7	92.8
House mouse	0.0210	0.00868	2			0.0174	0.01	60.5	17.7	36	4.50	7.10	0.244	28.1
Norway rat	0.282	0.0678	2			0.320	0.05	64.1	14.2	37	3.83	6.04	2.78	41.1
Muskrat	0.681	0.191	12	117	21	1.04	0.13	65.7	18.0	21	8.83	13.9	15.5	81.4
Ehrenberg's mole-rat	0.135	0.0319	13			0.176	0.03	63.4	15.0	38	3.99	6.30	1.39	43.6
Eurasian beaver	15.3	1.29	14	64.6	22	17.6	0.96	69.5	12.4	22	3.37	5.31	133	103
Human	70.0	4.17	15	75.2	39	59.7	2.31	71.2	14.7	39	4.65	7.34	715	172

1 McNab, 1984
2 Klieber, 1947
3 McNab, 1980
4 Frey, 1980
5 Gusztak Msc thesis, 2008
6 Hindle et al., 2003
7 McIntyre et al., 2002
8 Campbell unpublished
9 Ochoa-Acuna et al., 2009
10 Williams et al., 2001
11 Kanwisher & Sundnes, 1965
12 Campbell et al., 1998
13 Haim & Izhaki, 1993
14 MacArthur, 1989
15 Blakemore & Jennett, 2001
16 Campbell unpublished
17 McIntyre et al., 2002
18 Lenfant, 1970
19 Ponganis et al., 1993
20 Ridgway & Johnston, 1966
21 MacArthur et al., 2001
22 Mckean & Carlton, 1977
23 Parer and Metcalf, 1967
24 Dhindsa et al., 1972
25 Bartels et al., 1979
26 Lydersen et al., 1992
27 Ponganis et al., 1997
28 Bristow et al., 1977
29 Dhindsa et al., 1974a
30 Novy et al., 1973
31 Ullery et al., 1965
32 Brix et al., 1989
33 Reed et al., 2000
34 Dhindsa et al., 1974b
35 Neuberger & Niven, 1951
36 Maclean & Lee, 1973
37 Dhindsa et al., 1981
38 Ar et al., 1977
39 Kanstrup & Ekblom, 1984

Table A7 Volume of oxygen (O2) stored in the muscles of mammalian species and the duration muscle O2 could support basal metabolic rate (BMR). Measured BMR was used where possible. If data was not available then BMR was calculated from the allometric equation 3.03M 0.72 (White et al., 2009) these data are highlighted in blue text. Myoglobin (Mb) contents are maximum values reported for skeletal muscle, cardiac values used to allow inclusion of a few species are highlighted in green text. Average maximum dive duration data and associated body mass were used in conjunction with Mb net charge in order to model average maximum dive durations (see **Chapter 5**). Species highlighted with red text have incomplete Mb sequence and diving species are indicated by blue boxes.

Species	Body mass (kg)	Measured BMR (ml O_2 s^{-1})	Ref	Average Body mass (kg)	Allometry BMR (ml O_2 s^{-1})	Skeletal muscle Mb (g 100 g^{-1})	Ref	$VO_{2muscle}$/ BMR (s)	Average maximum dive duration (s)	Body mass dive time (kg)	Ref	Mb net charge pH 6.5
Echidna	2.73	0.120	1	4.22	0.343	1.35	21	134				2.40
Platypus	1.30	0.130	2	1.76	0.183	1.60	22	69.8	60.0	1.25	55	2.29
African elephant	3670	118	3	2420	33.3	0.46	23	62.4				2.12
Armadillo	3.51	0.240	4	4.00	0.330	0.85	24	53.9				-0.06
Etruscan shrew	0.00220	0.00194	5	0.00180	0.00129	0.19	25	0.948				
Water shrew	0.0146	0.0194	6	0.0125	0.00520	0.60	9	2.46	18.2	0.0148	6	2.65
Short-tailed shrew	0.0243	0.0319	7	0.0169	0.00645	0.30	9	0.845				0.15
Star-nosed mole	0.0509	0.0306	8	0.0455	0.0132	1.44	8	13.0	24.6	0.0502	8	3.15
Coast mole	0.0641	0.0229	9	0.0612	0.0163	1.21	8	19.2				1.16
Little brown bat	0.00520	0.00722	10	0.00796	0.00375	0.52	26	1.63				-2.33
Grey seal				193	5.38	5.40	27	846	1270	217	56	4.34
Baikal seal				28.5	1.36	6.70	28	614				5.34
Harbour seal	86.8	8.37	11	107	3.51	4.97	29	225	381	86.5	56	4.34
Ringed seal	46.6	3.92	11	75.8	2.74	7.18	30	372	745	46.4	56	4.34
Harp seal	150	9.51	11	130	4.04	9.70	31	665	1200	76.5	57	4.34
Hooded seal				375	8.68	10.40	31	1960	3120	129	58	4.34
Bearded seal				248	6.43				812	350	56	4.21
Weddell seal	389	20.7	12	367	8.54	7.24	32	620	2090	415	56	4.34
Elephant seal				655	13.0	7.90	33	1740	2520	655	56	4.21
California sea lion	73.0	8.86	12	111	3.61	4.90	34	1760	463	111	56	4.22
Steller sea lion				273	6.91	4.90	35	845				4.22
Australian sea lion				300	7.39	2.70	36	478	360	125	56	4.22
Northern fur seal				169	4.88	3.48	37	524	366	37.0	56	4.22
Walrus				1060	18.3	2.96	37	747	762	1900	56	4.08
Raccoon	4.82	0.607	13	7.32	0.510	0.29	38	10.1				1.27
American mink				1.03	0.125				32.0	0.648	59	1.27
River otter				7.61	0.524				35.0	7.00	56	2.27
Polar bear	181	6.19	14	225	6.00				39.0	350	60	1.99
Dog	20.8	1.83	3	12.9	0.768	0.61	39	30.2				1.54
Cat	3.00	0.367	3	3.41	0.294	0.30	26	10.7				1.97
Horse				250	6.48	0.80	40	135				1.76
Alpaca				80.0	2.85	0.51	41	62.4				
Pig	150	6.46	3	86.2	3.01	0.45	40	45.6				-0.01
Cow	445	16.3	3	269	6.83	0.60	23	71.5				1.84
Water buffalo				478	10.3	0.39	42	79.3				0.85
Sheep	46.6	3.12	3	33.8	1.53	0.35	23	22.8				0.56
Fin-back whale				62500	345	3.73	43	2950	174	56300	56	3.01
Grey whale				27880	193				688	31800	56	3.72
Sei whale				36667	235	0.91	44	619	900	23600	56	3.72
Blue whale				136000	604	0.84	45	825	468	101000	45	
Minke whale				7235	73.1				266	7000	56	4.01
Bowhead				110000	519	3.54	46	3280	3300	79400	56	3.72
Pygmy sperm whale				356	8.35	4.33	46	804				4.31
Sperm whale				29000	199	5.67	43	3610	3920	51700	56	4.22
Northern bottlenose whale				6650	68.8	6.34	43	2670	3300	4750	56	5.10
Cuvier's beaked whale				3752	45.6	4.32	46	1550	5220	2110	56	5.10
Narwhal				1200	20.1	7.87	46	2060	812	989	56	4.04
Beluga				852	15.7	3.44	46	816	820	1600	56	
Harbour porpoise	28.5	5.38	15	61.98	2.37	4.03	47	93.1	231	48.0	56	4.05
Dall's porpoise				119.90	3.82				167	50.0	56	4.05
Long-fin pilot whale				2960.00	38.4				1140	1250	56	4.04
Bridled dolphin				68.33	2.55	2.54	39	297	242	80.0	56	4.04
Saddleback dolphin				68.20	2.54	3.55	46	415				4.04
Bottlenose dolphin	148.6	16.1	12	179.50	5.11	2.66	43	107	480	200	56	4.04
Rabbit	3.37	0.385	3	1.52	0.165	0.17	48	6.49				0.32
House mouse	0.0210	0.00868	3	0.02	0.00657	0.19	49	2.01				-0.18
Norway rat	0.282	0.0678	3	0.32	0.0535	0.31	48	5.63				0.33
Muskrat	0.681	0.191	16	1.04	0.125	1.38	50	28.5	120	0.910	61	3.61
Ehrenberg's mole-rat	0.135	0.0319	17	0.18	0.0348	0.40	51	7.39				0.27
Eurasian beaver	15.3	1.29	18	17.63	0.961	1.30	52	67.0	900	17.6	62	4.62
Guinea pig	0.629	0.0961	19	0.73	0.0968	0.68	53	19.3				0.27
Human	70.0	4.17	20	58.70	2.31	0.65	54	47.3	48.0	70.0	56	0.66

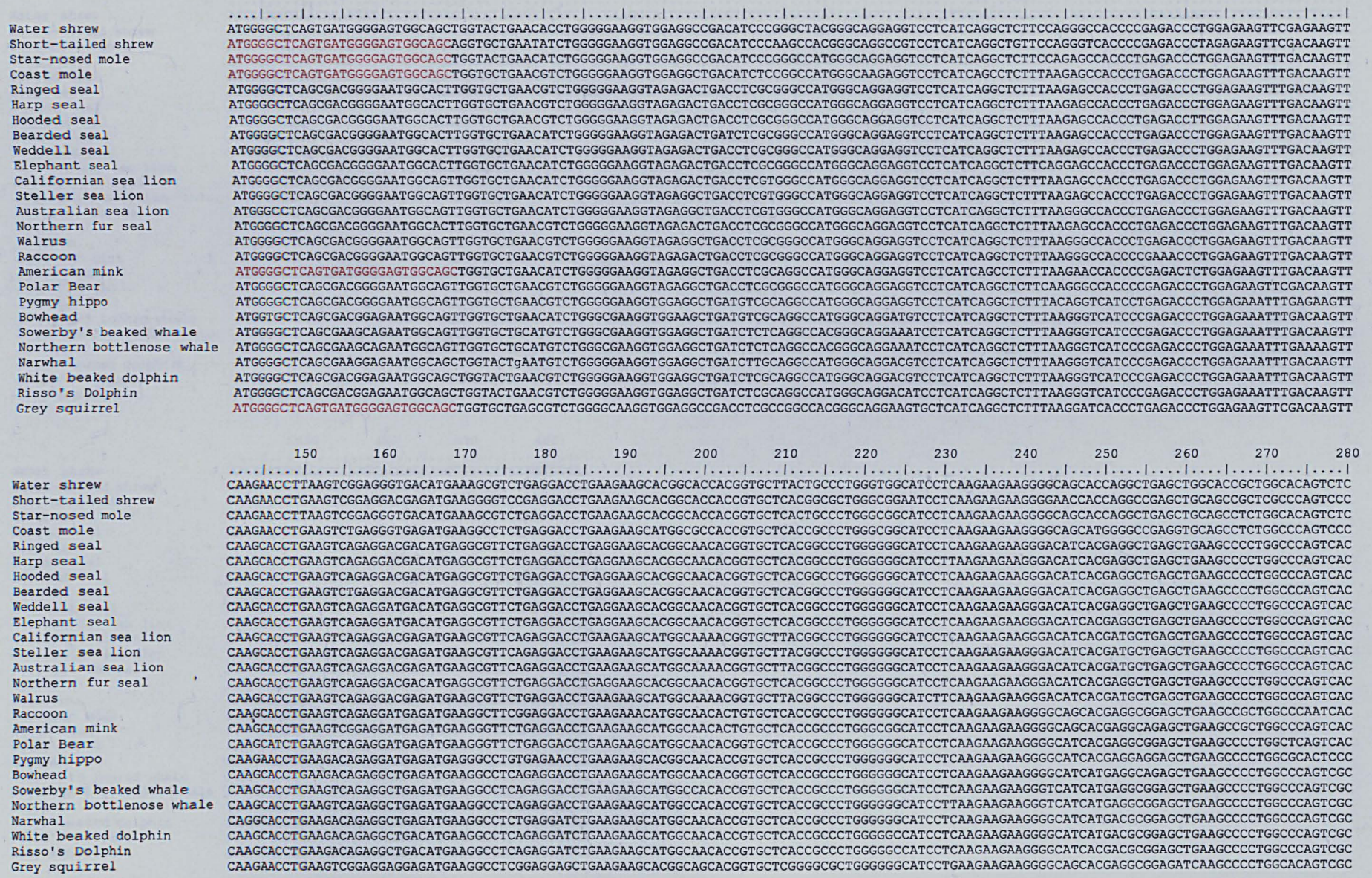
1 McNab, 1984
2 Grant & Dawson, 1978
3 Kleiber, 1947
4 McNab, 1980
5 Frey, 1980
6 Gusztak Msc thesis, 2008
7 Hindle et al., 2003
8 McIntyre et al., 2002
9 Campbell unpublished
10 Geiser, 1988
11 Ochoa-Acuna et al., 2009
12 Williams et al., 2001
13 Mogas et al., 1993
14 Watts et al., 1987
15 Kanwisher & Sundnes, 1965
16 Campbell et al., 1998
17 Haim & Izhaki, 1993
18 MacArthur, 1989
19 Arends & McNab, 2001
20 Blakemore & Jennett, 2001
21 Hochachka et al., 1984
22 Evans et al., 1994
23 Lawrie, 1952
24 Seab and Burns., 1976
25 Peters et al., 1999
26 Schuder et al., 1979
27 Reed et al., 1994
28 Neshumova et al., 1983
29 Blessing & Hartschen-Niemeyer, 1969
30 O'Brien et al., 1992
31 Lestyk et al., 2009
32 Kanatous et al., 2008
33 Hassrick et al., 2010
34 Weise & Costa, 2007
35 Richmond et al., 2006
36 Fowler et al., 2007
37 Lenfant et al., 1970
38 This study
39 Castellini & Somero, 1981
40 Lawrie, 1950
41 Reynafarje et al., 1975
42 Dosi et al., 2006
43 Scholander, 1940
44 Tawara, 1950
45 Croll et al., 2001
46 Noren & Williams, 2000
47 Blessing, 1972
48 Bailey & driedzic, 1992
49 Ordway & Garry, 2004
50 MacArthur et al., 2001
51 Ar et al., 1977
52 McKean & Carlton, 1977
53 Leniger-Follert & Lubbers, 1973
54 Blessing, 1971
55 Kruuk, 1993
56 Halsey et al., 2006
57 Folkow et al., 2004
58 Schreer & Kovacs, 1997
59 Hays et al., 2007
60 Dyck & Romberg, 2007
61 MacArthur, 1992
62 Irving & Orr, 1935

Table A8 Myoglobin sequences used to calculate the average myoglobin molecular weight. Molecular weight from ExPASy is based on amino acid sequence only.

Species	Accession	Molecular weight	Source
Sperm whale	P02185	17,331	ExPASy
Horse	P68082	17,083	ExPASy
Saddleback dolphin	P68276	17,188	ExPASy
Dwarf sperm whale	P02184	17,368	ExPASy
Harbour seal	P68080	17,428	ExPASy
Harbour porpoise	P68278	17,232	ExPASy
Hubb's beaked whale	P02183	17,267	ExPASy
Grey seal	P68081	17,428	ExPASy
Hedgehog	P02156	17,115	ExPASy
Aardvark	P02164	17,144	ExPASy
Plains viscacha	P04250	17,011	ExPASy
Human	P02144	17,184	ExPASy
Pig	P02189	17,085	ExPASy
River otter	P11343	17,168	ExPASy
Mole rat	A9JIH7	17,077	ExPASy
Northern gundi	P20856	17,075	ExPASy
Spiny rat	P04249	16,999	ExPASy
Weasel lemur	P02169	17,074	ExPASy
Hanuman langur	P68085	17,148	ExPASy
Tree shrew	P02165	17,099	ExPASy
Dog	P63113	17,337	ExPASy
Mouse	P04247	17,070	ExPASy
Badger	P02157	17,097	ExPASy
Muskrat	P32428	17,268	ExPASy
Rat	Q9QZ76	17,157	ExPASy
Rabbit	P02170	17,221	ExPASy
Thick-tailed galago	P02168	17,102	ExPASy
Eygptian fruit bat	P02163	17,079	ExPASy
	Average	**17,173**	

Table A9 Measured muscle mass percentages used to calculate the average muscle mass used throughout this study. A = adult, F = female

Species	muscle mass %	Age/ sex	Source
Harp seal	25.6	A/F	Lestyk et al., 2009
Hooded seal	28.0	A/F	Lestyk et al., 2009
California sea lion	37.0	?	Kooyman & Ponganis, 1998
Weddell seal	35.0	?	Kooyman & Ponganis, 1998
Atlantic bottlenose dolphin	30.0	?	Kooyman & Ponganis, 1998
Sperm whale	34.0	?	Kooyman & Ponganis, 1998
Rat (Fisher-344)	35.6	?	Delp et al., 1998
Human	36.0	A/F	Marieb & Hoehn, 2007
Rhesus monkey	39.6	A/F	Grand, 1977
Cat	38.3	A	Grand, 1977
Koala	34.0	A	Grand & Barboza, 2001
Sloth	23.0	A	Grand & Barboza, 2001
Bovids (Av. 8 species)	41.5	A/F	Grand, 1997
Average	**33.66**		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Short-tailed shrew ATGGGGCTCAGTGATGGGGAGTGGCAGCAGGTGCTGAATATCTGGGGGAAGGTGGAGGCCGACATCCCAAGCCACGGGCAGGCCGTCCTCATCAGGCTGTTCCAGGGTCACCCCGAGACCCTAGAGAAGTTCGACAAGTT
Star-nosed mole ATGGGGCTCAGTGATGGGGAGTGGCAGCTGGTACTGAACATCTGGGGGAAGGTGGAGGCCGACATCCCGGGCCATGGGCAGGAGGTCCTCATCAGGCTCTTCCAGAGCCACCCTGAGACCCTGGAGAAGTTTGACAAGTT
Coast mole ATGGGGCTCAGTGATGGGGAGTGGCAGCTGGTGCTGAACGTCTGGGGGAAGGTGGAGGCTGACATCTCCGGCCATGGGCAAGAGGTCCTCATCAGCCTCTTTAAGAGCCACCCTGAGACCCTGGAGAAGTTTGACAAGTT
Ringed seal ATGGGGCTCAGCGACGGGGAATGGCACTTGGTGCTGAACGTCTGGGGGAAGGTAGAGACTGACCTCGCGGGCCATGGGCAGGAGGTCCTCATCAGGCTCTTTAAGAGCCACCCTGAGACCCTGGAGAAGTTTGACAAGTT
Harp seal ATGGGGCTCAGCGACGGGGAATGGCACTTGGTGCTGAACGTCTGGGGGAAGGTAGAGACTGACCTCGCGGGCCATGGGCAGGAGGTCCTCATCAGGCTCTTTAAGAGCCACCCTGAGACCCTGGAGAAGTTTGACAAGTT
Hooded seal ATGGGGCTCAGCGACGGGGAATGGCACTTGGTGCTGAACGTCTGGGGGAAGGTAGAGACTGACCTCGCGGGCCATGGGCAGGAGGTCCTCATCAGGCTCTTTAAGAGCCACCCTGAGACCTTGGAGAAGTTTGACAAGTT
Bearded seal ATGGGGCTCAGCGACGGGGAATGGCACTTGGTGCTGAACATCTGGGGGAAGGTAGAGACTGATCTCGCGGGCCATGGGCAGGAGGTCCTCATCAGGCTCTTTAAGAGCCACCCTGAGACCCTGGAGAAGTTTGACAAGTT
Weddell seal ATGGGGCTCAGCGACGGGGAATGGCACTTGGTGCTGAACATCTGGGGGAAGGTAGAGACTGACCTCGCGGGCCATGGGCAGGAGGTCCTCATCAGGCTCTTTAAGAGCCACCCTGAGACCCTGGAGAAGTTTGACAAGTT
Elephant seal ATGGGGCTCAGCGACGGGGAATGGCACTTGGTGCTGAACATCTGGGGGAAGGTAGAGACTGACCTCGCGGGCCATGGGCAGGAGGTCCTCATCAGGCTCTTCAGGAGCCACCCTGAGACCCTGGAGAAGTTTGACAAGTT
Californian sea lion ATGGGGCTCAGCGACGGGGAATGGCAGTTGGTGCTGAACATCTGGGGGAAGGTAGAGACTGACCTCGTGGGCCATGGGCAGGAGGTCCTCATCAGGCTCTTTAAGAGCCACCCTGAGACCCTGGAGAAGTTTGACAAGTT
Steller sea lion ATGGGGCTCAGCGACGGGGAATGGCAGTTGGTGCTGAACATCTGGGGGAAGGTAGAGGCTGACCTCGTGGGCCATGGGCAGGAGGTCCTCATCAGGCTCTTTAAGAGCCACCCTGAGACCCTGGAGAAGTTTGACAAGTT
Australian sea lion ATGGGCCTCAGCGACGGGGAATGGCAGTTGGTGCTGAACATCTGGGGGAAGGTAGAGGCTGACCTCGTGGGCCATGGGCAGGAGGTCCTCATCAGGCTCTTTAAGGGCCACCCTGAGACCCTGGAGAAGTTTGACAAGTT
Northern fur seal ATGGGGCTCAGCGACGGGGAATGGCACTTGGTGCTGAACGTCTGGGGGAAGGTAGAGACTGACCTCGCGGGCCATGGGCAGGAGGTCCTCATCAGGCTCTTTAAGAGCCACCCTGAGACCCTGGAGAAGTTTGACAAGTT
Walrus ATGGGGCTCAGCGACGGGGAATGGCAGTTGGTGCTGAACGTCTGGGGGAAGGTAGAGGCTGACCTCGCGGGCCATGGGCAGGAGGTCCTCATCAGGCTCTTTAAGGGCCACCCTGAGACCCTGGAGAAGTTTGACAAGTT
Raccoon ATGGGGCTCAGCGACGGGGAATGGCAGTTGGTGCTGAACGTCTGGGGGAAGGTAGAGACTGACCTCGCGGGCCATGGGCAGGAGGTCCTCATCAGGCTCTTTAAGGGCCACCCCGAAACCCTGGAGAAGTTTGACAAGTT
American mink ATGGGGCTCAGTGATGGGGAGTGGCAGCTGGTGCTGAACATCTGGGGGAAGGTAGAGGCTGACCTCGCAGGCCATGGGCAGGAGGTCCTCATCAGCCTCTTTAAGAACCACCCCGAGACTCTGGAGAAGTTTGACAAGTT
Polar Bear ATGGGGCTCAGCGACGGGGAATGGCAGTTGGTGCTGAACGTCTGGGGGAAGGTAGAGGCTGACCTCGCGGGCCATGGGCAGGAGGTCCTCATCAGGCTCTTCAAGGGCCACCCCGAGACCCTGGAGAAGTTCGACAAGTT
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Northern bottlenose whale ATGGGGCTCAGCGAAGCAGAATGGCAGTTGGTGCTGCATGTCTGGGCGAAGGTGGAGGCTGATCTCTCAGGCCACGGGCAGGAAATCCTCATCAGGCTCTTTAAGGGTCATCCCGAGACCCTGGAGAAATTTGAAAAGTT
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Grey squirrel ATGGGGCTCAGTGATGGGGAGTGGCAGCTGGTGCTGAGCGTCTGGGGCAAGGTGGAGGCCGACCTCGCCGGCCACGGGCAGGAAGTGCTCATCAGGCTCTTTAAGGATCACCCTGAGACCCTGGAGAAGTTCGACAAGTT
150 160 170 180 190 200 210 220 230 240 250 260 270 280
Water shrew CAAGAACCTTAAGTCGGAGGGTGACATGAAAGCGTCTGAGGACCTGAAGAAGCACGGCACCACGGTGCTTACTGCCCTGGGTGGCATCCTCAAGAAGAAGGGGCAGCACCAGGCTGAGCTGGCACCGCTGGCACAGTCTC
Short-tailed shrew CAAGAACCTGAAGTCGGAGGACGAGATGAAGGGGTCCGAGGACCTGAAGAAGCACGGCACCACCGTGCTCACGGCGCTGGGCGGAATCCTCAAGAAGAAGGGGGAACCACCAGGCCGAGCTGCAGCCGCTCGCCCAGTCCC
Star-nosed mole CAAGAACCTTAAGTCGGAGGGTGACATGAAAGCGTCTGAGGACCTGAAGAAGCACGGCACCACGGTGCTCACTGCCCTGGGCGGCATCCTCAAGAAGAAGGGGCAGCACCAGGCTGAGCTGCAGCCTCTGGCACAGTCTC
Coast mole CAAGAACCTGAAGTCTGAGGGTGAGATGAAGGCCTCTGAGGACCTGAAGAAGCATGGCGCCACCGTGCTCACCGCCCTGGGCGGCATCCTCAAGAAGAAGGGGCGAGCATGGGGCCGAGGTGCAGCCTCTGGCCCAGTCCC
Ringed seal CAAGCACCTGAAGTCAGAGGACGACATGAGGCGTTCTGAGGACCTGAGGAAGCACGGCAACACGGTGCTCACGGCCCTGGGGGGCATCCTCAAGAAGAAGGGACATCACGAGGCTGAGCTGAAGCCCCTGGCCCAGTCAC
Harp seal CAAGCACCTGAAGTCAGAGGACGACATGAGGCGTTCTGAGGACCTGAGGAAGCACGGCAACACGGTGCTCACGGCCCTGGGGGGCATCCTTAAGAAGAAGGGACATCACGAGGCTGAGCTGAAGCCCCTGGCCCAGTCAC
Hooded seal CAAGCACCTGAAGTCAGAGGACGACATGAGGCGTTCTGAGGACCTGAGGAAGCACGGCAACACGGTGCTCACGGCCCTGGGGGGCATCCTCAAGAAGAAGGGACATCACGAGGCTGAGCTGAAGCCCCTGGCCCAGTCAC
Bearded seal CAAGCACCTGAAGTCTGAGGACGACATGAGGCGTTCTGAGGACCTGAGGAAGCACGGCAACACGGTGCTCACGGCCCTGGGGGGCATCCTCAAGAAGAAGGGGACATCACGAGGCTGAGCTGAAGCCCCTGGCCCAGTCAC
Weddell seal CAAGCACCTGAAGTCAGAGGATGACATGAGGCGTTCTGAGGACCTGAGGAAGCACGGCAACACGGTGCTCACGGCCCTGGGGGGCATCCTCAAGAAGAAGGGACATCACGAGGCTGAGCTGAAGCCCCTGGCCCAGTCAC
Elephant seal CAAGCACCTGAAGTCAGAGGATGACATGAGGCGTTCTGAGGACCTGAGGAAGCACGGCAACACGGTGCTCACGGCCCTGGGGGGCATCCTCAAGAAGAAGGGACATCACGAGGCTGAGCTGAAGCCCCTGGCCCAGTCAC
Californian sea lion CAAGCACCTGAAGTCAGAGGACGAGATGAAGCGTTCAGAGGACCTGAAGAAGCATGGCAAAACGGTGCTTACGGCCCTGGGGGGCATCCTCAAGAAGAAGGGACATCACGATGCTGAGCTGAAGCCCCTGGCCCAGTCAC
Steller sea lion CAAGCACCTGAAGTCAGAGGACGAGATGAAGCGTTCAGAGGACCTGAAGAAGCATGGCAAAACGGTGCTTACGGCCCTGGGGGGCATCCTCAAGAAGAAGGGACATCACGATGCTGAGCTGAAGCCCCTGGCCCAGTCAC
Australian sea lion CAAGCACCTGAAGTCAGAGGACGAGATGAAGCGTTCAGAGGACCTGAAGAAGCATGGCAAAACGGTGCTTACGGCCCTGGGGGGCATCCTCAAGAAGAAGGGACATCACGATGCTGAGCTGAAGCCCCTGGCCCAGTCAC
Northern fur seal CAAGCACCTGAAGTCAGAGGACGACATGAGGCGTTCTGAGGACCTGAGGAAGCACGGCAACACGGTGCTCACGGCCCTGGGGGGCATCCTCAAGAAGAAGGGACATCACGAGGCTGAGCTGAAGCCCCTGGCCCAGTCAC
Walrus CAAGCACCTGAAGTCAGAGGACGAGATGAAGCGTTCTGAGGACCTGAAGAAGCATGGCAAAACGGTGCTTACGGCCCTGGGGGGCATCTTCAAGAAGAAGGGGACATCACGATGCTGAGCTGAAGCCCCTGGCCCAGTCAC
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American mink CAAGCACCTGAAGTCGGAGGATGAGATGAAGGGTTCTGAGGACCTGAAGAAGCATGGCAACACTGTGCTCACCGCCCTGGGCGGCATCCTCAAGAAGAAGGGGCAGCACGAGGCAGAGCTGAAGCCGCTGGCCCAGTCAC
Polar Bear CAAGCATCTGAAGTCAGAGGATGAGATGAAGGGTTCTGAGGACCTGAAGAAGCATGGCAACACGGTGCTCACCGCCCTGGGGGGCATCCTCAAGAAGAAGGGGCATCACGAGGCGGAGCTGAAGCCCCTGGCTCAGTCAC
Pygmy hippo CAAGAACCTGAAGACAGAGGATGAGATGAGGGCCTGTGAGAACCTGAAGAAGCACGGCAACACCGTGCTCACCGCCCTGGGGGGCATCCTCAAGAAGAAGGGGCATCACGAGGAGGAGCTGAAGCCCCTGGCGCACTCCC
Bowhead CAAGCACCTGAAGACAGAGGCTGAGATGAAGGCCTCAGAGGACCTGAAGAAGCATGGCAACACGGTGCTCACCGCCCTGGGGGGCATCCTCAAGAAGAAGGGGCATCATGAGGCAGAGCTGAAGCCCCTGGCCCAGTCGC
Sowerby's beaked whale CAAGCACCTGAAGTCAGAGGCTGAGATGAAGGCCTCAGAGGACCTGAAGAAGCATGGCCACACCGTGCTCACCGCCCTGGGGGGCATCCTCAAGAAGAAGGGTCATCATGAGGCGGAGCTGAAGCCCCTGGCCCAGTCGC
Northern bottlenose whale CAAGCACCTGAAGTCAGAGGCTGAGATGAAGGCCTCAGAGGACCTGAAGAAGCATGGCCACACCGTGCTCACCGCCCTGGGGGGCATCCTTAAGAAGAAGGGTCATCATGAGGCGGAGCTGAAGCCCCTGGCCCAGTCGC
Narwhal CAGGCACCTGAAGACAGAGGCTGAGATGAAGGCCTCTGAGGATCTGAAGAAGCATGGCAACACCGTGCTCACCGCCCTGGGGGGCATCCTCAAGAAGAAGGGGCATCATGACGCGGAGCTGAAGCCCCTGGCCCAGTCGC
White beaked dolphin CAAGCACCTGAAGACAGAGGCTGACATGAAGGCCTCAGAGGATCTGAAGAAGCATGGCAACACCGTGCTCACCGCCCTGGGGGCCATCCTCAAGAAGAAGGGGCATCATGACGCGGAGCTGAAGCCCCTGGCCCAGTCGC
Risso's Dolphin CAAGCACCTGAAGACAGAGGCTGACATGAAGGCCTCAGAGGATCTGAAGAAGCATGGCAACACCGTGCTCACCGCCCTGGGGGCCATCCTCAAGAAGAAGGGGCATCACGACGCGGAGCTGAAGCCCCTGGCCCAGTCGC
Grey squirrel CAAGAACCTGAAGTCGGAGGAGGAGATGAAGGCCTCGGAGGAGCTGAAGAAGCACGGCAGCACGGTGCTCGGGGCGCTGGGGGGCATCCTGAAGAAGAAGGGGCAGCACGAGGCGGAGATCAAGCCCCTGGCACAGTCGC

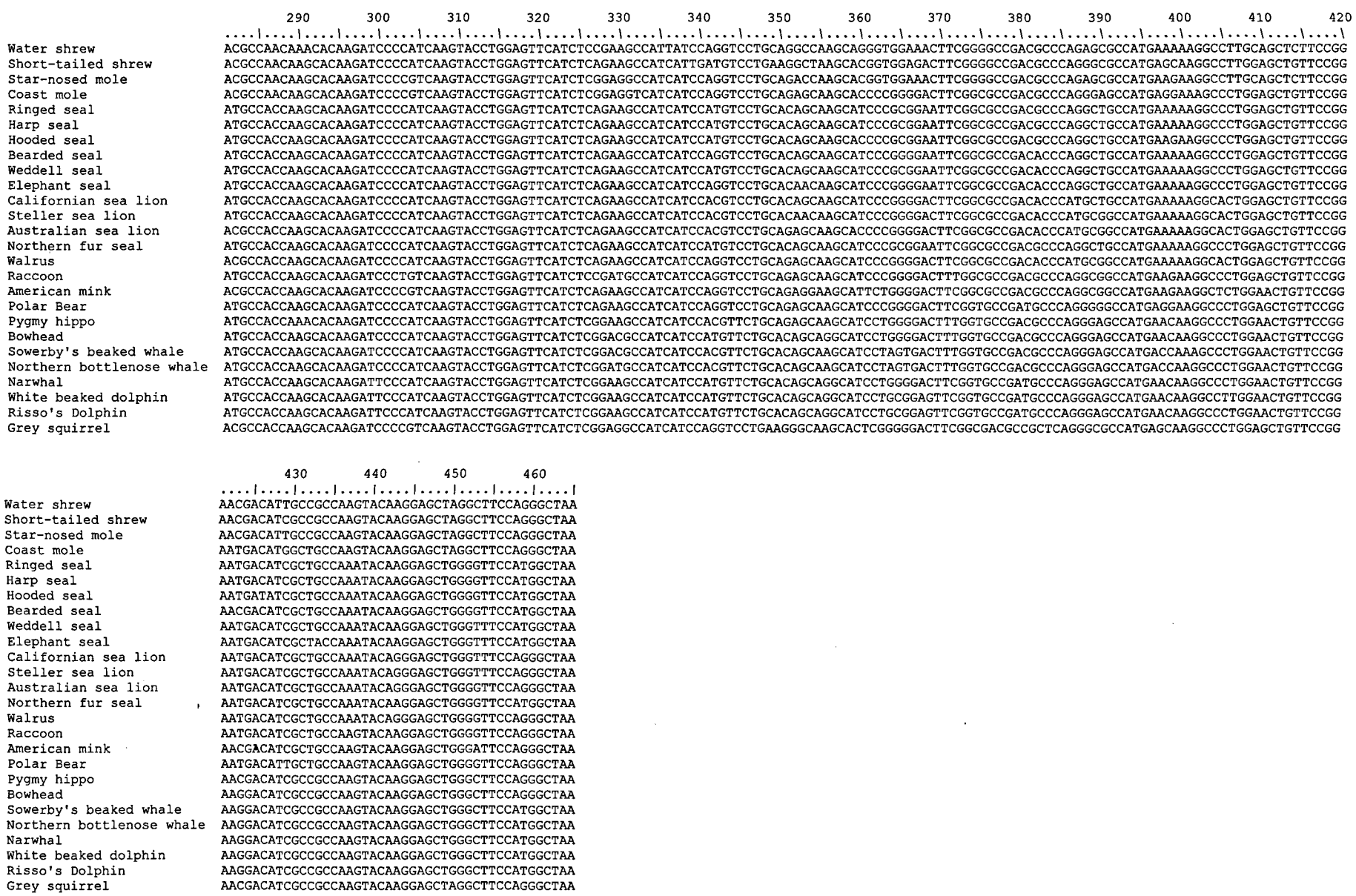

Figure A1 Mammalian myoglobin nucleotide alignment for species newly sequenced in this study. Red text indicates nucleotides that were influenced by the 5' forward primer taken from Bianchi et al., (2005) which overlaps the coding sequence. Tissue samples from these species either ran out or subsequent tissues were too degraded to determine the 5' end, once a primer had been established in the 5' end un-translated region (UTR). However the Bianchi et al. primer was also used to initially sequence the 5' end of the water shrew, when the water shrew was re-sequenced with the 5' UTR primer, sequence did not alter. This region is highly conserved among mammalian species and the influence of the forward primer does not affect amino acid sequence in terms of charge and Mb buffer value

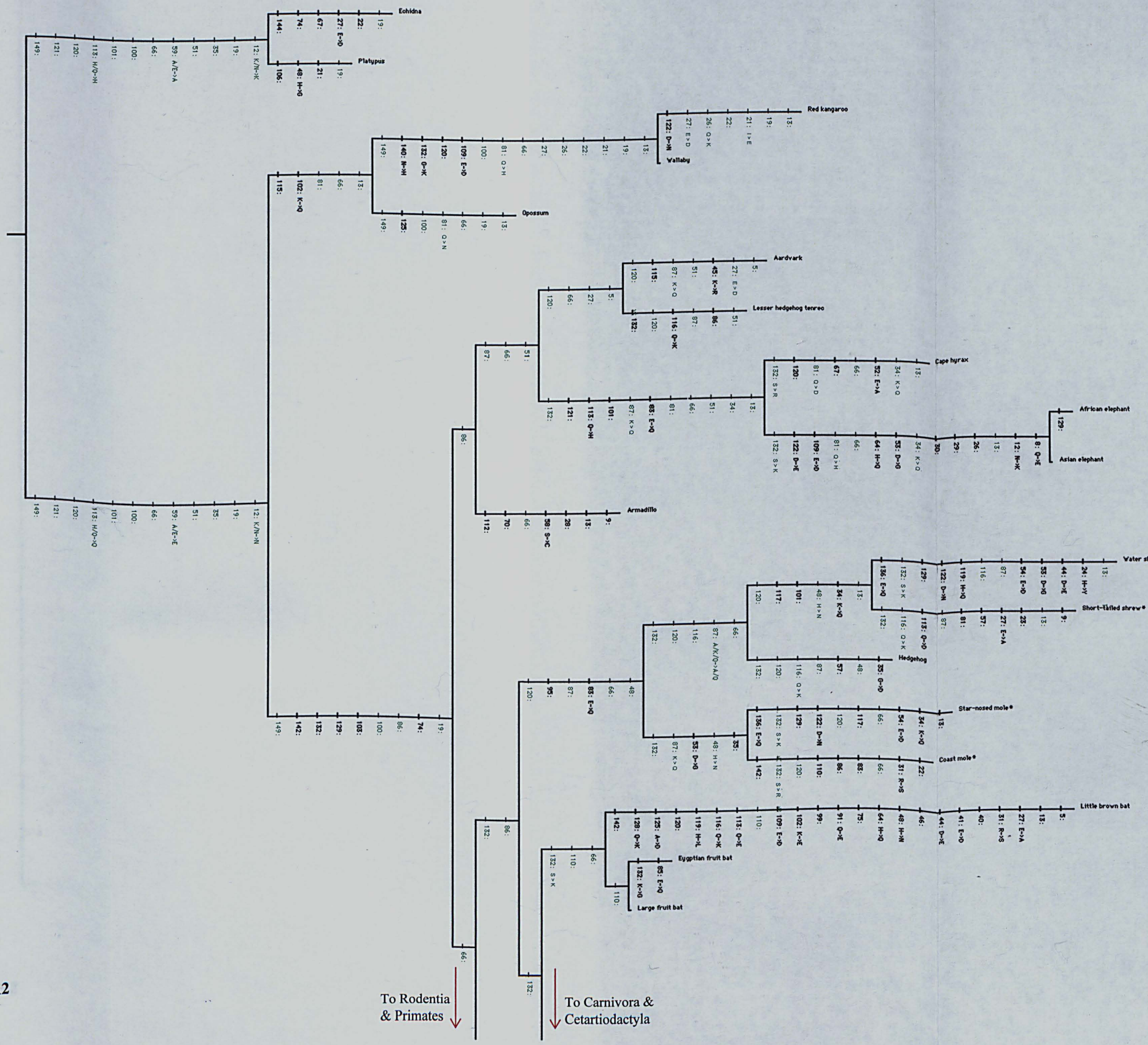
African elephant
Asian elephant
Red kangaroo
Wallaby
Cape hyrax
Aardvark
Lesser hedgehog tenrec
Opossum
Echidna
Platypus
Armadillo
Water shrew*
Short-tailed shrew*
Hedgehog
Star-nosed mole*
Coast mole*
Little brown bat
Eygptian fruit bat
Large fruit bat
To Rodentia & Primates
To Carnivora & Cetartiodactyla

Figure A2

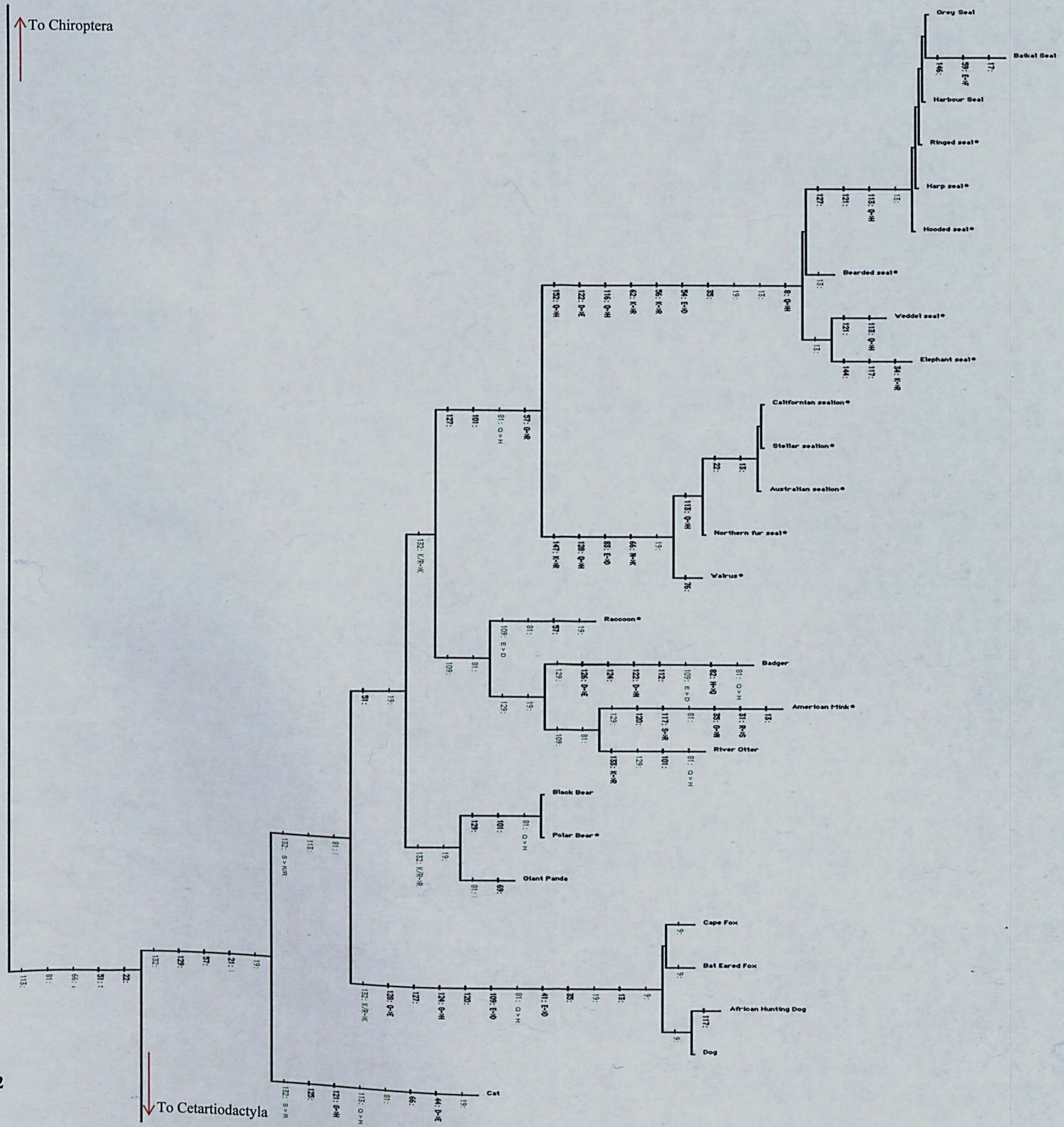
To Chiroptera
Grey Seal
Baikal Seal
Harbour Seal
Ringed seal*
Harp seal*
Hooded seal*
Bearded seal*
Weddel seal*
Elephant seal*
Californian sealion*
Stellar sealion*
Australian sealion*
Northern fur seal*
Walrus*
Raccoon*
Badger
American Mink*
River Otter
Black Bear
Polar Bear*
Giant Panda
Cape Fox
Bat Eared Fox
African Hunting Dog
Dog
Cat
To Cetartiodactyla

Figure A2

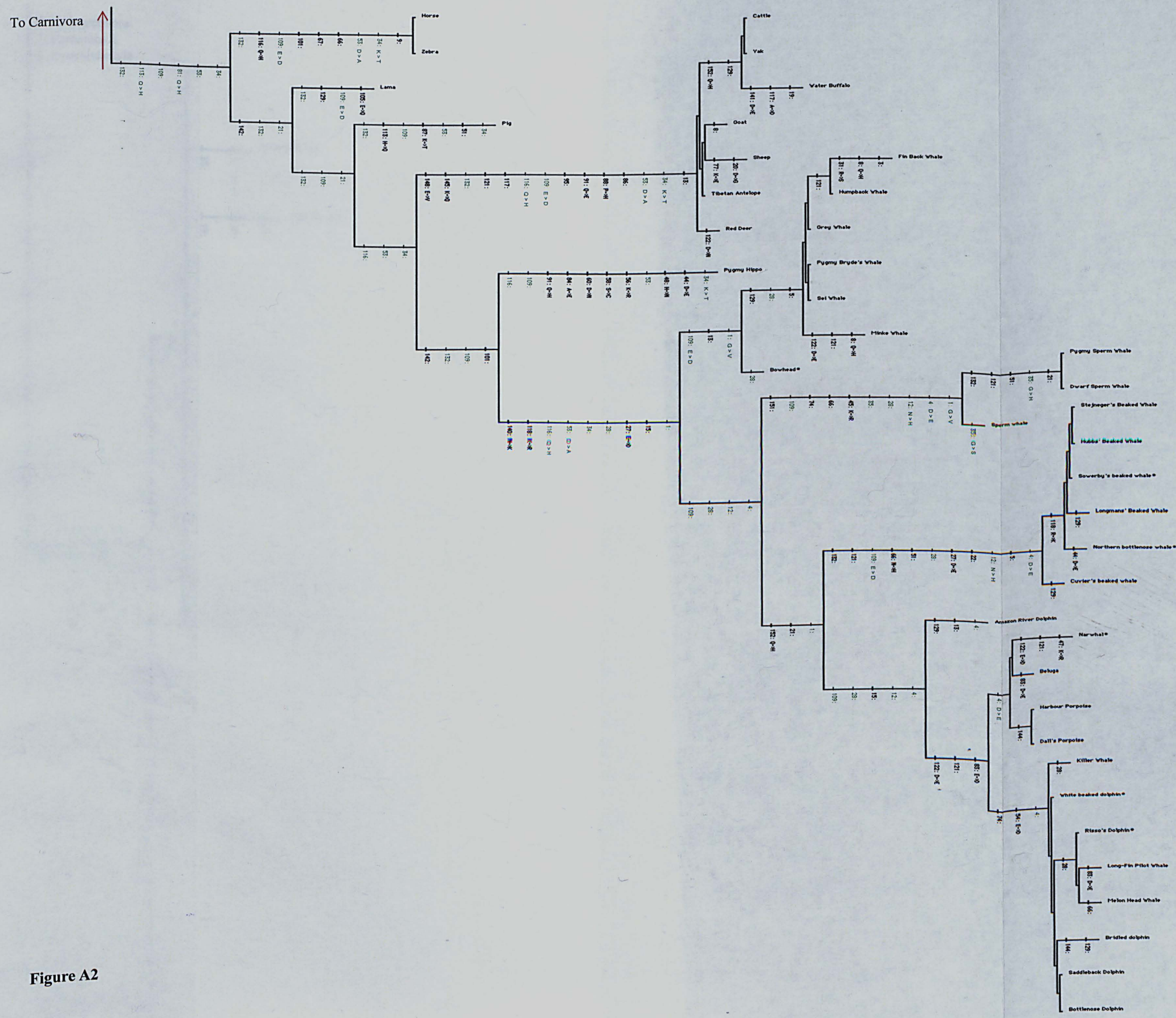
To Carnivora
Horse
Zebra
Lama
Pig
Cattle
Yak
Water Buffalo
Goat
Sheep
Tibetan Antelope
Red Deer
Pygmy Hippo
Bowhead*
Fin Back Whale
Humpback Whale
Grey Whale
Pygmy Bryde's Whale
Sei Whale
Minke Whale
Pygmy Sperm Whale
Dwarf Sperm Whale
Sperm whale
Stejneger's Beaked Whale
Hubbs' Beaked Whale
Sowerby's beaked whale*
Longmans' Beaked Whale
Northern bottlenose whale*
Cuvier's beaked whale
Amazon River Dolphin
Narwhal*
Beluga
Harbour Porpoise
Dall's Porpoise
Killer Whale
White beaked dolphin*
Risso's Dolphin*
Long-Fin Pilot Whale
Melon Head Whale
Bridled dolphin
Saddleback Dolphin
Bottlenose Dolphin

Figure A2

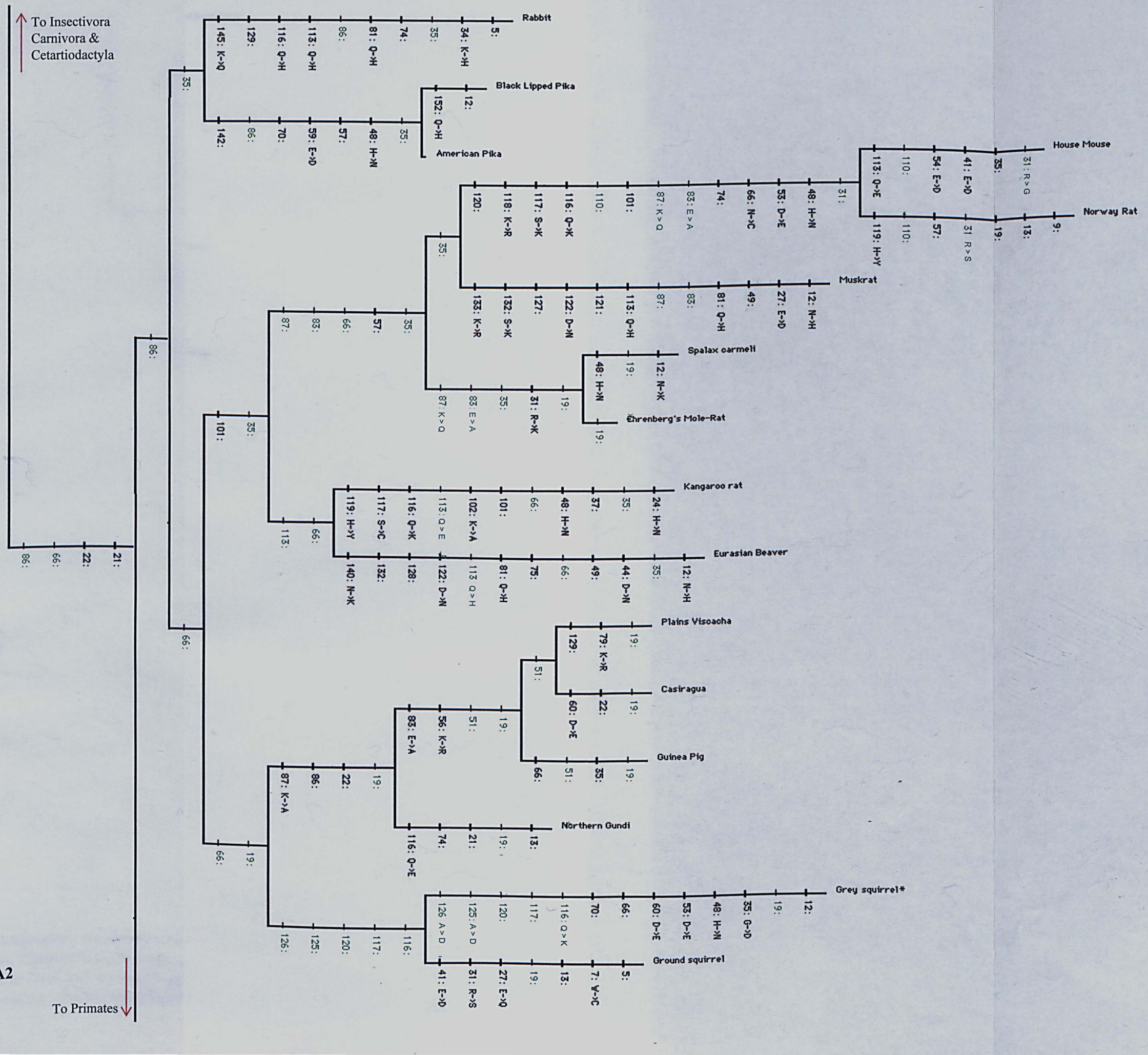
To Insectivora Carnivora & Cetartiodactyla
To Primates
Rabbit
Black Lipped Pika
American Pika
House Mouse
Norway Rat
Muskrat
Spalax carmeli
Ehrenberg's Mole-Rat
Kangaroo rat
Eurasian Beaver
Plains Viscacha
Casiragua
Guinea Pig
Northern Gundi
Grey squirrel*
Ground squirrel

Figure A2

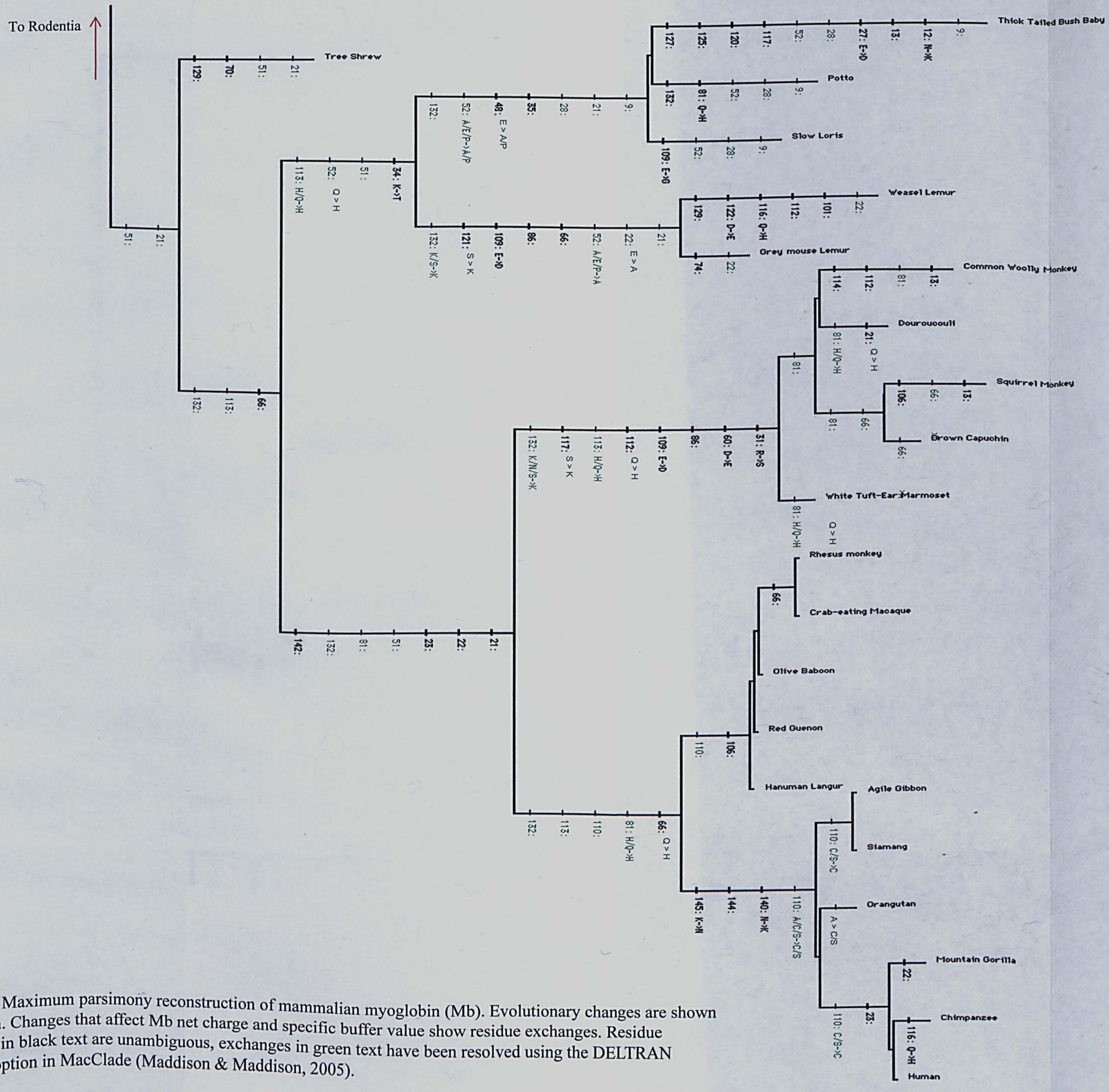

Figure A2 Maximum parsimony reconstruction of mammalian myoglobin (Mb). Evolutionary changes are shown by position. Changes that affect Mb net charge and specific buffer value show residue exchanges. Residue exchanges in black text are unambiguous, exchanges in green text have been resolved using the DELTRAN resolving option in MacClade (Maddison & Maddison, 2005).

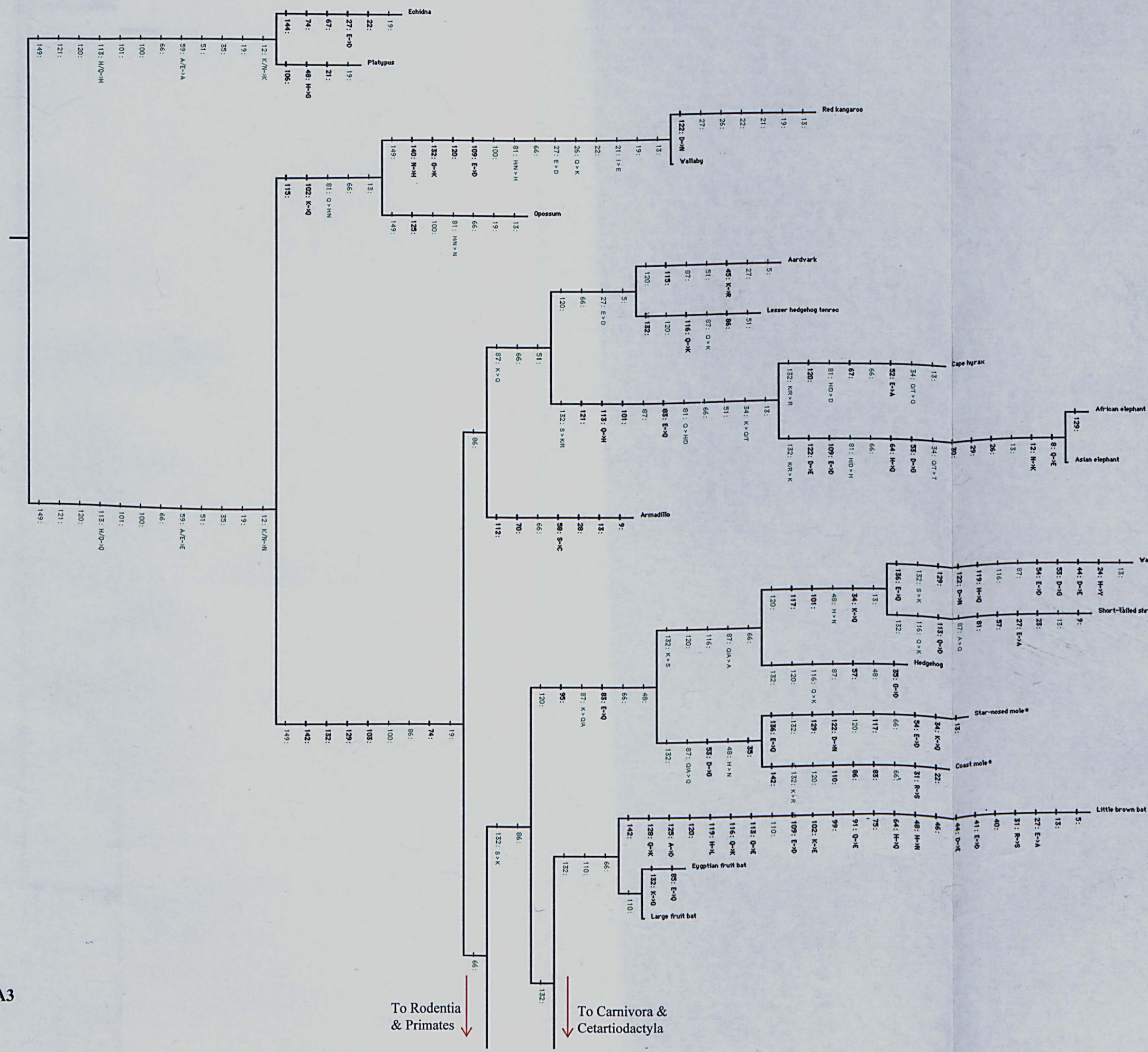
Echidna
Platypus
Red kangaroo
Wallaby
Opossum
Aardvark
Lesser hedgehog tenreo
Cape hyrax
African elephant
Asian elephant
Armadillo
Water shrew*
Short-tailed shrew*
Hedgehog
Star-nosed mole*
Coast mole*
Little brown bat
Egyptian fruit bat
Large fruit bat
To Rodentia & Primates
To Carnivora & Cetartiodactyla

Figure A3

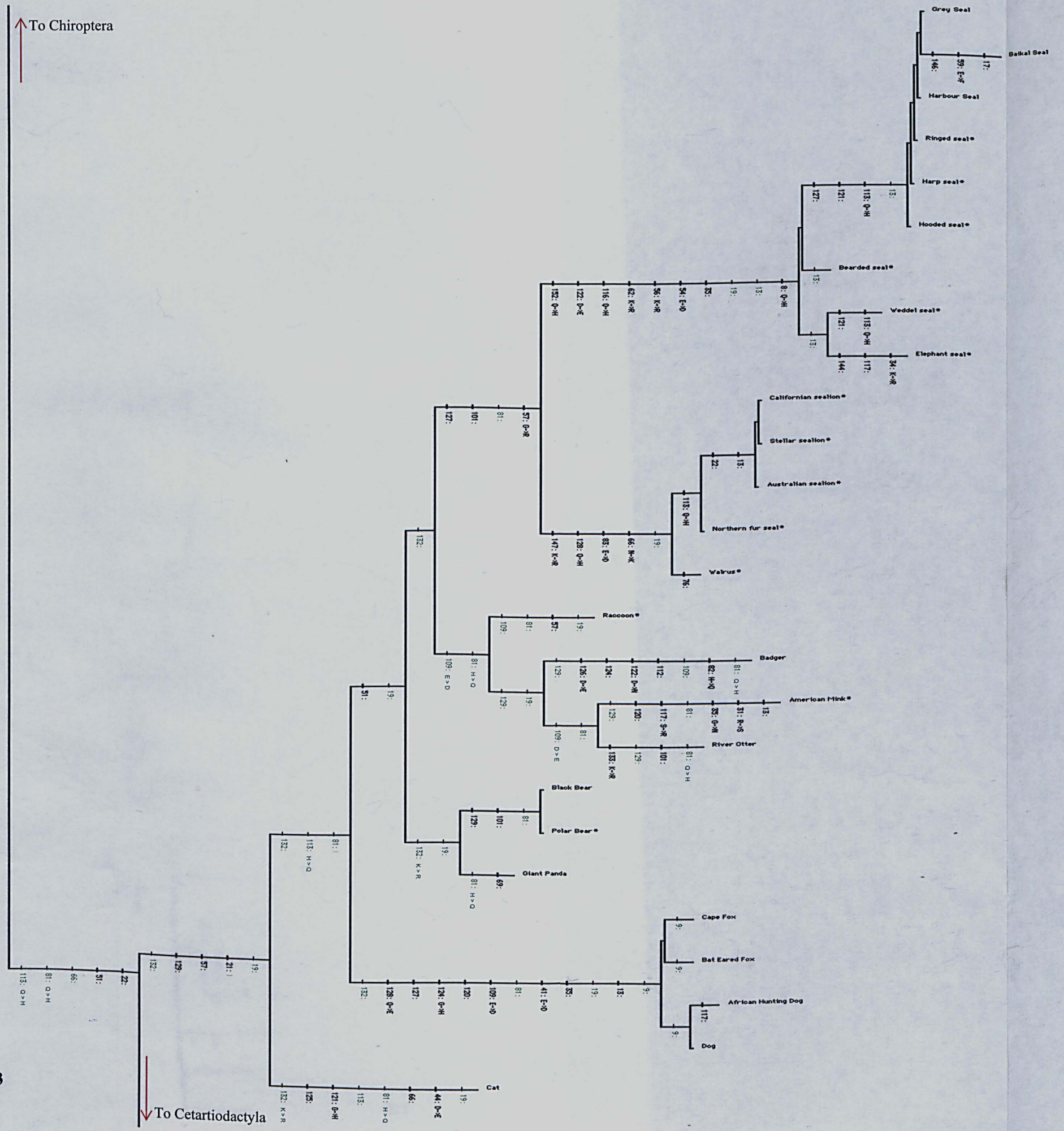
To Chiroptera
Grey Seal
Baikal Seal
Harbour Seal
Ringed seal*
Harp seal*
Hooded seal*
Bearded seal*
Weddel seal*
Elephant seal*
Californian sealion*
Stellar sealion*
Australian sealion*
Northern fur seal*
Walrus*
Raccoon*
Badger
American Mink*
River Otter
Black Bear
Polar Bear*
Giant Panda
Cape Fox
Bat Eared Fox
African Hunting Dog
Dog
Cat
To Cetartiodactyla

Figure A3

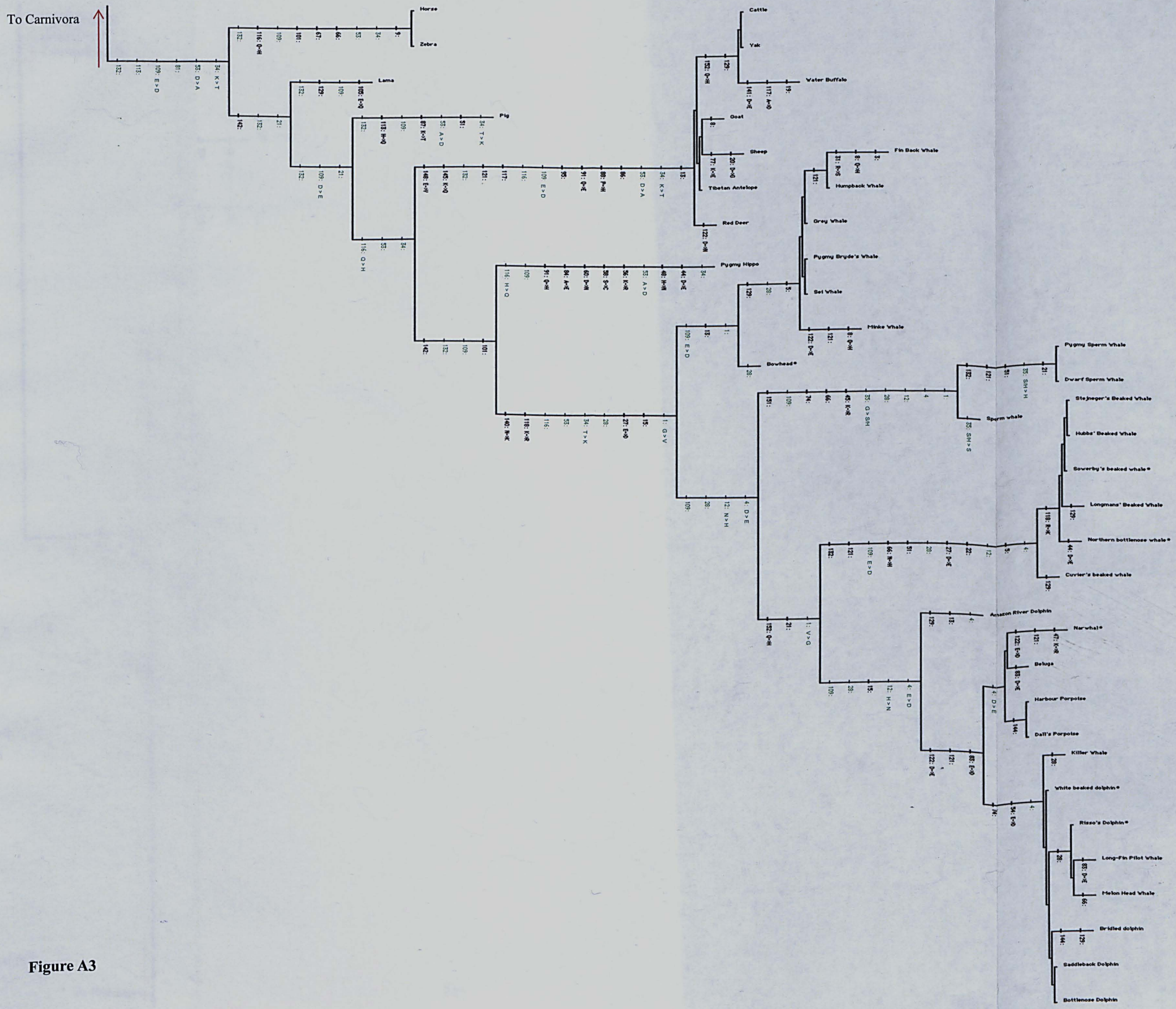
To Carnivora
Horse
Zebra
Lama
Pig
Cattle
Yak
Water Buffalo
Goat
Sheep
Tibetan Antelope
Red Deer
Pygmy Hippo
Bowhead*
Fin Back Whale
Humpback Whale
Grey Whale
Pygmy Bryde's Whale
Sei Whale
Minke Whale
Pygmy Sperm Whale
Dwarf Sperm Whale
Sperm whale
Stejneger's Beaked Whale
Hubbs' Beaked Whale
Sowerby's beaked whale*
Longmans' Beaked Whale
Northern bottlenose whale*
Cuvier's beaked whale
Amazon River Dolphin
Narwhal*
Beluga
Harbour Porpoise
Dall's Porpoise
Killer Whale
White beaked dolphin*
Risso's Dolphin*
Long-Fin Pilot Whale
Melon Head Whale
Bridled dolphin
Saddleback Dolphin
Bottlenose Dolphin

Figure A3

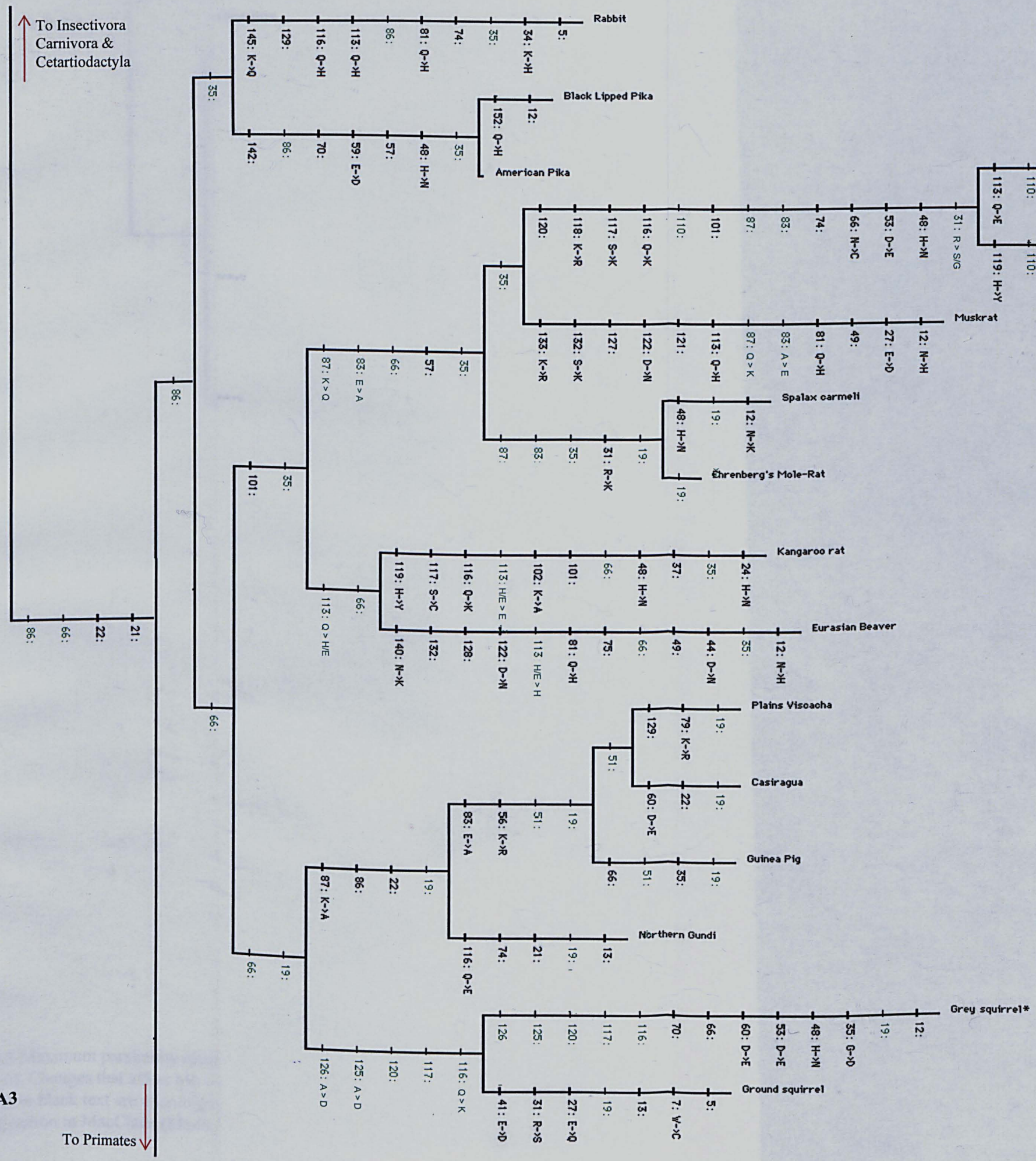
To Insectivora Carnivora & Cetartiodactyla
To Primates
Rabbit
Black Lipped Pika
American Pika
House Mouse
Norway Rat
Muskrat
Spalax carmeli
Ehrenberg's Mole-Rat
Kangaroo rat
Eurasian Beaver
Plains Viscacha
Casiragua
Guinea Pig
Northern Gundi
Grey squirrel*
Ground squirrel

Figure A3

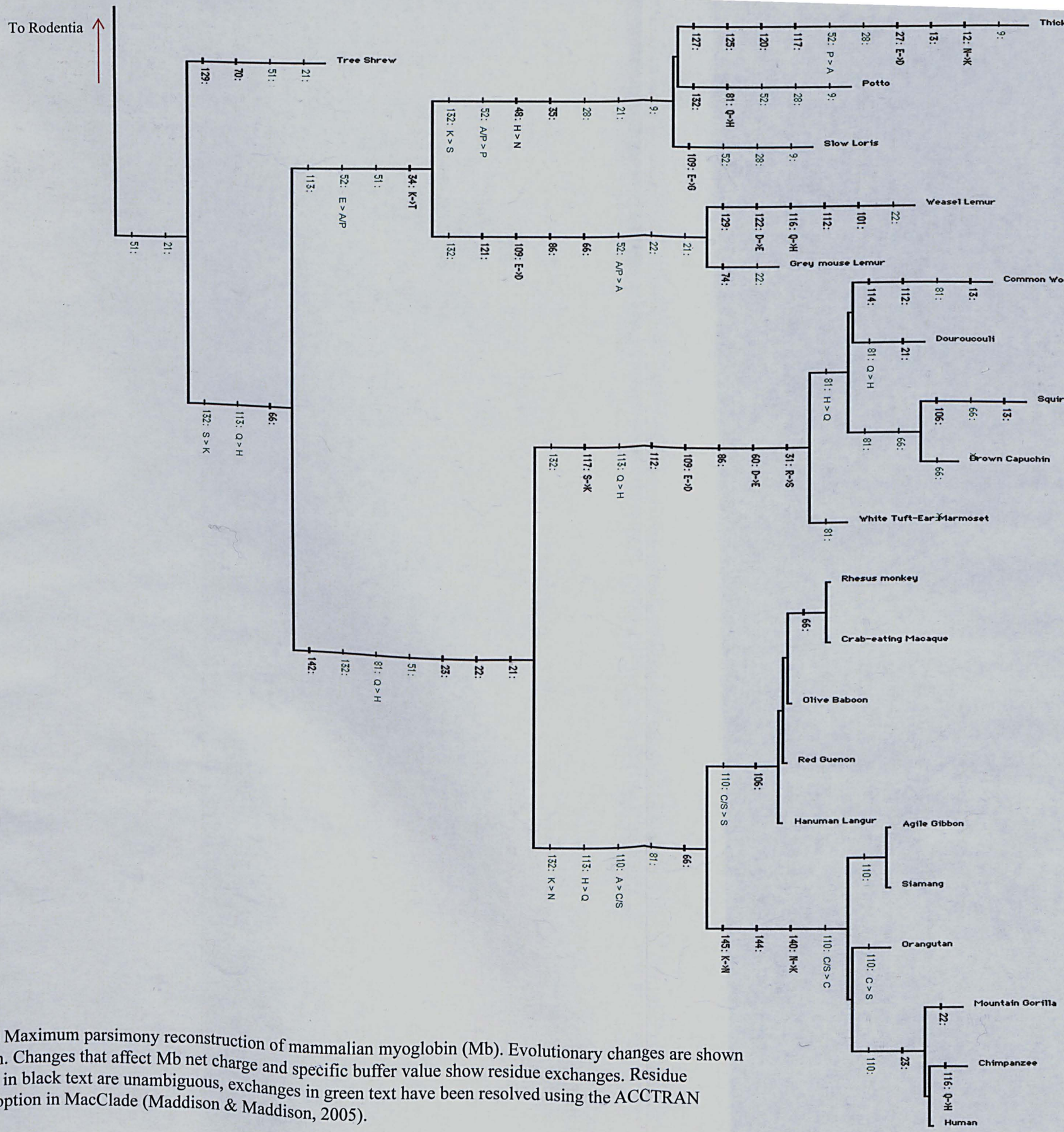

Figure A3 Maximum parsimony reconstruction of mammalian myoglobin (Mb). Evolutionary changes are shown by position. Changes that affect Mb net charge and specific buffer value show residue exchanges. Residue exchanges in black text are unambiguous, exchanges in green text have been resolved using the ACCTRAN resolving option in MacClade (Maddison & Maddison, 2005).