Evolution of an extreme hemoglobin phenotype contributed to the sub-Arctic specialization of extinct Steller's sea cows

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

[1]Department of Biological Sciences, University of Manitoba, Winnipeg, R3T 3N2, Canada
[2]Department of Biology, Zoophysiology, Aarhus University, DK-8000 Aarhus, Denmark

***Corresponding author:**
Kevin L. Campbell (email: Kevin.Campbell@umanitoba.ca; phone: +1-204-474-6397)

Key words: ancient DNA, hemoglobin, molecular evolution, oxygen binding, thermal adaptation, protein biochemistry, paleophysiology, Sirenia

Abstract

The extinct Steller's sea cow (*Hydrodamalis gigas*; †1768) was a whale-sized marine mammal that manifested profound morphological adjustments to exploit the harsh coastal climate of the North Pacific. Yet despite first-hand accounts of their biology, little is known regarding underlying physiological specializations to this extreme environment. Here, the adult-expressed hemoglobin (Hb) of this species is shown to harbor a fixed amino acid replacement at an otherwise invariant position (β/δ82Lys→Asn) that is predicted to profoundly alter multiple aspects of Hb function. To unravel the functional and evolutionary consequences of this substitution, we recombinantly synthesized Hb proteins of Steller's sea cow, a *H. gigas* β/δ82Asn→Lys Hb mutant, the dugong (*Dugong dugon*), the last common dugongid ancestor, and the Florida manatee (*Trichechus manatus*). Our detailed functional analyses demonstrate that the Hb–O_2 affinity of Steller's sea cow evolved to become less affected by temperature compared to other sea cows. This phenotype presumably safeguarded O_2 delivery to cool peripheral tissues and largely arises from a reduced intrinsic temperature sensitivity of the *H. gigas* protein owing to the β/δ82Lys→Asn substitution. We further confirm that this same exchange also underlies the secondary evolution of a reduced blood–O_2 affinity phenotype that is moreover unresponsive to the intraerythrocytic allosteric effector 2,3-diphosphoglycerate. This radical modification, which augments O_2 offloading by the protein, and presumably impacted maternal/fetal O_2 exchange, is the first documented example of this phenotype among mammals. Notably, this replacement also increases protein solubility and is consistent with increased Hb concentrations within both the adult and pre-natal circulations that may have contributed to the elevated metabolic (thermoregulatory) requirements and fetal growth rates associated with their cold adaptation.

Introduction

During periods of submergence apnea when atmospheric gas exchange has ceased, the underwater foraging time by mammals is dictated by onboard oxygen stores and the efficiency of their use. Thus, evolutionary increases in oxygen stores, in the form of increased hemoglobin (Hb) and myoglobin—located within erythrocytes and skeletal/cardiac muscle, respectively—are nearly ubiquitous among aquatic species[1]. Notable exceptions to this rule are extant sirenians (sea cows), a group of strictly aquatic, (sub)tropical herbivores encompassing only four members, three species of manatee (family Trichechidae) and the dugong, *Dugong dugon* (family Dugongidae). While sirenians are proficient divers, they do not exhibit the strongly elevated body O_2 stores or an enhanced dive reflex common to other lineages of marine mammals[2,3]. Rather, previous work revealed that the sirenian's secondary transition to aquatic life coincided with a rapid evolution of their Hb encoding genes due, in part, to gene conversion events with a neighboring globin pseudogene[4]. The resulting high blood–O_2 affinity phenotype presumably allows extant sea cows to maximize O_2 extraction from the lungs during submergence while curbing the rate of O_2 offloading, thus lowering overall metabolic intensity and fostering a prolonged breath-hold capacity[4].

While the relatively weak thermoregulatory capacity of extant sea cows confine them to (sub)tropical waters[5,6], fossil evidence and first-hand accounts of the sub-Arctic Steller's sea cow (*Hydrodamalis gigas*) provide tantalizing insights into both the biological and morphological adaptations of this titanic sirenian to the harsh coastal conditions of the North Pacific, where they persisted from the Miocene until their demise in 1768[7–9]. The retrieval of ancient genetic material from museum specimens has since been instrumental in clarifying the phylogenetic affinities and population history of this species, while providing additional details regarding the evolution of key morphological and physiological attributes[4,10–15] (Fig. 1A). For example, characterization of "resurrected" Steller's sea cow recombinant Hb demonstrated that the blood–O_2 affinity of this lineage secondarily decreased following their divergence from dugongs in the mid Oligocene[4]. Although sirenians do not possess the capacity for non-shivering thermogenesis due to pseudogenization of the *UCP1* gene[11], this affinity shift in Steller's sea cow Hb would have promoted increased O_2 offloading that was speculated to fuel an increased metabolism to help cope with exposure to cold sub-Arctic waters. This decrease in Hb–O_2 affinity was hypothesized to arise from a highly unusual 82Lys→Asn exchange in the chimeric β-type (β/δ) chain encoding the Hb

of this species[4]. Data mined from more recent ancient DNA studies[10,14] further confirms that this substitution was fixed in the last remaining Steller's sea cow population (Fig. 1B). This replacement is intriguing not only because β82Lys is invariant among known mammalian Hbs, but because human variants with substitutions at this position display profound alterations in both structural and functional properties[16–19]. The human Hb Providence (β82Lys→Asn) variant in particular has been well characterized as asparagine at this position progressively undergoes post-translational deamidation to form aspartic acid (Asp), which over the life-time of the erythrocyte, constitutes ca. two-thirds of the Hb Providence proteins[20–22]. Both isoforms exhibit a decreased inherent Hb–O_2 affinity, a markedly reduced sensitivity to allosteric effectors (e.g. 2,3-diphosphoglycerate [DPG], Cl^-, and H^+, which preferentially bind and stabilize the deoxy-state protein, reducing Hb–O_2 affinity), and an increased Hb oxidative stability and solubility[16,17,20,23,24]. Notably, the 82Lys→Asn replacement is expected to benefit an aquatic lifestyle in cold marine climates if this exchange induced parallel changes in Hb–O_2 affinity and Hb solubility of Steller's sea cow Hb. However, a reduction of anionic effector binding may be non-adaptive as it is predicted to detrimentally increase the effect of temperature on Hb–O_2 binding and release.

The weak covalent bond between O_2 and the heme iron, which absorbs free energy upon breakage, dictates an inverse relationship between Hb–O_2 affinity and temperature[25]. In temperate and Arctic endotherms this inherent attribute of Hb potentially impedes O_2 delivery to the limbs and flukes, which are maintained at substantively lower temperatures to minimize heat loss and hence daily energy requirements[26]. Accordingly, these heterothermic mammals generally possess Hbs whose O_2 binding properties are less affected by temperature than the Hbs of non-cold-adapted species, thereby maintaining sufficient O_2 offloading in the face of decreasing tissue temperatures. This reduction in thermal sensitivity (the overall enthalpy of oxygenation, ΔH'), appears to predominantly arise from an increased interaction between allosteric effectors and the Hb moiety (an exothermic process), which releases additional heat to assist with deoxygenation[25]. Hence, the β/δ82Lys→Asn replacement, which deletes integral binding sites for both the heterotropic ligands Cl^- and DPG[17], is expected to maladaptively increase the effect of temperature on O_2 uptake and release in the blood of the Arctic Steller's sea cow.

To unravel the combined effects of evolved amino acid replacements on hemoglobin function in relation to the extreme thermal biology of the extinct Steller's sea cow, we synthesized recombinant Hb proteins of this species together with those of the dugong (*Dugong dugon*) and

Florida manatee (*Trichechus manatus latirostris*), and measured their O_2 binding properties, relative solubilities, responses to allosteric effectors, and thermal sensitivities. We also synthesized a *H. gigas* β/δ82Asn→Lys Hb mutant to assess the specific effects of this exchange, together with the Hb of the last common ancestor shared between the dugong and Steller's sea cow (from ~21.2 million years ago; Fig. 1A) in order to assess the directionality of evolved physicochemical changes in Hb function.

Results and Discussion

O_2 Affinity of Sirenian Hbs. Measured O_2-equilibrium curves of the five examined Hbs revealed marked differences in intrinsic O_2 affinity (Figs. 2A, S1, S2 and Table S1). In the absence of allosteric effectors (pH 7.2, 37°C), the P_{50} (the O_2 tension resulting in 50% Hb–O_2 saturation) of Steller's sea cow Hb (P_{50} = 8.8 mmHg) is ~2 times higher than that of dugong (3.5 mmHg), ancestral dugongid (4.3 mmHg), and manatee (5.4 mmHg) Hbs under the same conditions (Figs. 2A, S1 and Table S1). We used site directed mutagenesis to revert this amino acid to the ancestral state (β/δ82Asn→Lys) to test whether the low intrinsic O_2 affinity of *H. gigas* Hb arises from this substitution. These experiments reveal that the increased intrinsic P_{50} of Steller's sea cow Hb predominantly arises from β/δ82Asn, as the β/δ82Asn→Lys mutant exhibits an intrinsic P_{50} similar to that of the ancestral dugongid (Fig. 2A, S2). Of note, the O_2 affinity of dugong, ancestral dugongid, and manatee Hbs was reduced in the presence of Cl^- and DPG (P_{50} = 10.2, 9.9, and 10.9 mmHg, respectively) by a similar degree to that of Asian elephant Hb[27]. This finding extends previous studies conducted on sirenian Hbs[4,28,29], and reveals that the high O_2 affinity of dugong and manatee blood is not attributable to decreased allosteric effector sensitivity. Conversely, Steller's sea cow Hb was shown to be markedly less responsive to allosteric effectors, as only a moderate reduction in O_2–affinity was observed in the presence of Cl^- and DPG (P_{50} = 14.3 mmHg). When the effects of these allosteric effectors were measured individually, Steller's sea cow Hb exhibits markedly reduced DPG, Cl^-, and H^+ (Bohr) effects relative to those of the dugongid ancestor and extant sirenians (Fig. 2C-E; Table S1). These data confirm that a high intrinsic (i.e. in the absence of allosteric effectors) Hb–O_2 affinity is an ancient sirenian trait that likely aided the transition of the group to the aquatic environment, and that Hb–O_2 (and hence whole blood) affinity was secondarily reduced in the Steller's sea cow lineage[4].

The single most distinct feature of *H. gigas* Hb is the lack of a discernable effect of DPG on P_{50} ($\Delta\log P_{50}^{(0.2\text{ mM DPG} - 0.0\text{ mM DPG})} = 0.02$ at 37°C and pH 7.2; Fig. 2C and Table S1), relative to the Hbs of the ancestral dugongid (0.27) and the extant manatee and dugong (0.14 and 0.30, respectively). This intracellular effector generally occurs in equimolar concentrations to Hb[30] and strongly influences the O_2 affinity of most mammalian Hbs via its electrostatic interactions with the α-NH_2 group of 1Val of the β_2 chain and 2His, 82Lys, and 143His of β_1 and β_2 chains[31] (Fig. S3). Unlike the other DPG-binding residues, which form single bonds with this organophosphate, β82Lys is strongly cationic within the physiological pH range, enabling DPG to form three bonds with $\beta_1$82Lys and two with $\beta_2$82Lys[31]. Presumably arising from its indispensable role in DPG binding, this residue is uniformly conserved in mammalian Hbs (Fig. S3), with the exception of several heterozygous adult human HbA carriers with substitutions at this position[18,19,21]. Given that none of the other six β/δ-chain replacements that evolved on the Steller's sea cow branch (Fig. S4) are implicated in DPG binding, the replacement of a positive charge at β/δ82 in Steller's sea cow Hb by a neutral (Asn) residue is consistent with its lack of DPG sensitivity. This conclusion is further supported by our measurements on the Steller's sea cow β/δ82Asn→Lys mutant, which show that reversion to the ancestral state restores the DPG effect to the same level observed in ancestral dugongid Hb (Fig. 2C). In contrast, and likely owing to the high DPG sensitivity of human HbA (0.53)[20], Hb Providence-Asn exhibits a notably higher DPG effect (0.08) than the *H. gigas* protein[17,20]. Accordingly, the whole blood O_2 affinity of Hb Providence carriers is ~5 mmHg higher than that of the general population (P_{50} = ~21 mmHg vs. ~26 mmHg, respectively)[20,23], whereas the results presented here provide strong evidence that the blood O_2 affinity of Steller's sea cows would have been lower than that of extant sirenians. These observations emphasize that the phenotypic effect of specific amino acid substitutions are conditional on the genetic background in which they occur[32].

The insensitivity of *H. gigas* Hb to DPG it notable as it would have markedly reduced their capacity to modulate blood–O_2 affinity *in vivo* (e.g., seasonally), and is the first demonstrated example of this phenotype among mammals. While eastern moles (*Scalopus aquaticus*) are a possible exception[33], feliformid carnivores, ruminants, and two species of lemurs have also traditionally been placed in this category[30] despite the fact their Hbs are moderately responsive to DPG in the absence of Cl^-[17,34–36]. Nonetheless, red blood cell DPG concentrations of these 'DPG insensitive' species are markedly reduced relative to mammals whose Hb–O_2 affinity is regulated

by DPG (<0.1-1.0 mM vs. 4-10 mM, respectively), presumably owing to the energetic cost of DPG synthesis (that bypasses production of one of the two ATP molecules generated via glycolysis within the erythrocytes)[30,33]. Accordingly, Steller's sea cow blood likely also contained low levels of this organophosphate.

Hb Solubility. Ectopic expression of human Hb Providence β82Asp mutants in *E. coli* has been shown to improve soluble protein production by 47-116% relative to the expression of human Hb variants not carrying this substitution[24]. Consistent with this observation, we found that Steller's sea cow Hb is more soluble than those of other sirenians and the engineered *H. gigas* β/δ82Asn→Lys mutant (Fig. 2F and S5). While the precise mechanism underlying this phenomenon is unknown, both β82Asn and β82Asp have been shown to strongly reduce irreversible oxidative damage of nearby β93Cys that initiates Hb denaturation[16,37,38]. These β82 replacements thereby presumably decrease the rate of Hb turnover and increase the half-life of the protein[38], and may be the underlying cause of mild polycythemia in humans carrying this substitution[20,21]. Blood with an elevated O_2 carrying capacity is typical of most mammalian divers, where it increases onboard O_2 stores and extends dive times[1], but is not observed in extant sirenians[29,39,40]. However, solubility driven increases in blood Hb levels resulting from the β82Lys→Asn exchange would have allowed Steller's sea cows to maintain an elevated rate of tissue O_2 delivery to meet their metabolic demands during extended underwater foraging intervals. Although this species was presumably unable to completely submerge[9,41], this conjecture is corroborated by Steller's account that, "*they keep their heads always under water* [foraging], *without regard to life and safety*"[9].

Thermal Sensitivity. The invariant energy change associated with forming the weak covalent bond between O_2 and the heme iron (i.e. the enthalpy of heme oxygenation; ΔH^{O_2}) is exothermic (-59 kJ mol^{-1} O_2)[42], and only moderately opposed by the endothermic solubilization of O_2 (ΔH^{H_2O}; 12.55 kJ mol^{-1} O_2), resulting in an inverse relationship between temperature and Hb–O_2 affinity. However, the heat of the T→R conformational change ($\Delta H^{T\rightarrow R}$), and the oxygenation-linked dissociation of H^+ (ΔH^{H+}), Cl^- (ΔH^{Cl-}), and DPG (ΔH^{DPG}) may offset this relationship, such that the overall enthalpy of Hb oxygenation (ΔH') can become greatly minimized or even endothermic[25,43]. By facilitating adequate oxygenation of cool peripheral tissues, Hbs with

numerically low ΔH' values are interpreted to be adaptive for cold-tolerant, regionally heterothermic mammals. The evolution of this phenotype has predominantly been attributed to the formation of additional heterotropic ligand binding sites on the protein moiety, as has previously been demonstrated for the woolly mammoth, *Mammuthus primigenius*[25,27]. Conversely, Steller's sea cow Hb that lacks heterotropic binding of DPG and displays lower Bohr (H^+) and Cl^- effects than all other sirenian Hbs measured (Fig. 2; Table S1), exhibits a ΔH' value (-18.8 kJ mol^{-1} O_2; Fig. 2B) that is close to that of mammoth Hb (-17.2 kJ mol^{-1} O_2)[27]. This convergence largely arises from the inherently low ΔH of stripped Steller's sea cow Hb (-34.2 kJ mol^{-1} O_2) relative to dugong, ancestral dugongid, and manatee Hbs (range: -50.1 to -58.6 kJ mol^{-1} O_2) at pH 7.8—where oxygenation-linked binding of protons is minimal—and indicates that structural differences modifying the T→R transition largely underlie the low thermal sensitivity of *H. gigas* Hb. Recent studies that have similarly shown that a large positive $\Delta H^{T\rightarrow R}$ may similarly contribute to the low ΔH' of deer mouse, cow, shrew, and mole Hbs[33,44–47] suggesting that this mechanism of temperature adaptation may be more widespread than previously appreciated. Our experiments with the Steller's sea cow β82Asn→Lys mutant further implicate substitutions at this position as a key factor underlying the inherently low ΔH of the protein, as this modified protein displays a greatly increased ΔH in the absence of allosteric effectors relative to the wild-type *Hydrodamalis* protein (Fig. 2B). Interestingly, despite these inherent ΔH differences between the mutant and wild-type Steller's sea cow Hbs, their ΔH' values are indistinguishable in the presence of allosteric effectors (Fig. 2B). These data suggest that β82Asn uncouples thermal sensitivity from DPG concentration, permanently conferring the *H. gigas* protein with a numerically low ΔH' by genetic assimilation while simultaneously eliminating the energetic cost of DPG production within the red blood cells.

Paleophysiology of Steller's sea cows. The posthumously published behavioral and anatomical accounts of the last remaining *H. gigas* population by naturalist Georg Wilhelm Steller while stranded on Bering Island (55°N, 166°E) in 1741/1742 provide a rich tapestry to interpret the paleophysiology of this colossal marine herbivore. For example, their protective thick bark-like hide and extensive blubber layer give credence to the extreme nature of their shallow rocky and (during winter) ice strewn habitat[10]. Here, as Steller[9] remarked, they used fingerless, bristle-covered forelimbs for support and to shear "*algae and seagrasses from the rocks*", which they

masticated "*not with teeth, which they lack altogether, but with*" large, ridged keratinized pads located on the upper palate and lower mandible. Although they became visibly thin during winter when "*their spinous processes can be seen*", Steller[9] noted that "*(t)hese animals are very voracious, and eat incessantly*" such that their stupendous stomach ("*6 feet* [1.8 m] *long, 5 feet* [1.5 m] *wide*") and enormous intestines—which measured a remarkable 5,958 inches (~151.5 m) from esophagus to anus, equivalent to "*20½ times as long as the whole animal*"—are constantly "*stuffed with food and seaweed*". These observations of a proportionally larger gut[41] are consistent with relatively high energetic requirements relative to extant manatees, which owing to their low metabolic intensity become cold stressed and die if chronically exposed to water temperatures below 15°C[48]. Reductions in insulative blubber thickness during the winter months would have compounded the rate of heat loss of these behemoths to sub-zero degree Celsius air and water, though may have been compensated for by arteriovenous anastomoses that regulated blood flow to the skin, and by countercurrent rete supplying the flippers and tail flukes, the latter of which are well developed in manatees and presumably other sirenians[49,50]. These structures conserve thermal energy by promoting profound cooling at the appendages and periphery[26], and presumably underlie the low thermal dependence of Steller's sea cow Hb relative to those of extant sea cows.

Reductions in blood–O_2 affinity accompanying the *H. gigas* β/δ82Lys→Asn substitution is expected to have further augmented tissue O_2 delivery with only negligible effects on lung O_2 uptake, thereby helping to fuel increased thermogenesis to maintain a stable core temperature. The latter was presumably supplemented by a substantive heat increment arising from fermentation and other post-prandial processes[50]. Although the attendant increase in the rate of O_2 consumption would have mandated a reduction in breath-hold endurance—likely reflecting the relatively short submergence times (4 to 5 minutes) observed by Steller[9]—our results suggests this may be been counteracted by an elevated Hb concentration (blood–O_2 carrying capacity) that was potentially coupled to a greater lung volume[41]. Underwater foraging times were presumably further defended by key components of the dive reflex, namely bradycardia and peripheral vasoconstriction. Indeed, Steller inadvertently was the first to (indirectly) describe this phenomenon as he observed his crew hunting the animals with spears and knives, "*the blood from the wounded back spurted up like a fountain. As long as he kept his head under water the blood did not flow out, but as soon as he raised his head to breathe the blood leaped forth anew*".

A final compelling aspect of Steller's sea cow evolution was their immense increase in size—up to 11,000 kg in mass and 10 m in length—relative to extant sirenians[7]. While it is unfortunate Steller does not provide measurements of "*their tender little offspring*", Gerhard Friedrich Müller, who edited Steller's manuscript prior to publication, noted calves "*weighed 1200 pounds* [544 kg] *and upwards*"[51]. This value is ~10 to 50 times the mass of new born manatees and dugongs (~10-50 kg)[52] and is suggestive of rapid prenatal growth during the ~1 year gestational period proposed by Steller[9]. Given that all mammals except ruminants and anthropoid primates express the same Hb isoform during both the fetal and post-natal stages of development, the novel DPG insensitive phenotype of *H. gigas* is particularly notable in this regard owing to its potential impact on maternal/fetal O_2 delivery. In the vast majority of mammalian species, the O_2 affinity of fetal blood is elevated relative to that of the maternal circulation[30]. This increase is typically achieved via maintenance of low intracellular DPG concentrations in fetal blood cells; exceptions are the above ruminants and anthropoid primates which instead express a higher O_2 affinity fetal-specific hemoglobin isoform[30]. However, an elevated fetal blood O_2 affinity is expected to lower blood-to-tissue PO_2 gradients within the fetal circulation (i.e. lowered O_2 offloading potential), which together with the immersion foraging strategy and high maternal blood O_2 affinity of manatees and dugongs may promote intermittent fetal hypoxia, which has experimentally been shown to reduce the birth weight of rat pups[53,54]. By contrast, and owing to the inability of Steller's sea cows Hb to respond to DPG in either the fetal or adult circulations, this species would represent a rare example (feloids and eastern moles are others) in which fetal and maternal blood have the same (lower) O_2 affinity. However, by stipulating O_2 offloading occurs at a relatively high PO_2, the DPG insensitive Steller's sea cow Hb phenotype may have increased both placental O_2 transfer and fetal O_2 delivery. Together with increases in Hb solubility/reduced susceptibility to oxidative damage arising from β/δ82Lys→Asn that conceivably also elevated the O_2 carrying capacity of fetal blood, these attributes presumably fostered an enhanced fetal growth rate of these immense sirenians. The resulting increased thermal inertia and relatively low surface-area-to-volume ratio following birth, together with an adaptively reduced Hb thermal sensitivity and thick a 'bark-like' skin arising from inactivation of lipoxygenase genes[10], were presumably central components of Steller's sea cows successful exploitation of the harsh sub-Arctic marine environments of the North Pacific.

Methods and Materials

Construction of Recombinant Hb Expression Vectors. The adult-expressed Hb genes (HBA and HBB/HBD) of the Florida manatee, dugong, and Steller's sea cow, and the most recent common ancestor shared by Steller's sea cow and the dugong ('ancestral dugongid') have previously been determined[4]. As the *H. gigas* β/δ82Lys→Asn exchange is not known to occur in any living species, we mined recently deposited genomes for 13 additional Steller's sea cows (PRJNA484555, PRJEB43951) to test for the prevalence of this replacement in the population. Briefly, we first searched SRA files of each specimen using the megablast function against a known *H. gigas HBB/HBD* gene sequence (GenBank accession #: MK562081). All hits were then downloaded, trimmed of adapters and low quality regions using BBDuk (Joint Genome Institute), and assembled to *H. gigas HBB/HBD* using Geneious Prime 2019 software (Biomatters Ltd, Auckland, New Zealand).

Globin sequences for the above four species were optimized for expression in *E. coli* and synthesized *in vitro* by GenScript (Piscataway, NJ). The resulting gene cassettes were digested with restriction enzymes and tandemly ligated into a custom Hb expression vector[54] using a New England BioLabs Quick Ligation Kit as recommended by the manufacturer. Chemically competent JM109 (DE3) *E. coli* (Promega) were prepared using a Z-Competent *E. coli* Transformation Kit and Buffer Set (Zymo Research). We also prepared a *H. gigas* β/δ82Asn→Lys Hb mutant via site-directed mutagenesis on the Steller's sea cow Hb expression vector by whole plasmid amplification using mutagenic primers and Phusion High-Fidelity DNA Polymerase (New England BioLabs), phosphorylation with T4 Polynucleotide Kinase (New England BioLabs), and circularization with an NEB Quick Ligation Kit (New England BioLabs). All site-directed mutagenesis steps were performed using the manufacture's recommended protocol.

Hb expression vectors were co-transformed into JM109 (DE3) chemically competent *E. coli* alongside a plasmid expressing methionine aminopeptidase[55], plated on LB agar containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml), and incubated for 16 hours at 37°C. A single colony from each transformation was cultured in 50 ml of 2xYT broth for 16 hours at 37°C while shaking at 200 rpm. Post incubation, 5 ml of the culture was pelleted by centrifugation and plasmid DNA was isolated using a GeneJET Plasmid Miniprep Kit (Thermo Scientific). The plasmid sequence was verified using BigDye 3.1 sequencing chemistry and an ABI3130 Genetic Analyzer.

The remainder of the culture was supplemented with glycerol to a final concentration of 10%, divided into 25 ml aliquots and stored at -80°C until needed for expression.

Expression and Purification of Recombinant Hb. 25 ml of starter culture (above) was added to 1250 ml of TB media containing ampicillin (100 µg/ul) and kanamycin (50 µg/ul) and distributed evenly amongst five 1 L Erlenmeyer flasks. Cultures were grown at 37°C while shaking at 200 rpm until the absorbance at 600 nm reached 0.6-0.8. Hb expression was induced by supplementing the media with 0.2 mM isopropyl β-D-1-thiogalactopyranoside, 50 µg/ml of hemin and 20 g/L of glucose and the culture was incubated at 28°C for 16 hours while shaking at 200 rpm. Once expression had completed, dissolved O_2 was removed by adding sodium dithionite (1 mg/ml) to the culture, which was promptly saturated with CO for 15 minutes. Bacterial cells were then pelleted by centrifugation and Hb purified by ion exchange chromatography according to Natarajan et al.[55]

It should be noted that the β82Asn residue of human Hb Providence is relatively uncommon in that it slowly undergoes post-translational deamination *in vivo* to form aspartic acid, with the latter residue comprising ~67-75% in mature mixed blood[20,22]. While it is unknown to what degree *H. gigas* β/δ82Asn was catalyzed into Asp in nature, O_2 binding data (see below) of this species was collected from freshly purified recombinant samples for which only one peak—presumably β/δ82Asn—was resolved during chromatography (data not shown). Additionally, this reaction is dependent on the local protein environment[56], specifically two nearby residues β143His and β83Gly[22]. Importantly, the latter residue was replaced by β/δ83Ser on the Steller's sea cow branch (Fig. 1B), which is expected to slow (but not stop) the rate of deamidation[55]. Regardless, since the two Hb Providence isoforms have similar O_2 affinities and functional properties[17,20,23] it is unlikely that presence of β/δ82Asp in Steller's sea cow blood would meaningfully alter the results and interpretations presented herein.

Functional Analyses of Hbs. O_2-equillibrium curves for Hb solutions (0.25 mM heme in 0.1 M HEPES buffers) were measured at 25 and 37°C using the thin film technique described by Weber[57]. Hb solutions varied in their pH (6.9, 7.4, and 7.9), chloride concentration (0 or 0.1 M KCl), and organic phosphate concentration (0 or 2-fold molar excess of DPG relative to tetrameric Hb concentrations) in order to test the influence of these cofactors on Hb function. Each Hb solution

was sequentially equilibrated with three to five different oxygen tensions (PO_2) that result in Hb–O_2 saturations between 30 to 70%. Hill plots (log[fractional saturation/[1-fractional saturation]] vs logPO_2) constructed from these measurements were used to determine the PO_2 (P_{50}) and the cooperativity coefficient (n_{50}) at half saturation, from the χ-intercept and slope of these plots, respectively. By this method, the r^2 determination coefficients for the fitted curves exceed 0.995 and the standard errors (SEM) are less than 3% of the P_{50} and n_{50} values[47]. P_{50} values at 25 and 37°C were used to assess the thermal sensitivity of sirenian Hbs by calculating the apparent enthalpy of oxygenation using the van't Hoff isochore:

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

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

Solubility assay. Ammonium sulfate was added to Hb solutions (0.074±0.004 mM Hb_4) to generate final concentrations that ranged from 0 to 3.5 M. These solutions were incubated for 60 minutes at 37°C and the remaining soluble Hb was measured via Drabkin's reagent, according to manufacturer's instructions (Sigma-Aldrich).

Acknowledgements

We thank Mike Gaudry and Elin Ellebæk Petersen for technical assistance, Chandrasekhar Natarajan for providing us with a hemoglobin expression plasmid, and J. Storz for constructive feedback on an earlier version of this manuscript. Authorization to use paintings by C. Buell was kindly provided by J. Gatesy. This study was supported by NSERC (Canada) Discovery and Accelerator Supplement Grants (K.L.C.; RGPIN/238838-2011, RGPIN/412336-2011, and RGPIN/06562-2016), an NSERC Postgraduate Scholarship (A.V.S), the Faculty of Science and Technology, Aarhus University (REW), and the Danish Council for Independent Research (AF; DFF - 4181-00094).

References

1. Ponganis, P. J. Diving mammals. *Compr. Physiol.* **1**, 447–465 (2011).
2. Blessing, M. H. Studies on the concentration of myoglobin in the sea-cow and porpoise. *Comp. Biochem. Physiol. A* **41**, 475–480 (1972).
3. Scholander, P. F. & Irving, L. Experimental investigations on the respiration and diving of the florida manatee. *J. Cell. Comp. Physiol.* **17**, 169–191 (1941).
4. Signore, A. V. *et al.* Emergence of a chimeric globin pseudogene and increased hemoglobin oxygen affinity underlie the evolution of aquatic specializations in Sirenia. *Mol. Biol. Evol.* **36**, 1134–1147 (2019).
5. Gallivan, G. J., Kanwisher, J. W. & Best, R. C. Heart rates and gas exchange in the Amazonian manatee (*Trichechus inunguis*) in relation to diving. *J. Comp. Physiol. B* **156**, 415–423 (1986).
6. Marsh, H., O'Shea, T. J. & Reynolds III, J. E. *Ecology and conservation of the Sirenia: dugongs and manatees*. (2011).
7. Domning, D. P. An ecological model for late tertiary sirenian evolution in the North Pacific Ocean. *Systemat. Biol.* **25**, 352–362 (1976).
8. Stejneger, L. How the great northern sea-cow (Rytina) became exterminated. *Am. Nat.* **21**, 1047-1054 (1887).
9. Steller, G. W. De bestiis marinis [The beasts of the sea]. *Novi Commentarii Acad. Sci. Imp. Petropoli.* **2**, 289-398 (1751).
10. Le Duc, D. *et al.* Genomic basis for skin phenotype and cold adaptation in the extinct Steller's sea cow. *Sci. Adv.* **8**, eabl6496 (2022).
11. Gaudry, M. J. *et al.* Inactivation of thermogenic UCP1 as a historical contingency in multiple placental mammal clades. *Sci. Adv.* **3**, e1602878 (2017).

12. Mirceta, S. *et al.* Evolution of mammalian diving capacity traced by myoglobin net surface charge. *Science* **340**, 1234192 (2013).

13. Sharko, F. S. *et al.* Molecular phylogeny of the extinct Steller's sea cow and other Sirenia species based on their complete mitochondrial genomes. *Genomics* **111**, 1543–1546 (2019).

14. Sharko, F. S. *et al.* Steller's sea cow genome suggests this species began going extinct before the arrival of Paleolithic humans. *Nat. Commun.* **12**, 2215 (2021).

15. Springer, M. S. *et al.* Interordinal gene capture, the phylogenetic position of Steller's sea cow based on molecular and morphological data, and the macroevolutionary history of Sirenia. *Mol. Phylo. Evol.* **91**, 178–193 (2015).

16. Abraham, B. *et al.* Isolated Hb Providence β82Asn and β82Asp fractions are more stable than native HbA0 under oxidative stress conditions. *Biochemistry* **50**, 9752–9766 (2011).

17. Bonaventura, J. *et al.* Hemoglobin providence. Functional consequences of two alterations of the 2,3-diphosphoglycerate binding site at position beta 82. *J. Biol. Chem.* **251**, 7563–7571 (1976).

18. Ikkala, E. *et al.* Hb Helsinki: A variant with a high oxygen affinity and a substitution at a 2, 3-DPG binding site (β82 [EF6] Lys→ Met). *Acta Haematol.* **56**, 257–275 (1976).

19. Sugihara, J. *et al.* Hemoglobin Rahere, a human hemoglobin variant with amino acid substitution at the 2,3-diphosphoglycerate binding site. Functional consequences of the alteration and effects of bezafibrate on the oxygen bindings. *J. Clin. Invest.* **76**, 1169–1173 (1985).

20. Bardakjian, J. *et al.* A new case of Hemoglobin Providence (α2 β2 82 (Ef6) Lys → Asn or Asp) discovered in a french caucasian family. Structural and functional studies. *Hemoglobin* **9**, 333–348 (1985).

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

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

23. Charache, S. *et al.* Postsynthetic deamidation of hemoglobin Providence (beta 82 Lys replaced by Asn, Asp) and its effect on oxygen transport. *J Clin Invest* **59**, 652–658 (1977).

24. Weickert, M. J., Pagratis, M., Glascock, C. B. & Blackmore, R. A mutation that improves soluble recombinant hemoglobin accumulation in *Escherichia coli* in heme excess. *App. Enviro. Microbiol.* **65**, 640–647 (1999).

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

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

27. Campbell, K. L. *et al.* Substitutions in woolly mammoth hemoglobin confer biochemical properties adaptive for cold tolerance. *Nat. Genet.* **42**, 536–540 (2010).

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

29. Farmer, M., Weber, R. E., Bonaventura, J., Best, R. C. & Domning, D. Functional properties of hemoglobin and whole blood in an aquatic mammal, the Amazonian manatee (*Trichechus inunguis*). *Com.p Biochem. Physiol. A* **62**, 231–238 (1979).

30. Bunn, H. F. Regulation of hemoglobin function in mammals. *Amer. Zool.* **20**, 199–211 (1980).

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

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

33. Campbell, K. L. *et al.* Molecular basis of a novel adaptation to hypoxic-hypercapnia in a strictly fossorial mole. *BMC Evol. Biol.* **10**, 214 (2010).

34. Fronticelli, C., Bucci, E. & Razynska, A. Modulation of oxygen affinity in hemoglobin by solvent components: Interaction of bovine hemoglobin with 2,3-diphosphoglycerate and monatomic anions. *J. Mol. Biol.* **202**, 343–348 (1988).

35. Janecka, J. E. *et al.* Genetically based low oxygen affinities of felid hemoglobins: lack of biochemical adaptation to high-altitude hypoxia in the snow leopard. *J. Exp. Biol.* **218**, 2402–2409 (2015).

36. Perutz, M. F., Fermi, G., Poyart, C., Pagnier, J. & Kister, J. A novel allosteric mechanism in haemoglobin. Structure of bovine deoxyhaemoglobin, absence of specific chloride-binding sites and origin of the chloride-linked Bohr effect in bovine and human haemoglobin. *J. Mol. Biol.* **233**, 536–545 (1993).

37. Jana, S., Strader, M. B. & Alayash, A. I. The Providence mutation (βK82D) in human hemoglobin substantially reduces βCysteine 93 oxidation and oxidative stress in endothelial cells. *Int. J. Mol. Sci.* **21**, 9453 (2020).

38. Strader, M. B. *et al.* Engineering oxidative stability in human hemoglobin based on the Hb providence (βK82D) mutation and genetic cross-linking. *Biochem. J.* **474**, 4171–4192 (2017).

39. Wong, A. W., Lanyon, J. M., Sneath, H. L., Leggatt, G. R. & Woolford, L. Comparison of i-STAT® with traditional laboratory analysers in the measurement of blood analytes from field captured dugongs (*Dugong dugon*). *Aqua. Mam.* **44**, 19–31 (2018).

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

41. Domning, D. P. What Can We Infer About the Behavior of Extinct Sirenians? in *Ethology and Behavioral Ecology of Sirenia* (ed. Marsh, H.) 1–17 (Springer International Publishing, 2022).

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

43. Weber, R. E., Campbell, K. L., Fago, A., Malte, H. & Jensen, F. B. ATP-induced temperature independence of hemoglobin-O_2 affinity in heterothermic billfish. *J. Exp. Biol.* **213**, 1579–1585 (2010).

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

45. Jensen, B., Storz, J. F. & Fago, A. Bohr effect and temperature sensitivity of hemoglobins from highland and lowland deer mice. *Comparative Biochemistry and Physiology, Part A* **195**, 10–14 (2016).

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

47. Weber, R. E., Fago, A. & Campbell, K. L. Enthalpic partitioning of the reduced temperature sensitivity of O_2 binding in bovine hemoglobin. *Comp. Biochem. Physiol. A* **176**, 20–25 (2014).

48. O'Shea, T. J., Beck, C. A., Bonde, R. K., Kochman, H. I. & Odell, D. K. An analysis of manatee mortality patterns in Florida, 1976-81. *J. Wildl. Manag.* **49**, 1–11 (1985).

49. Rommel, S. A. & Caplan, H. Vascular adaptations for heat conservation in the tail of Florida manatees (*Trichechus manatus latirostris*). *J. Anat.* **202**, 343–353 (2003).

50. Marshall, C. D., Sarko, D. K. & Reep, R. L. Morphological and Sensory Innovations for an Aquatic Lifestyle. in *Ethology and Behavioral Ecology of Sirenia* (ed. Marsh, H.) 19–65 (Springer International Publishing, 2022).

51. Müller, G. F. *Voyages from Asia to America, for Completing the Discoveries of the North West Coast of America.* London: Thomas Jefferys (1761).

52. Odell, D. K. Sirenian Life History. in *Encyclopedia of Marine Mammals (Second Edition)* (eds. Perrin, W. F., Würsig, B. & Thewissen, J. G. M.) 1019–1021 (Academic Press, 2009).

53. Hebbel, R. P., Berger, E. M. & Eaton, J. W. Effect of increased maternal hemoglobin oxygen affinity on fetal growth in the rat. *Blood* **55**, 969–974 (1980).

54. Bauer, C., Jelkmann, W. & Moll, W. High oxygen affinity of maternal blood reduces fetal weight in rats. *Respir. Physiol.* **43**, 169–177 (1981).

55. Natarajan, C. *et al.* Expression and purification of recombinant hemoglobin in *Escherichia coli*. *PLoS ONE* **6**, e20176 (2011).

55. Robinson, N. E. Protein deamidation. *Proc. Nat. Acad. Sci.* **99**, 5283–5288 (2002).

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

57. Heritage, S., & Seiffert, E.R. Total evidence time-scaled phylogenetic and biogeographic models for the evolution of sea cows (Sirenia, Afrotheria). *PeerJ* **10**, e13886 (2022).

Figure Legends

Figure 1. Evolution of notable morphological and genetic attributes within Sirenia. A) Phenotypic innovations contributing to the unique biology of Steller's sea cows are mapped along the sirenian phylogeny (red bars) with the underlying genetic causes shown in brackets where known. Note that the red bars do not represent the dating of these traits and that their placement order is arbitrary. Divergence dates in millions of years (Mya) are based on Heritage and Seiffert (2022)[57]. The ancestral dugongnid (Anc. dugongid) is represented by a late-Oligocene *Metaxytherium spp*. Images by Carl Buell, and are used with permission. B) Partial nucleotide/amino acid alignment of the sirenian β/δ-globin gene in the central region of the 2,3-diphosphoglycerate binding pocket of hemoglobin. The G→C mutation underlying the otherwise invariant amino acid substitution (β/δ82Lys→Asn) of Steller's sea cow Hb is apparent in all 16 individuals for which sequence data is available. Values in brackets next to each *H. gigas* specimen represent the depth of sequence coverage for this nucleotide. Dots represent sequence identity with *T. manatus*.

Figure 2. Biochemical properties of hemoglobins (Hbs) from manatee (*T. manatus*), dugong (*D. dugon*), ancestral dugongid (Anc. dugongid), Steller's sea cow β/δ82Asn→Lys mutant (*H. gigas* β82K), and wild-type Steller's sea cow (*H. gigas*). All values were measured at 37°C and pH 7.2, unless otherwise noted. A) Oxygen tensions at half O_2 saturation (P_{50}) in the absence (stripped) and presence of allosteric cofactors (0.2 mM 2,3-diphosphoglycerate (DPG) and 100 mM KCl). B) The enthalpy of oxygenation (ΔH) between 25 and 37°C in stripped Hb and in the presence of allosteric cofactors (0.2 mM DPG and 100 mM KCl). C) The effect of DPG on sirenian Hbs determined from $\log P_{50}^{(0.2\ \mathrm{mM\ DPG})} - \log P_{50}^{(\mathrm{stripped})}$. D) The effect of chloride on sirenian Hbs determined from $\log P_{50}^{(100\ \mathrm{mM\ KCl})} - \log P_{50}^{(\mathrm{stripped})}$. E) The Bohr effect of sirenian Hbs in the presence of allosteric cofactors (0.2 mM DPG and 100 mM KCl), as calculated from $\Delta \log P_{50}/\Delta pH$ over the pH range 6.9 and 7.8. F). The relative solubility of sirenian Hbs is denoted by the percentage of Hb protein precipitated by the addition of 3M ammonium sulfate.

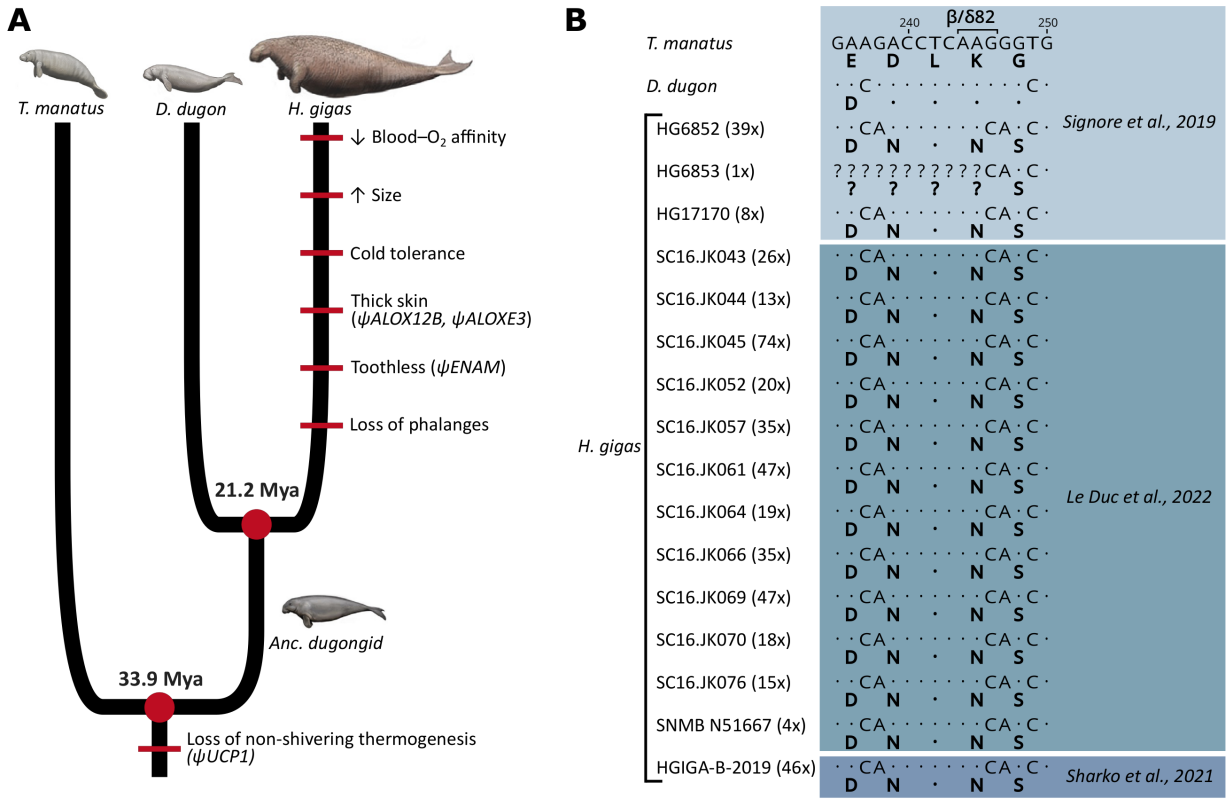
A
T. manatus
D. dugon
H. gigas
↓ Blood–O2 affinity
↑ Size
Cold tolerance
Thick skin
(ψALOX12B, ψALOXE3)
Toothless (ψENAM)
Loss of phalanges
21.2 Mya
Anc. dugongid
33.9 Mya
Loss of non-shivering thermogenesis
(ψUCP1)
B
β/δ82
240
250
T. manatus
GAAGACCTCAAGGGTG
E D L K G
D. dugon
D
HG6852 (39x)
HG6853 (1x)
HG17170 (8x)
SC16.JK043 (26x)
SC16.JK044 (13x)
SC16.JK045 (74x)
SC16.JK052 (20x)
SC16.JK057 (35x)
SC16.JK061 (47x)
SC16.JK064 (19x)
SC16.JK066 (35x)
SC16.JK069 (47x)
SC16.JK070 (18x)
SC16.JK076 (15x)
SNMB N51667 (4x)
HGIGA-B-2019 (46x)
H. gigas
Signore et al., 2019
Le Duc et al., 2022
Sharko et al., 2021

Figure 1.

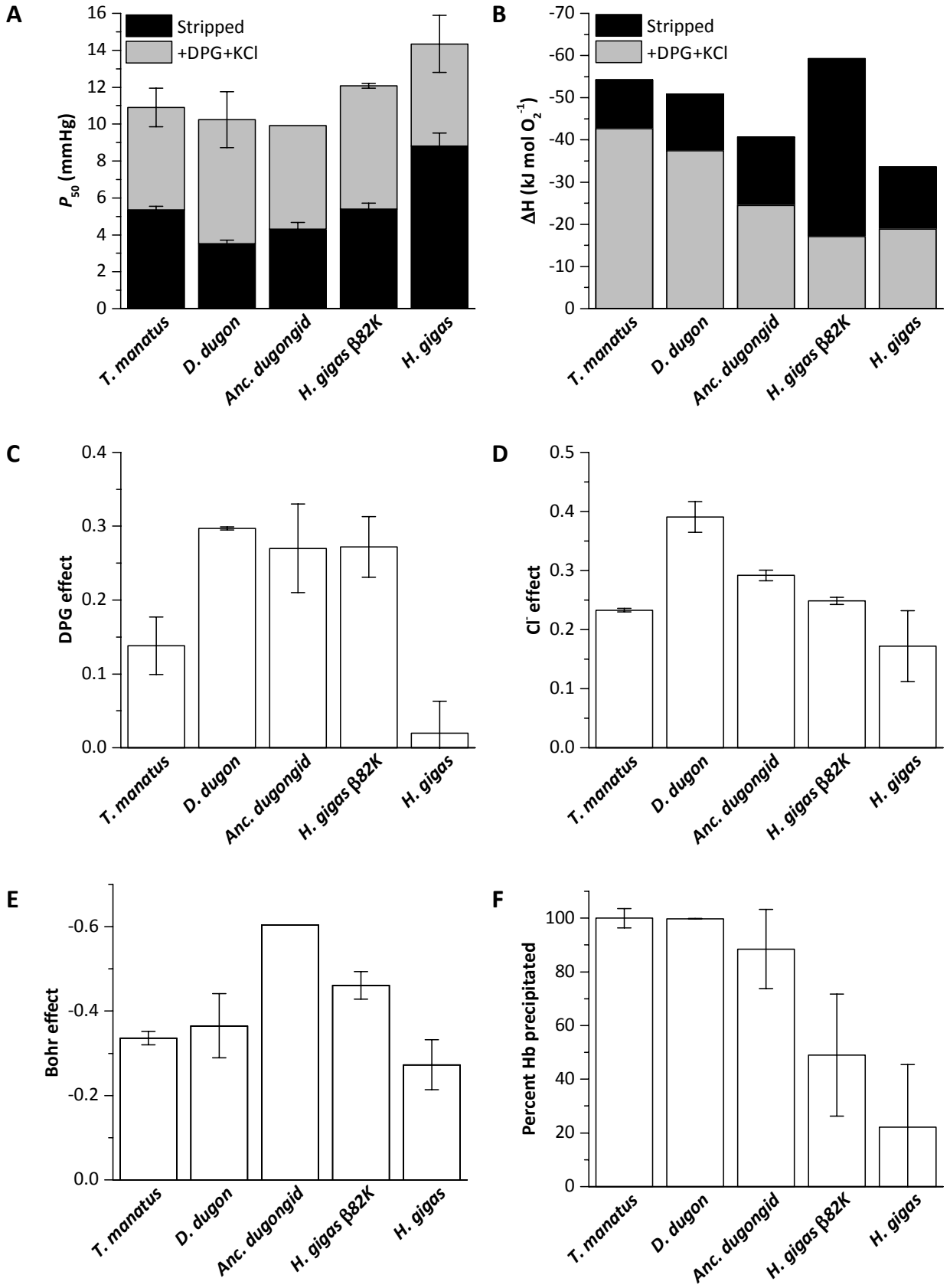
A
Stripped
+DPG+KCl
P_{50} (mmHg)
0
2
4
6
8
10
12
14
16
T. manatus
D. dugon
Anc. dugongid
H. gigas β82K
H. gigas
B
Stripped
+DPG+KCl
ΔH (kJ mol O_2^{-1})
0
-10
-20
-30
-40
-50
-60
-70
T. manatus
D. dugon
Anc. dugongid
H. gigas β82K
H. gigas
C
DPG effect
0.0
0.1
0.2
0.3
0.4
T. manatus
D. dugon
Anc. dugongid
H. gigas β82K
H. gigas
D
Cl^- effect
0.0
0.1
0.2
0.3
0.4
0.5
T. manatus
D. dugon
Anc. dugongid
H. gigas β82K
H. gigas
E
Bohr effect
0.0
-0.2
-0.4
-0.6
T. manatus
D. dugon
Anc. dugongid
H. gigas β82K
H. gigas
F
Percent Hb precipitated
0
20
40
60
80
100
T. manatus
D. dugon
Anc. dugongid
H. gigas β82K
H. gigas

Figure 2.