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RESEARCH ARTICLE

Relationship between red blood cell lifespan and endogenous carbon monoxide in the common bottlenose dolphin and beluga

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Abstract

Certain deep-diving marine mammals [i.e., northern elephant seal (*Mirounga angustirostris*), Weddell seal (*Leptonychotes weddellii*)] have blood carbon monoxide (CO) levels that are comparable with those of chronic cigarette smokers. Most CO produced in humans is a byproduct of heme degradation, which is released when red blood cells (RBCs) are destroyed. Elevated CO can occur in humans when RBC lifespan decreases. The contribution of RBC turnover to CO concentrations in marine mammals is unknown. Here, we report the first RBC lifespans in two healthy marine mammal species with different diving capacities and heme stores, the shallow-diving bottlenose dolphin (*Tursiops truncatus*) and deep-diving beluga whale (*Delphinapterus leucas*), and we relate the lifespans to the levels of CO in blood and breath. The belugas, with high blood heme stores, had the longest mean RBC lifespan compared with humans and bottlenose dolphins. Both cetacean species were found to have three times higher blood CO content compared with humans. The estimated CO production rate from heme degradation indicates some marine mammals may have additional mechanisms for CO production, or delay CO removal from the body, potentially from long-duration breath-holds.

NEW & NOTEWORTHY This is the first study to determine the red blood cell lifespan in a marine mammal species. High concentrations of carbon monoxide (CO) were found in the blood of bottlenose dolphins and in the blood and breath of belugas compared with healthy humans. Red blood cell turnover accounted for these high levels in bottlenose dolphins, but there may be alternative mechanisms of endogenous CO production that are contributing to the CO concentrations observed in belugas.

gasotransmitter; hematology; isotopes; osmotic fragility; physiology

INTRODUCTION

In healthy humans, ~80% of endogenous carbon monoxide (CO) is produced from the degradation of heme from hemoglobin inside red blood cells (RBCs) that are naturally turned over by the reticuloendothelial system or from ineffective erythropoiesis in the bone marrow (1, 3). The process begins with senescent RBCs being engulfed by macrophages, releasing intracellular hemoglobin. The globin units from hemoglobin are then subsequently broken down into its amino acid components and recycled, whereas the heme is oxidized by heme oxygenase enzymes (HO-1, HO-2), producing equimolar concentrations of CO, ferrous iron, and biliverdin. Biliverdin is reduced by biliverdin reductase to bilirubin and excreted from the body (Fig. 1*A*). The endogenously produced CO will bind to heme proteins in the body, such as hemoglobin to form carboxyhemoglobin (COHb).

Endogenous CO production in healthy adult humans results in less than 1% COHb (4). In certain diseases associated with increased RBC and heme turnover, endogenous CO levels in the blood and breath are elevated. For example, individuals with sickle cell disease have an RBC lifespan of ~2 wk, resulting in mean COHb of 2.4% (6, 7). Adult females can almost double their CO production during the progesterone phase of the menstrual cycle most likely due to hepatic heme catabolism (8). End-tidal CO (ETCO) measurements are useful to obtain a noninvasive measurement of CO in the body, and to investigate the quantity and rate of removal, as the primary route for the gas to leave the body is via the lungs (4, 9). Healthy adult humans have an average ETCO value of ~1.8 ± 0.7 ppm (5). Whereas, patients with sickle cell

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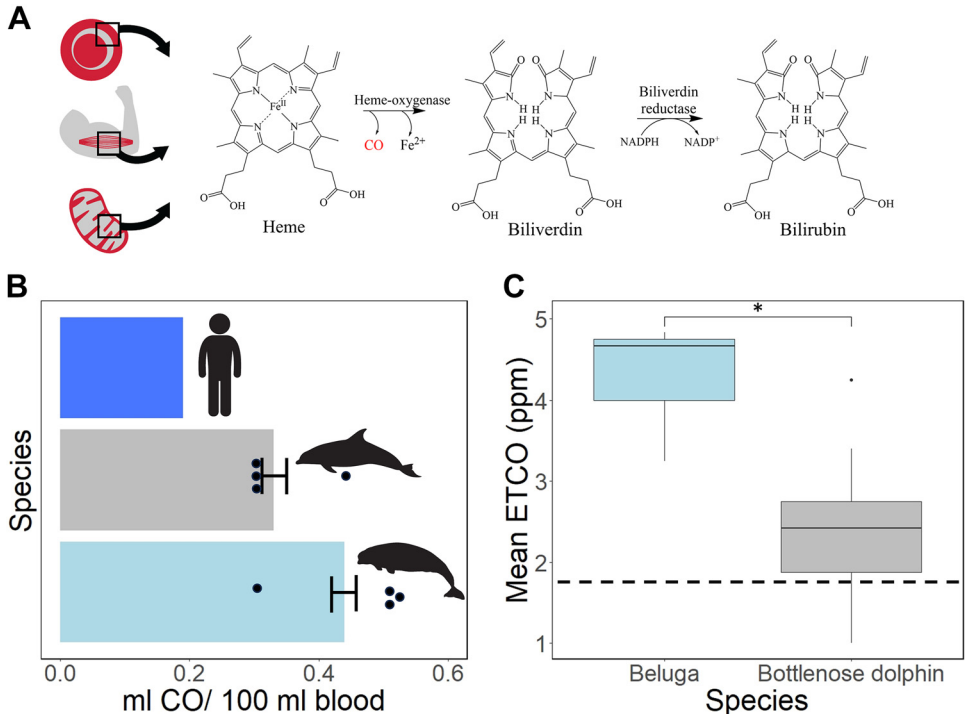

Figure 1. Carbon monoxide (CO) mechanism of production and levels in the blood and breath. *A*: endogenous CO is produced through the heme oxygenase-dependent breakdown of heme molecules that come from sources such as hemoglobin in red blood cells, myoglobin in muscle, and other hemoproteins (e.g., cytochromes, neuroglobin). About 80% of the CO produced in the body is from the breakdown of hemoglobin in red blood cells (3). Heme is cleaved by heme oxygenase enzymes to form biliverdin, producing CO and ferrous iron in the process. Biliverdin is then typically reduced to bilirubin via biliverdin reductase. *B*: volume of CO in blood (mL of CO·100 mL^{-1}·blood) for humans (4), bottlenose dolphins ($n = 4$, 2 males, 2 females) and belugas ($n = 4$, 2 males, 2 females). Individuals are represented as black dots. *C*: box plot representing the mean and interquartile range of the end-tidal carbon monoxide (ETCO) of the beluga ($n = 4$, 2 males, 2 females) and bottlenose dolphin ($n = 4$, 2 males, 2 females). *Significant difference between the two species ($P < 0.05$). Dotted line represents the mean ETCO (ppm) for an adult human ~1.8 ppm (5).

disease or thalassemia had average ETCO values of 6.4 ± 2.9 and 3.6 ± 1.5 ppm, respectively (5, 10).

The average healthy adult human breaks down and produces ~200 billion RBCs every day, making them the most abundant and frequently produced cell in the body (11). Newly produced RBCs will circulate in the bloodstream for ~120 days, until they are removed by the reticuloendothelial system, primarily within the spleen and liver (12–18). RBCs must exhibit extreme flexibility as they travel through the splenic sinusoids to avoid removal from the circulatory system. Structural proteins of the RBC membrane, including Band 3, α-spectrin, β-spectrin, protein 4.1, tropomyosin, tropomodulin, adducin, stomatin, and dematin, work together to form a flexible, yet strong net around the RBC (19–21). Certain membrane proteins may be more important to different species by allowing the RBC to withstand specific environmental or physiological challenges. For example, llamas (*Lama glama*), a high-altitude adapted camelid, have RBCs with a unique elliptical shape and three times the concentration of Band 3 compared with humans, indicating a need for increased anion transport to facilitate efficient carbon dioxide transport (22). Moreover, the llama RBCs were resistant to several conditions that caused human RBCs to form spicules (22). The quantity and types of proteins present in RBC membranes have an influence on the overall fragility of the cell. An osmotic fragility test for RBCs determines the lowest salinity at which 50% lysis occurs and complements our understanding of how membrane protein composition can impact strength and flexibility.

Guanacos (*Lama guanicoe*), another camelid species, have the longest reported mammalian RBC lifespan with a median RBC survival of 225 days and camelid RBCs have been found to withstand extreme hypotonic exposure by swelling over twice their size before rupture (22). The RBC lifespan positively correlates with body mass for a variety of terrestrial mammalian species (23, 24). The allometric relationship of RBC lifespan is hypothesized to be related to the mass-specific metabolic rate of species. Within this relationship, larger animals consume less oxygen per tissue mass, consequently forming fewer reactive oxygen species (ROS) that can damage cell membranes, proteins, DNA, etc., via oxidative stress. This damage can impede the flexibility of RBCs, preventing passage through microvasculature by disrupting cytoskeletal proteins of the membrane, eventually leading to their removal from circulation (25, 26).

Marine mammals are well known for large variations in body mass between species as well as continually experiencing repetitive ischemia/reperfusion (I/R) events in several tissues from diving, resulting in routine exposure to oxidative stress (27–30). For example, ringed seals (*Pusa hispida*) have higher superoxide production rates in the heart, kidney, and muscle tissue, when compared with similarly sized terrestrial mammals (28). Also, deep-diving pygmy and dwarf sperm whales (*Kogia breviceps* and *Kogia sima*, respectively) have higher superoxide production rates in pectoral muscle, heart, liver, lung, and kidney when compared with the shallow-diving bottlenose dolphins (27). Marine mammals have been found to express high concentrations of antioxidant enzymes to protect against repetitive exposure to high concentrations of ROS (27, 31–35). It has recently been suggested that increased endogenous CO production may be an additional anti-inflammatory and antioxidant mechanism that certain marine mammal species utilize to combat injuries from I/R events (36).

Although CO is known to be a toxic gas at high concentrations, low to moderate concentrations of the gas have been demonstrated to exert cytoprotective effects against I/R injuries following organ transplants in both mice (*Mus musculus*)

and rats (*Rattus rattus*) (37). Specifically, the gas directly inhibits the production of proinflammatory cytokines and the signaling cascade that leads to apoptosis (38, 39). Importantly, certain deep-diving marine mammal species with high quantities of whole body heme stores from hemoglobin and myoglobin have been found to have elevated CO concentrations in their blood; these levels support the hypothesis that naturally high concentrations of endogenous CO may serve a protective mechanism against damaging effects associated with oxidative stress, inflammation, and/or apoptosis (36, 40, 41).

To date, two deep-diving pinniped species, Weddell seals and northern elephant seals, are known to have consistently elevated blood CO levels that are not attributable to a known morbidity (40, 41). Weddell seals were found to have between 2.5 and 17 times the CO in their blood when compared with human nonsmokers (41). Adult northern elephant seals had mean COHb levels of 8.7% in venous blood, similar to levels found in chronic cigarette smokers (40). The source of this CO is currently unknown but is hypothesized to be related to their large heme stores in blood and the rates at which the stores are turned over. In this study, the relationship between RBC lifespan and endogenous CO levels in blood and exhaled breath is examined in two species of marine mammals. These species were chosen based on their differences in dive durations, heme storage capacity, and body mass. The beluga whale is a deep diver, has high heme storage, and a large body mass, whereas the bottlenose dolphin is considered to be a shallow diver and has a lower heme storage capacity and body mass (42–44). These differences allow for a comparison of factors that may influence red blood cell lifespan and endogenous CO levels.

MATERIALS AND METHODS

Red Blood Cell Lifespan

Data collection took place from 2020 to 2022 on four healthy adult bottlenose dolphins (BDs) and four healthy adult belugas housed at SeaWorld facilities (Table 1). The methods for this study were reviewed and approved by UNCW Institutional Research and Animal Welfare Committee before data collection. Veterinary oversight throughout the project ensured the safety and comfort of the animals included in this study.

To measure RBC lifespan, we used the common amino acid glycine that has nitrogen labeled with the heavy nitrogen isotope (^{15}N-labeled glycine, 98%; No. NLM-202, Cambridge Isotope Laboratories). The glycine is administered orally and is utilized by the animal in the production of new proteins, such as hemoglobin (Fig. 2*A*). This results in newly synthesized hemoglobin being enriched in the heavy nitrogen isotope (^{15}N) when compared with older hemoglobin, and this can be detected through isotope ratio mass spectrometry (IRMS). The enriched RBCs are expected to circulate in the body for the duration of the red blood cell lifespan and then be removed from the body by the reticuloendothelial system. This process of production, circulation, and removal will be reflected in the change in δ^{15}N values of blood samples collected after the ingestion of the glycine (Fig. 2*B*).

The ratio between the light isotope (^{14}N) and the heavy isotope (^{15}N) was measured through IRMS, and the values obtained were expressed in delta notation as follows:

$$\delta^{15}\mathrm{N}\,(‰) = \left(\frac{R\,\text{sample}}{R\,\text{standard}} - 1\right) \times 1,000 \qquad (1)$$

where R is the ratio of the heavy isotope (^{15}N) over the light isotope (^{14}N) for the heme samples collected from animals and for the standard (N_2 gas).

Animals voluntarily presented their flukes to veterinary staff for routine collection of fasted mixed-venous blood samples (3–10 mL) from the central fluke veins into Vacutainers with lithium heparin or EDTA to prevent clot formation. A blood sample was taken before administration of glycine to measure the baseline values of δ^{15}N in heme from hemoglobin (45). The ^{15}N-labeled glycine dosage was based on the individuals' body mass following the dose given to adult humans in the study by Khera et al. (~20 mg·kg^{-1}) (45). The glycine was preweighed and packaged into cellulose capsules and administered orally with the animal's food. We confirmed the increase in δ^{15}N compared with baseline within a month of glycine ingestion. A smaller dose was given to two of the bottlenose dolphins (BD-3 and BD-4: ~6–7 mg·kg^{-1}) to assess if this lower dose would achieve adequate enrichment, defined by a doubling of the δ^{15}N values from baseline. This dose did reach adequate enrichment to distinguish it from baseline values within 1 mo (Fig. 2*C*). Previous studies have shown that the RBC lifespan estimates are not affected by the dose if adequate enrichment is achieved (45). The first postglycine blood sample was collected within 24 h after consumption of glycine, and additional blood samples were obtained every 2–4 weeks afterward, for up to 301 days in the beluga and 204 days in the bottlenose dolphin. The whole blood samples were stored at −80°C until heme extraction.

Whole blood was thawed and heme was extracted and lyophilized for 24–48 h (46). Heme was then stored at room temperature in a desiccator until analysis (45, 46). Each

Table 1. *Animal information*

Animal ID	Species	Sex	Mass, kg	Dose ^{15}N Enriched-Glycine, mg·kg^{-1}
BD-1	Bottlenose dolphin	Male	250	24
BD-2	Bottlenose dolphin	Female	211	24
BD-3	Bottlenose dolphin	Male	267	6
BD-4	Bottlenose dolphin	Female	230	7
BW-1	Beluga	Female	574	20
BW-2	Beluga	Male	1091	17
BW-3	Beluga	Female	612	20
BW-4	Beluga	Male	855	20

Identification, species, sex, mass, and dose of nitrogen-15 (^{15}N) enriched-glycine for each animal.

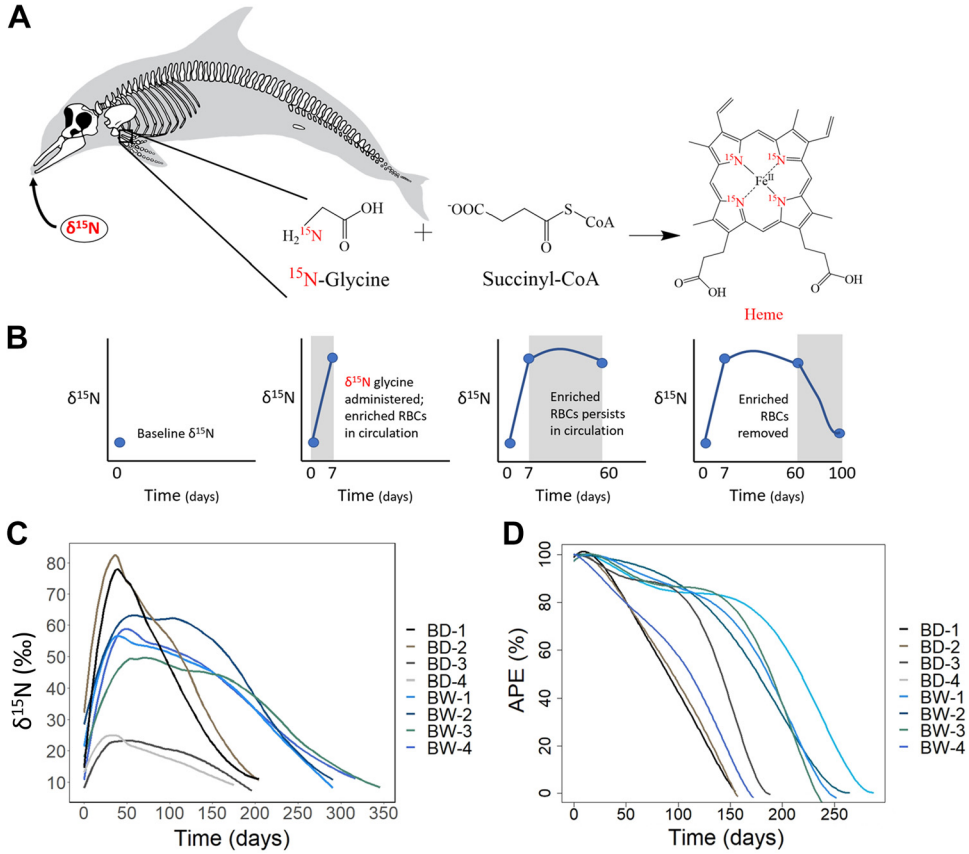

Figure 2. Measurement of the red blood cell lifespan through enriched glycine. *A*: enriched glycine (^{15}N-labeled glycine) is orally ingested by animals, where the glycine combines with succinyl coenzyme-A within the bone marrow to form new heme molecules within red blood cells. *B*: plots depicting the expected rise in δ^{15} nitrogen of heme after ingestion of glycine and fall of δ^{15} of heme after all δ^{15} nitrogen-enriched heme has been incorporated into newly produced hemoglobin. *C*: average δ^{15} curve for each individual from this study. Bottlenose dolphins (n = 4, 2 males, 2 females) are indicated in gray/black and belugas (n = 4, 2 males, 2 females) are indicated in green/blue. *D*: a fifth-order polynomial set to the atom percent excess (APE) for each individual from this study. Bottlenose dolphins (n = 4, 2 males, 2 females) are indicated in gray/black and belugas are indicated in green/blue (n = 4, 2 males, 2 females).

lyophilized heme sample was then analyzed in triplicate with a Costech 1040 elemental analyzer connected to a Thermo Finnigan Delta V Plus isotope ratio mass spectrometer. Median RBC survival, mean RBC survival, half-median RBC survival, and mean RBC age was calculated for each individual animal following the studies by Khera et al. (45) and Lindsell et al. (47). Atom Percent Excess (APE) was determined by subtracting the average δ^{15}N before administration of ^{15}N-labeled glycine from the average maximum δ^{15}N values for each animal postadministration. A normalized survival curve was created for each individual by setting the maximum value at 100% and adjusting all points relative to the maximum for a relative percent APE (%APE). A fifth-order polynomial was fit to the corrected survival curve and the mean RBC survival was determined by finding the area under the curve using R (RStudio, v.4.1.3) (Supplemental Table S1). The median survival is defined as the point at which the %APE falls to 50%, and the half-median survival is half of this value. The mean RBC age is calculated using a life table approach and is reflective of the mean age of RBCs present in a population at any time (45).

Hematology and Serum Chemistry

Blood was voluntarily collected from the fluke for all animals. Blood was collected into EDTA for complete blood counts and into a Thrombin Rapid Serum Tube for blood chemistry. The serum tube was allowed to sit for 15 min and then centrifuged to separate serum. Blood chemistry was analyzed with Beckman Coulter AU480 analyzer. Blood smears were prepared using the Giemsa-Wright staining technique and analyzed with Sysmex XNV Hematology analyzer (Table 2).

CO Production from RBC Turnover

The volume (in mL) of CO produced per hour was evaluated for all animals in the red blood cell lifespan section. This was calculated by first estimating the total moles of blood heme in the animals from a measured hemoglobin concentration (g·100 mL^{-1}), measured body mass (kg), and estimated mass-specific blood volume (mL·kg^{-1}) based on previous studies in bottlenose dolphins and belugas (Table 3, Supplemental Methods S1) (42, 44). The total moles of blood heme were multiplied by the heme turnover rate, determined from the reciprocal of the mean RBC survival, to obtain moles of heme degraded per hour. This value was converted into milliliters of CO produced per hour by multiplying by the volume a mole of gas occupies at standard temperature and pressure (22,400 mL). Because of the large differences in mass-specific heme stores and body mass between the two species from this study, we also predicted CO production rates as a function of their total body hemoglobin (mL CO·h^{-1}·g^{-1}·10^{-4}) and body mass (mL CO·h^{-1}·kg^{-1}·10^{-3}). These values are compared with the whole body CO production for adult male humans previously measured (48). The human values were corrected to reflect the amount of CO produced solely from RBC turnover, assuming ~80% of CO production is from RBC turnover (2).

Table 2. *Complete blood counts and hematological parameters for all individuals*

Animal ID	BD-1	BD-2	BD-3	BD-4	BW-1	BW-2	BW-3	BW-4
Total bilirubin, mg·dL^{-1}	0.2	0.1	0.2	0.6	0.3	0.2	0.2	0.2
Iron, μg·dL^{-1}	75	97	111	220	271	323	301	230
RBC, million cells·μL^{-1}	3.35	3.13	3.44	4.12	3.25	2.88	2.92	3.31
Hb, g·dL^{-1}	14.9	13.2	14.0	16.5	22.2	20.8	18.9	19.1
Hct, %	45	39	40	48	55	51	47	50
MCV, fL	132.6	125.7	116.2	115.4	169.0	177.0	160.2	151.5
MCH, pg	44.4	42.3	40.7	40.1	68.0	72.0	64.5	57.9
MCHC, g·dL^{-1}	33.5	33.6	35.0	34.7	41.0	41.0	40.3	38.2
RDW, %	13.8	13.7	15.1	14.5	13.8	14.6	9.4	12.1
WBC, 10^3/μL	5.28	6.54	4.08	3.55	5.60	4.47	7.79	6.41
Retic, %	1.22	2.26	0.80	1.11	1.10	0.60	1.36	0.65
Sodium, mEq·L^{-1}	152	153	155	154	146	149		151
Cholesterol, mg·dL^{-1}	222	165	176	225	214	237	206	273

Hb, hemoglobin; Hct, hematocrit; MCH, mean corpuscular hemoglobin, MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RBC, red blood cell; RDW, red cell distribution width; Retic, reticulocyte; WBC, white blood cell.

Osmotic Fragility

Osmotic fragility was tested by incubation of RBCs in hypotonic to isotonic saline concentrations (0–0.9% NaCl wt/vol) for all belugas and all bottlenose dolphins (49). Whole blood samples (3 mL) were collected into Vacutainers containing EDTA and shipped overnight with icepacks. Samples were analyzed within 24 h of collection. Previous studies on stored RBCs suggest storage up to 14 days at 4 ± 2°C resulted in the same fragility curve for fresh human RBCs (50). Washed RBCs (10 μL) were added to the hypotonic solutions (1 mL) and allowed to sit for 1 h at room temperature. The solutions were gently centrifuged (2,000 rpm, 5 min) and percent hemolysis was calculated by quantifying the hemoglobin concentration in the supernatant using a spectrophotometer at 540 nm (SpectraMax iD5) using the following equation:

$$\%\,\text{hemolysis} = 100\left(\frac{A_{x\%} - A_{0.9\%}}{A_{0.0\%} - A_{0.9\%}}\right) \quad (2)$$

where A represents the absorbance at 0.9%, 0.0%, and at each concentration (x%).

RBC Membrane Proteins

RBC membrane proteins were evaluated for all animals in the red blood cell lifespan section. Isolated RBCs were lysed with 5 mM sodium phosphate buffer, pH 8.0, and centrifuged at 12,000 *g* for 5 min. The supernatant containing free hemoglobin was removed and the process was repeated until the supernatant was clear. RBC membranes (ghosts) pelleted at the bottom and were white to light pink (51, 52). Protein concentration of ghosts was determined with the Bradford assay using bovine serum albumin as the standard (53). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE: 10%) was used to separate the following membrane proteins: α-spectrin, β-spectrin, ankyrin, Band 3, Band 4.1, Band 4.2, actin, and stomatin ($W = 10$, $P = 0.69$) (Bio-Rad, TGX FastCast Acrylamide Kit, 10%). Ghosts were diluted with an equal volume Laemmli sample buffer (125 mM Tris·HCl buffer, pH 6.8, containing 2% SDS, 0.05% 2-mercaptoethanol, and 0.01% bromophenol blue), heated to 70°C for 5 min, and then centrifuged at 1,000 *g* for 1 min at 4°C. Approximately 5 μg of ghosts were loaded into each well. Protein ladders (5 μL) were loaded into wells on either side of the ghosts (Bio-Rad, Precision Plus Protein Dual Color Standards). The gels were stained with 0.1% Coomassie Blue dye in 50% methanol, 10% glacial acetic acid for 30 min, and destained in two steps with 50% methanol and 10% acetic acid for 1 h and then with 7% methanol, 10% acetic acid for 1 h.

Bands and lanes were quantified using BioRad Gel Doc Image Lab 6.1. Lanes were manually created, and bands were

Table 3. *Calculation of CO production rate variables*

ID	Mean RBC Survival, days	Hb, g·dL^{-1}	Blood Volume, mL·kg^{-1}	Body Mass, kg	CO Production Rate, mL CO·h^{-1}	CO Production Rate kg^{-1}, mL CO·h^{-1}·kg^{-1} 10^{-3}	CO Production Rate TBHB^{-1}, mL CO·h^{-1}·g^{-1} 10^{-4}
BW-1	171	22.2	128	523	5.0	9.5	3.3
BW-2	172	20.8	128	1,091	7.1	8.8	3.3
BW-3	196	18.9	128	612	4.3	7.0	2.9
BW-4	169	19.1	128	855	9.6	8.2	3.4
BD-1	90	14.9	71	250	1.7	6.7	6.3
BD-2	101	13.2	71	210	1.1	5.3	5.6
BD-3	131	14.0	71	267	1.2	4.3	4.4
BD-4	92	16.5	71	230	1.7	7.3	6.2
				Average ± SD BW	6.5 ± 2.4	8.4 ± 1.0	3.2 ± 0.2
				Average ± SD BD	1.4 ± 0.3	5.9 ± 1.3	5.6 ± 0.9

The CO production rate (mL CO·h^{-1}) and CO production rate relative to total body hemoglobin (TBHB) (mL CO·h^{-1}·g^{-1} 10^{-4}) for each individual is calculated from mean RBC survival (days), hemoglobin concentration (g·dL^{-1}), blood volume (mL·kg^{-1}), and body mass (kg). The averages and standard deviation for belugas (BW) and bottlenose dolphins (BD) are listed for the CO production rate and CO production rate TBHB^{-1}.

automatically detected with maximum sensitivity. Any missed bands were manually detected, and all band ranges were manually adjusted to reflect band density. The disk size was set using the ladder lane for each gel and applied to all the lanes. Lane percent was used to record the percentage of each band in the RBC membrane.

End-Tidal Carbon Monoxide

A custom-made breath collection device with a series of one-way valves was used to capture end-tidal samples (Fig. 3*A*). Briefly, animals [10 bottlenose dolphins (9 females, 1 male) and 5 belugas (3 females, 2 males) located at SeaWorld San Diego] were first desensitized with the device being gently placed near or over the blowhole (Fig. 3, *B* and *C*). Animals were then trained to breathe normally at the surface without the collection device and then immediately exhale fully into the collection device upon command. A CPR mask at the device's base ensured atmospheric air or water did not enter the breath collection device when the animals were exhaling. During exhalation into the device, a series of one-way valves opened to allow air to pass through, and then closed at the end of exhalation, trapping ~750 mL of end-tidal sample. Using a modified aquarium pump, the sample was then pushed directly into a CO_2 analyzer (Model No. 17515, VacuMed, Ventura, CA) and a portable carbon monoxide breathalyzer (±5%) (ToxCO: Covita, Santa Barbara, CA) to quantify end-tidal CO_2 ($ETCO_2$) and end-tidal CO (ETCO). Only samples which had a $ETCO_2$ of at least 35 mmHg CO_2 were considered to represent a true end-tidal sample and used for ETCO analysis (54). The $ETCO_2$ analyzer had undergone a daily two-point calibration (0.3 mmHg and 76 mmHg) before sample analysis. The ETCO analyzer had also undergone a daily two-point calibration (0 and 50 ppm CO) before sample analysis. ETCO data collection was conducted before the RBC lifespan experiments during an earlier sample collection period from 2014 to 2015. Some individuals from the RBC lifespan were included in breath collection (BD-2, BD-3, BD-4, BW-2, BW-4), whereas others were not (BD-1, BW-1, BW-4).

Carboxyhemoglobin

Gas chromatography (Peak Performer 1 RCP, 910-Series, Peak Laboratories) was used on mixed-venous blood collected from fluke veins to determine the percent carboxyhemoglobin (COHb) in blood for all animals in the red blood cell lifespan section (55). Briefly, heparinized mixed-venous whole blood (1 μL) collected from the veins of the flukes for RBC lifespan measurements was combined with 20 μL of 100 $g \cdot L^{-1}$ potassium ferricyanide and 10 $g \cdot L^{-1}$ saponin dissolved in 0.1 M potassium phosphate, pH 7.4 in CO-free amber glass vials (2 mL) with a rubber septum. The vials were allowed to sit for 10 min on ice and then analyzed for CO content (mL CO·100 mL·$blood^{-1}$). Hemoglobin concentration was obtained using Beckman Coulter AU480 analyzer, and the values were used to calculate carboxyhemoglobin as shown below:

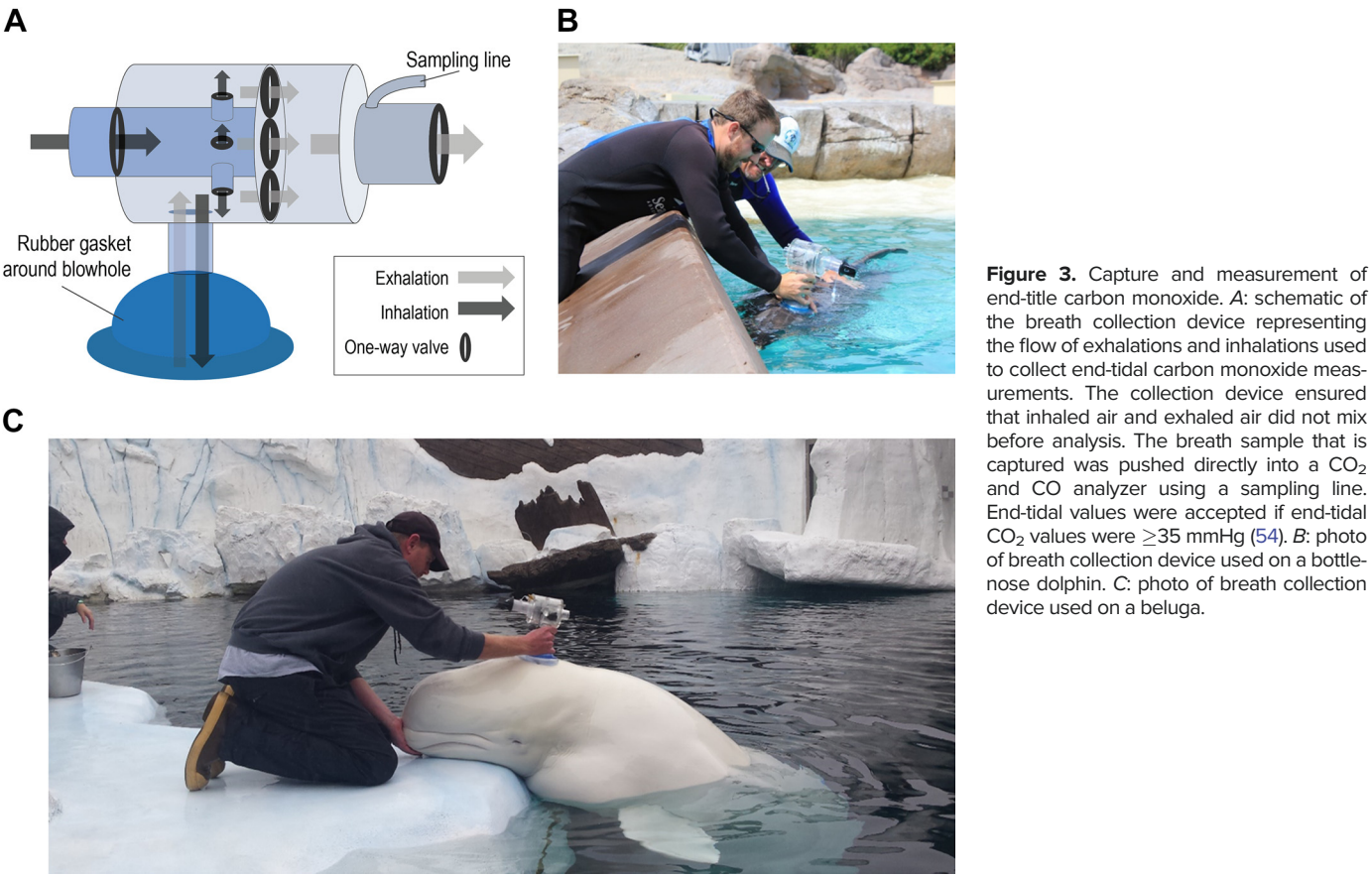

Figure 3. Capture and measurement of end-title carbon monoxide. *A*: schematic of the breath collection device representing the flow of exhalations and inhalations used to collect end-tidal carbon monoxide measurements. The collection device ensured that inhaled air and exhaled air did not mix before analysis. The breath sample that is captured was pushed directly into a CO_2 and CO analyzer using a sampling line. End-tidal values were accepted if end-tidal CO_2 values were ≥35 mmHg (54). *B*: photo of breath collection device used on a bottlenose dolphin. *C*: photo of breath collection device used on a beluga.

$$\text{COHb} = \left(\frac{\text{mL CO}}{100\,\text{mL blood}} \times 100\right)/([\text{Hb}] \times 1.34)$$

where 1.34 is the Hüfner factor expressing the binding capacity of CO to hemoglobin (mL CO·g Hb^{-1}) (55). In healthy and nonhealthy humans, it has been reported a small arteriovenous difference in COHb, with slightly higher (0.1–0.25%) values in arterial blood (56–59). Therefore, the COHb values obtained from mixed venous samples from this study should represent the average blood COHb values for healthy individuals of these species.

Statistical Analysis

All statistics were performed using R (v.4.1.3) or Excel (v.2204). A Mann–Whitney test was performed to detect differences between species for mean RBC age, median RBC survival, half-median RBC survival, mean RBC survival, mean ETCO, max ETCO, 50% hemolysis, COHb, mL CO per 100 mL of blood, and each identifiable band from the 10% SDS-PAGE (Supplemental Table S2).

RESULTS

Carboxyhemoglobin

There were no significant differences between the mean COHb (%) of bottlenose dolphins (1.6 ± 0.1%) and belugas (1.7 ± 0.3%) ($W = 10$, $P = 0.69$). There were also no significant differences between the mean mL CO per 100 mL of blood between the bottlenose dolphins (0.3 ± 0.1 mL CO·100·mL $blood^{-1}$) and belugas (0.4 ± 0.3 mL CO·100 mL·$blood^{-1}$) ($W = 14$, $P = 0.11$) (Fig. 1*B*).

End-Tidal Carbon Monoxide

Mean ETCO ($W = 32$, $P = 0.02$) and max ETCO ($W = 44.5$, $P = 0.02$) were significantly higher in belugas compared with bottlenose dolphins (Fig. 1*C*). Mean ETCO for belugas was 4.3 ± 0.7 ppm and 2.4 ± 1.0 ppm for bottlenose dolphins. The maximum ETCO measured for belugas was 5.6 ± 1.5 ppm and 3.4 ± 1.3 ppm for bottlenose dolphins.

Red Blood Cell Lifespan

Maximum enrichment in $\delta^{15}N$ occurred within 14–36 days after ingestion of the initial dose of ^{15}N-labeled glycine for all animals. A flat plateau period of $\delta^{15}N$ values was observed in most individuals following maximum enrichment (Fig. 2*C*). This flat plateau period was followed by an exponential decline in $\delta^{15}N$ values in all belugas and one dolphin (BD-3). In contrast, the $\delta^{15}N$ in three bottlenose dolphins (BD-1, BD-2, and BD-4) immediately decreased after maximum enrichment and continued to decrease linearly. The corrected % APE curves have a similar appearance to the $\delta^{15}N$ curves (Fig. 2*D*, Supplemental Fig. S1).

Belugas have a significantly older mean RBC age ($W = 16$, $P = 0.029$), longer median RBC survival ($W = 16$, $P = 0.029$), longer half-median RBC survival ($W = 16$, $P = 0.029$), and longer mean RBC survival ($W = 16$, $P = 0.029$) than bottlenose dolphins (Table 4).

Hematology and Serum Chemistry

Hematology and serum chemistry values were within range of previously published values for bottlenose dolphins and belugas (60, 61).

CO Production from RBC Turnover

The bottlenose dolphin and beluga have a calculated mean CO production rate from RBC and hemoglobin turnover that results in 1.4 ± 0.3 mL CO·h^{-1} and 6.5 mL ± 2.4 mL CO·h^{-1}, respectively (Table 3). When the production rate is normalized by the mass of each animal, the bottlenose dolphins produce an average of 5.9 ± 1.3 mL CO·h^{-1}·kg^{-1}·10^{-3} and belugas produce an average of 8.4 ± 1.1 mL CO·h^{-1}·kg^{-1}·10^{-3}. When the CO production rate is normalized by total body hemoglobin, the bottlenose dolphin produces 5.6 ± 0.9 mL CO·h^{-1}·g^{-1}·10^{-4} and the beluga produces 3.2 ± 0.2 mL CO·h^{-1}·g^{-1}·10^{-4}.

Osmotic Fragility

Osmotic fragility curves follow the expected sigmoid appearance for all individuals (Fig. 4*B*). The salinity resulting in 50% hemolysis was significantly higher in dolphins (0.58 ± 0.02% NaCl) compared with belugas (0.50 ± 0.2% NaCl) ($W = 0$, $P = 0.029$) (Fig. 4*C*).

RBC Membrane Proteins

There was no significant difference between α-spectrin ($W = 10$, $P = 0.69$), β-spectrin ($W = 6$, $P = 0.69$), ankyrin

Table 4. *RBC lifespan results*

Individual	Mean RBC Age, days	Median RBC Survival, days	Half-Median RBC Survival, days	Mean RBC Survival, days	Allometric RBC Lifespan Estimation, days
BW-1	91	187	94	172	157
BW-2	96	191	96	171	171
BW-3	93	220	110	196	159
BW-4	81	177	89	169	166
BD-1	47	88	44	90	141
BD-2	51	110	55	101	138
BD-3	59	140	70	131	142
BD-4	45	91	46	92	139
Means ± SD BW	90 ± 7*	194 ± 18*	97 ± 9*	177 ± 13*	163 ± 6
Means ± SD BD	50 ± 6	107 ± 24	54 ± 12	104 ± 19	140 ± 2

Mean red blood cell (RBC) age, median RBC survival, half-median RBC survival, mean RBC survival, and the allometric RBC lifespan determination based on the allometric relationship from terrestrial mammals from Vácha and Znojil (24) for all individuals in this study. The average and standard deviation for beluga (BW) and bottlenose dolphins (BD) are listed. *Significant differences for average values between species.

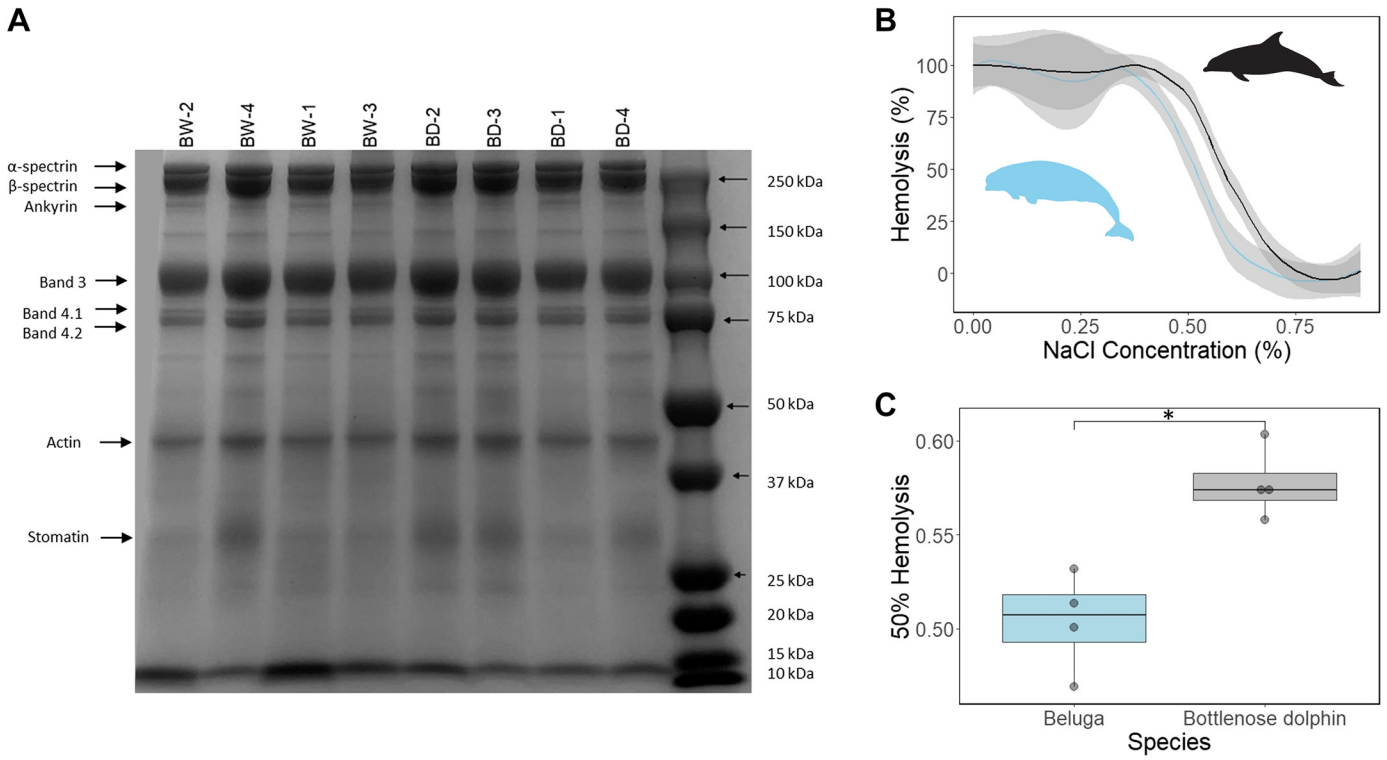

Figure 4. *A*: a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of red blood cell (RBC) ghosts for the belugas (BW-1, BW-2, BW-3, BW-4) and bottlenose dolphins (BD-1, BD-2, BD-3, BD-4). Band names are listed on the left-hand side and the ladder is listed on the right with the molecular weights. *B*: average hemolysis curve for bottlenose dolphins ($n = 4$, 2 male, 2 females) and belugas ($n = 4$, 2 males, 2 females). Belugas are indicated in blue and bottlenose dolphins are indicated in gray. The gray shadow represents the confidence interval (95%). *C*: box plot representing the 50% hemolysis point for bottlenose dolphins and belugas. The star above represents a significant difference between the two species ($P < 0.05$).

($W = 8.5$, $P = 1$), Band 3 ($W = 11$, $P = 0.49$), Band 4.1 ($W = 7$, $P = 0.89$), Band 4.2 ($W = 8$, $P = 1$), actin ($W = 6$, $P = 0.69$), and stomatin ($W = 10$, $P = 0.69$) between species (Fig. 4*A*, Table 5).

DISCUSSION

This study shows that the CO levels in the blood and end-tidal gas from two cetacean species are elevated compared with healthy adult humans (4). In fact, the mean blood COHb values from both bottlenose dolphins and belugas (1.6% and 1.7%, respectively) were two to three times greater than those found in healthy adult male humans (0.5–0.79%) (55, 64). The elevated values are similar to those seen in multiple disease states in humans including stable idiopathic pulmonary fibrosis (1.5 ± 0.5%) and cirrhosis with peritonitis (1.9 ± 0.2%) (62, 63). Despite this difference, blood CO levels in the two species of cetaceans are much lower than those found in deep-diving pinnipeds, which can be over 10% (40, 41). The mean ETCO observed in belugas (4.3 ± 0.7 ppm) is consistent with levels seen in humans with thalassemia (3.6 ± 1.5 ppm) and the lower end of values seen in cigarette smokers (3.8–32.6 ppm) (5). Whereas, the mean ETCO observed in the bottlenose dolphin (2.4 ± 1.0 ppm) is similar to those of healthy adult humans (1.8 ± 0.7 ppm) (5).

Table 5. *RBC membrane protein quantification*

Protein	Bottlenose Dolphin, %	Beluga, %
α-Spectrin	7.9 ± 0.7	8.3 ± 0.9
β-Spectrin	16.8 ± 1.0	16.0 ± 0.7
Ankyrin	3.0 ± 0.6	3.1 ± 0.3
Band 3	22.4 ± 0.9	22.9 ± 1.3
4.1	1.3 ± 0.1	1.3 ± 0.3
4.2	4.5 ± 0.5	4.6 ± 0.3
Actin	7.9 ± 0.9	7.8 ± 1.0
Stomatin	3.4 ± 0.5	3.4 ± 0.9

Mean band percent (%) plus and minus the standard deviation of the identifiable proteins within the red blood cell (RBC) membrane in bottlenose dolphin and beluga whale.

One can estimate the volume of CO produced per hour from RBC turnover by taking into account the mean RBC survival, hemoglobin concentration, body mass, and calculated mass-specific blood volume from each species (Tables 1 and 3) (42, 44). These calculations were first validated using published values of CO production in men, assuming an average blood volume of 70 mL·kg^{-1} and a 120-day lifespan (Supplemental Methods S1) (48). A 120-day RBC lifespan would produce 0.34 mL CO·h^{-1} in men, which is equivalent to the measured CO production rate attributed to RBC turnover (48). The bottlenose dolphins (1.4 mL CO·h^{-1}) and belugas (6.5 mL CO·h^{-1}) are respectively producing 4 and 19 times more CO per hour from RBC turnover than the average healthy human male (48). The increase in CO production compared with humans for bottlenose dolphins can most easily be explained by their greater blood heme stores. Bottlenose dolphins have a body mass and total blood heme stores that are 3–3.5 times greater than humans, meaning they are turning over a larger volume of RBCs per time and

producing more CO. Similarly, belugas have a 10-fold increase in body mass, a 50% greater Hb concentration, and a mass-specific blood volume that is two times greater than adult humans. This results in total blood heme stores that are >30 times higher than adult humans. However, the mean RBC lifespan in belugas (177 days) is ~1.5 times longer than adult humans (120 days), which results in a slower rate of heme turnover, and thus impacts their CO production rate (16, 17). When all these factors are considered, this helps to understand why the CO production rate in belugas is ~19 times higher than adult humans.

It is important to remember that the CO production rate reflects the total CO produced in an individual over time but will not necessarily be representative of the COHb or ETCO measurements, which are relative percents of CO. A large portion of endogenous CO will bind to hemoproteins, such as hemoglobin, which varies widely in concentration between species. This means a high CO production rate in a species with large total body hemoglobin stores may have the same COHb as a species with a low CO production rate and small total body hemoglobin. Therefore, to understand how CO production rates relate between species, we can normalize the CO produced per hour by the total body hemoglobin stores. This normalization results in dolphins producing 1.5 times as much CO for a given period (5.6 ± 0.9 mL $CO \cdot h^{-1} \cdot g^{-1} \cdot 10^{-4}$) compared with humans ($3.65 \pm 0.49$ mL $CO \cdot h^{-1} \cdot g^{-1} \cdot 10^{-4}$) (48). The greater normalized CO production rate in bottlenose dolphins explains why their COHb (1.6 ± 0.1%) is also higher than healthy adult male humans (0.5–0.79%) (Fig. 5) (55, 64). When the difference between total body hemoglobin stores are considered between humans and belugas, we find that the amount of CO produced from RBC turnover in belugas (3.2 ± 0.2 mL $CO \cdot h^{-1} \cdot g^{-1} \cdot 10^{-4}$) is actually comparable with that in healthy adult human males (3.65 ± 0.49 mL $CO \cdot h^{-1} \cdot g^{-1} \cdot 10^{-4}$) (48). Therefore, even though belugas produce 19 times more CO due to their greater body mass, hemoglobin concentration, and blood volume, they are maintaining a similar total body concentration of CO compared with humans due to their >30 times larger blood heme stores serving as a major sink for CO. We would therefore expect belugas to have similar COHb to humans (Fig. 5). The discrepancy between the observed and expected COHb values in belugas indicates there may be additional sources of CO not taken into account.

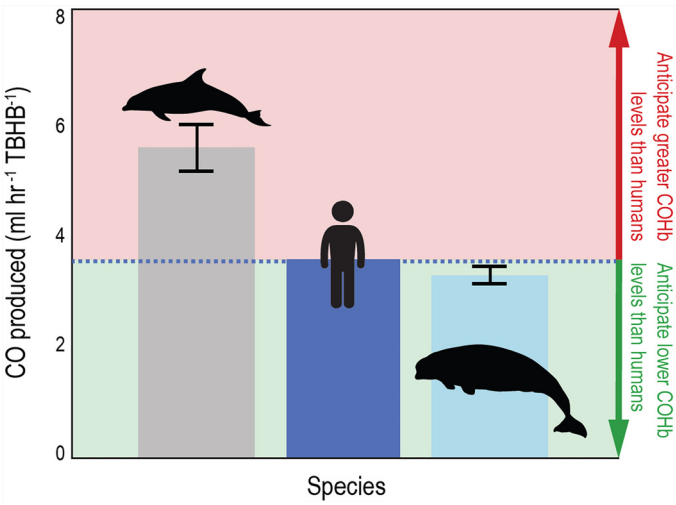

Figure 5. Expected carbon monoxide (CO) levels from mean red blood cell survival. Average CO produced per hour from the natural turnover of heme from hemoglobin inside red blood cells, normalized by total body hemoglobin stores (TBHB) for humans, bottlenose dolphins, and belugas. Dashed line represents the average CO produced by healthy human males from heme degradation via red blood cell turnover rates (48). Because of the high affinity of CO for heme in hemoglobin, the majority of CO that enters the body or is produced in the body will likely bind to hemoglobin, producing COHb. This figure illustrates the relationship of the CO production rate to TBHB and states that values above or below the dashed line would be expected to result in COHb values that are higher or lower than healthy adult humans.

The elevated ETCO values in belugas suggest there is a difference in the amount, and potentially the rate, of CO offloading in the lungs. It is possible that the longer average breath-hold duration (9–16 min) of belugas results in more CO diffusing into alveolar air, increasing the ETCO in belugas (42, 65–68). In contrast, the shorter duration breath holds (1 min) of the bottlenose dolphin would limit accumulation of CO in the lungs, resulting in low ETCO values (43). The northern elephant seal and Weddell seal also routinely experience long breath-hold durations from diving and terrestrial sleep apneas (40, 41). Previous studies have even shown that COHb values increase with age in northern elephant seals, when blood heme stores and breath hold durations are known to drastically increase (40). Interestingly, breath hold duration may not have an impact on COHb, which helps to explain why belugas and bottlenose dolphins have different ETCO but the same COHb. In patients with obstructive sleep apnea and elevated ETCO, the ETCO values from these patients were reduced with long-term continuous positive airway pressure therapy, but the treatment had no effect on the quantity of CO in blood (69, 70). Future studies should evaluate the relationship between CO levels in blood and breath, breath-hold durations, CO removal rate via respiration, and RBC turnover.

There are other potential sources of CO that could help explain elevations in COHb and/or ETCO. Higher CO production by lung epithelial cells or within the paranasal sinuses could be a potential cytoprotective mechanism to prevent inflammation associated with hypoxia and/or I/R events and would help to explain differential CO values in the blood and breath (71). In addition, the turnover of hemoproteins other than hemoglobin (e.g., cytochromes, myoglobin, neuroglobin) could contribute to resting CO levels seen in these species. Many marine mammals, including the two species from this study, have myoglobin concentrations in skeletal muscle that are over 10 times higher than that of human or rodent skeletal muscle (72). However, the myoglobin lifespan and turnover rate in these species are unknown. There is also some evidence that HO-1 may be more sensitive to an inflammatory challenge, such as in I/R, in marine mammals, increasing the breakdown of hemoproteins (73). Other minor sources (e.g., lipid-peroxidation, microbiome) of CO could also contribute to the levels we have seen in these two species (3, 74–78). Finally, the affinity of these species' hemoglobin for CO, compared with oxygen, is not known. Both species have a higher hemoglobin-oxygen affinity, compared with humans, which suggests their hemoglobin may also hold onto CO more tightly, causing a

slower release from the body, and a potential elevation in COHb and ETCO (79, 80).

The mass-specific CO production rates between the two species follows an inverse allometric relationship, with larger belugas having higher mass-specific CO production rates than the smaller bottlenose dolphins. The bottlenose dolphin produces 5.6 ± 1.3 mL $CO \cdot h^{-1} \cdot kg^{-1}$ 10^{-3} whereas the beluga produces 8.4 ± 1.0 mL $CO \cdot h^{-1} \cdot kg^{-1} \cdot 10^{-3}$ (Table 3). Both marine mammals in this study have much lower mass-specific CO production rates when compared with smaller mammals (i.e., rats and mice), that have mass-specific CO production rates of 12.5 ± 0.3 and 33.4 ± 1.5 mL $CO \cdot h^{-1} \cdot kg^{-1} \cdot 10^{-3}$, respectively (93). The duration of RBC survival in the bottlenose dolphin and beluga is consistent with the allometric relationship for several terrestrial mammals, with larger animals having longer RBC lifespans, that have been attributed to slower metabolic rates and decreased ROS production (24).

The RBC survival curves seen in three of the four bottlenose dolphins never reached a plateau phase; %APE continually decreased at a steady rate after the maximum value was reached. Similar studies in humans observed a flat $\delta^{15}N$ enrichment plateau followed by a rapid decline after day 80 (16, 45). This shape is indicative of age-dependent removal for RBCs where cells are generally removed from circulation once they reach a certain age (16). Studies have postulated that the absence of a plateau period is indicative of indiscriminate removal, whereby RBCs are removed at a fixed rate that is independent of the cells' age (81–84). The curve observed in three of the bottlenose dolphins is suggestive of a combination of indiscriminate and age-dependent removal. Similar RBC survival curves have been observed in healthy animals of other species such as Barbary sheep, guanaco, domestic piglets, and mice (81–85).

Despite no measurable difference between the RBC membrane proteins of the bottlenose dolphins and belugas, the bottlenose dolphins did have significantly greater osmotic fragility (50% hemolysis at 0.58 ± 0.2% NaCl) when compared with the belugas (50% hemolysis at 0.50 ± 0.2% NaCl). It should be noted that the anticoagulant present in the vacutainer used for blood collection may influence the osmotic fragility in certain species (86, 87). Therefore, care should be taken to compare osmotic fragility results across different species with different collection methods. The difference in membrane strength to an osmotic challenge between the bottlenose dolphin and beluga may be related to the difference in mean corpuscular volume (MCV). An inverse correlation between MCV and osmotic fragility has been observed previously, with smaller RBCs having a greater osmotic fragility (i.e., more sensitive to osmotic challenges) (88). The bottlenose dolphins in this study have an MCV of 115.4–132.6 fL and the belugas have a MCV of 151.5–160.2 fL (Table 2). Interestingly, marine mammal RBCs may have a greater osmotic fragility compared with terrestrial mammals, including humans (88). The MCV of both cetacean species are larger than humans (~90 fL), but they display greater osmotic fragility (humans: 50% hemolysis at ~0.39–0.45% NaCl in both EDTA and heparin) (86, 89). Early observations on Weddell seal blood marked that their large RBCs (142 ± 1 fL) easily lyse compared with human RBCs (41, 90). Therefore, a more in-depth investigation into the role of membrane proteins, lipids, and their interaction on RBC strength is warranted for marine mammal species. For example, certain marine mammals, including bottlenose dolphins and belugas, have relatively high levels of n-3 fatty acids (eicosapentaenoic acid) within their blood and an increase in dietary n-3 fatty acids has been shown to temporarily decrease osmotic fragility in humans (91, 92).

To date, there are now four documented marine mammal species with elevated concentrations of endogenous CO in the blood compared with humans and other terrestrial mammals. These include the northern elephant seal, the Weddell seal, the bottlenose dolphin, and beluga (40, 41). This study revealed the contribution of RBC and hemoglobin turnover toward the amount of CO produced in bottlenose dolphins and belugas, and highlights the potential impact of large heme stores, RBC turnover rates, and breath-holding on the amount of CO in blood and breath. Future studies investigating the regulation and role of naturally elevated endogenous CO should consider RBC lifespan measurements in other species with large variations in heme stores, hypoxia/ischemia exposure, and also investigate direct measurements of CO production and removal rates. In addition, future studies should aim to increase sample size to distinguish potential differences between sexes of the same species.

Perspectives and Significance

This is the first study to investigate the red blood cell lifespan in a marine mammal species as well as in the largest animal (1,091 kg). In addition, this study adds to the growing evidence of high concentrations of CO in animals routinely exposed to hypoxia, suggesting CO could be providing cytoprotection to certain tissues, or possibly regulating rates of oxygen consumption. Understanding how certain animals produce and maintain elevated levels of CO is becoming increasingly important as researchers attempt to use CO as a therapeutic tool in human and veterinary medicine. Although it was shown that most CO is endogenously produced through hemoglobin degradation from RBC turnover in humans, this study shows alternative mechanisms (i.e., breath holding, other hemoproteins, etc.) may provide higher amounts of endogenous CO in hypoxia-tolerant species. The extent to which alternative mechanisms contribute to endogenous CO production should be the focus of future research.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL DATA

Supplemental Methods S1, Supplemental Fig. S1, and Supplemental Tables S1 and S2: https://doi.org/10.5281/zenodo.10372637.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.B.P., L.A.H., T.L.S., T.R.R., J.S.L., and M.S.T. conceived and designed research; A.B.P. and J.S.L. performed experiments; A.B.P. analyzed data; A.B.P., L.A.H., S.T.K., T.L.S., P.J.P., and M.S.T. interpreted results of experiments; A.B.P. prepared figures; A.B.P. drafted manuscript; A.B.P., L.A.H., S.T.K., T.L.S., T.R.R., J.S.L., P.J.P., and M.S.T. edited and revised manuscript; A.B.P., L.A.H., S.T.K., T.L.S., T.R.R., J.S.L., P.J.P., and M.S.T. approved final version of manuscript.

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