UNIVERSITY OF CALIFORNIA, SAN DIEGO

Impacts of endogenous carbon monoxide (CO) and muscle workload on the utilization of blood oxygen in marine mammals

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Marine Biology

by

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The Dissertation of Michael Scott Tift is approved, and it is acceptable

in quality and form for publication on microfilm and electronically:

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Chair

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DEDICATION

To my loving and kind parents, and grandparents who have always supported my interests in the ocean, animals, and science. And to my beautiful and brilliant wife Andrea, who pushes me forward to achieve my dreams and shares my passion for science and understanding nature.

EPIGRAPH

“For such a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied.”

August Krogh

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ABSTRACT OF THE DISSERTATION

Impacts of endogenous carbon monoxide (CO) and muscle workload on the utilization of blood oxygen in marine mammals

by

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Doctor of Philosophy in Marine Biology

University of California, San Diego, 2016

Paul Ponganis, Chair

For air-breathing divers, oxygen stores represent crucial resources for survival. The primary oxygen store for deep-diving animals is the blood oxygen store, which is typically enlarged in diving species and is mainly comprised of hemoglobin. Efficient utilization of blood oxygen stores while diving is critical for the optimization of dive durations. During dives, animals exhibit the dive response which is a reduction in both heart rate and blood flow to peripheral tissues. However, animals also must increase muscle workload (swim) to reach depths and to return to the surface from a dive, which has the potential to interfere with heart rate and blood oxygen use during dives. The erythrocytes that carry hemoglobin have a limited lifespan, and are constantly turned over

in the body. The destruction of erythrocytes releases hemoglobin into circulation, where the heme portion of hemoglobin is degraded by heme oxygenase enzymes, and results in equimolar production of carbon monoxide (CO). Increased hemoglobin stores of marine mammals make them susceptible to elevated CO production. Due to the high affinity of hemoglobin for CO over oxygen, increased endogenous CO production could impact blood oxygen stores.

In this dissertation, I address the effect of muscle workload and endogenous CO on blood oxygen use in marine mammals. I highlight the lack of relationship between muscle workload and posterior vena caval oxygen depletion during deep dives of free-ranging California sea lions. I hypothesize this is a reflection of the extreme posterior peripheral vasoconstriction associated with the dive response. I also present the variation in flipper stroke rate patterns in sea lions, with prolonged glides during the descent and ascent of deep dives. I show that species with elevated hemoglobin stores (elephant seals and beluga whales) exhibit increased endogenous CO production. In adult elephant seals, this results in over 10% of hemoglobin stores being bound to CO. These levels of CO shift the oxygen-hemoglobin dissociation curve to the left, increasing hemoglobin-oxygen affinity, and increases oxygen content at the end of dives. This is the first report of endogenous CO leading to increased hemoglobin-oxygen affinity in a species adapted to tolerate chronic hypoxia.

CHAPTER 1:

Elevated carboxyhemoglobin in a marine mammal, the northern elephant seal

Michael S Tift, Paul J Ponganis, Daniel E Crocker

Abstract

Low concentrations of endogenous carbon monoxide (CO), generated primarily through degradation of heme from heme-proteins, have been shown to maintain physiological function of organs and to exert cytoprotective effects. However, high concentrations of carboxyhemoglobin (COHb), formed by CO binding to hemoglobin, potentially prevent adequate O_2 delivery to tissues by lowering arterial O_2 content. Elevated heme-protein concentrations, as found in marine mammals, are likely associated with greater heme degradation, more endogenous CO production and consequently elevated COHb concentrations. Therefore, we measured COHb in elephant seals, a species with large blood volumes and elevated hemoglobin and myoglobin concentrations. The levels of COHb were positively related to the total hemoglobin concentration. The maximum COHb value was 10.4% of total hemoglobin concentration. The mean value in adult seals was 8.7 ± 0.3% (mean ± s.e.m, N = 6), while juveniles and pups (with lower heme-protein contents) had lower mean COHb values of 7.6 ± 0.2% and 7.1 ± 0.3%, respectively (N = 9 and N = 9, respectively). Serial samples over several hours revealed little to no fluctuation in COHb values. This consistent elevation in COHb suggests the magnitude and/or rate of heme-protein turnover is much higher than in terrestrial mammals. The maximum COHb values from this study decrease total body O_2 stores by 7%, thereby reducing the calculated aerobic dive limit (cADL) for this species. However, the constant presence of elevated CO in blood may also protect against potential ischemia-reperfusion injury associated with the extreme breath-holds of elephant seals. We suggest the elephant seal represents an ideal model for understanding the potential cytoprotective effects, mechanisms of action and

evolutionary adaptation associated with chronically elevated concentrations of endogenously produced CO.

Introduction

Carbon monoxide (CO) was often thought to be a strictly toxic gas, depriving the body of oxygen (O_2) by binding to heme-proteins such as hemoglobin and forming carboxyhemoglobin (COHb) (Weaver, 2009). Deleterious symptoms (e.g. headache, nausea and shortness of breath) of CO-driven hypoxia are typically seen when COHb values reach $\geq$ 20% of total hemoglobin concentration, and death is associated with values of 50-80% (Stewart, 1975; Weaver, 2009). These COHb values drastically reduce blood-O_2 transport and O_2 storage capacity (decreased arterial O_2 content), thus limiting mitochondrial respiration. However, CO is also generated endogenously in low concentrations, and functions in neurotransmission and in protection of tissues and cells against inflammation, apoptosis and ischemia-reperfusion injuries (Snyder et al., 1998; Kevin and Laffey, 2008; Mustafa et al., 2009; Kajimura et al., 2010; Prabhakar, 2012). Therefore, low concentrations of CO can provide beneficial and therapeutic effects up to a specific concentration, at which elevated CO then leads to detrimental effects from reduced O_2 delivery. These relatively recent findings give CO a new functional perspective and emphasize the importance of understanding the biological effects of specific CO concentrations in the body which can be viewed as therapeutic.

Ironically, the primary source of endogenous CO comes from the breakdown of heme, which is an essential component of many heme-proteins (e.g. hemoglobin, myoglobin, and cytochrome-C) that transport O_2 or associate closely with aerobic

respiration. The breakdown of heme-proteins releases heme, which is then enzymatically degraded by heme-oxygenase (HO), resulting in equimolar production of free iron, CO and biliverdin (Coburn et al., 1963; Tenhunen et al., 1968). Biliverdin is then reduced to bilirubin via biliverdin reductase. These products (biliverdin and bilirubin) and bi-products (CO) of heme degradation have been shown to have a multitude of beneficial effects including: vasodilatation, antioxidative properties, attenuation of ischemia/reperfusion injury, inhibition of apoptosis and down regulation of the inflammatory response (Stocker et al., 1987; Barañano et al., 2002; Mustafa et al., 2009). It is these properties that have stimulated investigation of CO-therapy and the use of CO-releasing pharmaceuticals for future clinical applications (i.e. sepsis, organ transplants, heart failure, hypertension, inflammation and cancer) (Motterlini and Otterbein, 2010).

Considering that heme-proteins are the primary source of endogenous CO production, marine mammals, which have elevated blood volumes, hemoglobin content and myoglobin concentrations (Ponganis et al., 2011), potentially represent an excellent model for investigating elevated endogenous CO production. For example, in an early study by Pugh, there was an unexpected finding of elevated CO in the blood of Weddell seals (*Leptonychotes weddelli*, Gill) (Pugh, 1959). This study found mean CO levels in Weddell seal blood that were over six times the values seen in the blood of human non-smokers (Pugh, 1959). Bilirubin concentration in Weddell seal plasma was also elevated about three to four-fold that of human plasma. Similarly, bilirubin and biliverdin have been measured in the blood of adult and juvenile northern elephant seals (*Mirounga angustirostris,* Gill), yet the values are in the same range as those seen in healthy humans (Thorson and Le Boeuf, 1994; Dennery et al., 2001; Crocker, Unpublished).

Northern elephant seals have some of the highest known mammalian blood volumes (216 ml kg^{-1}) and hemoglobin concentrations (25 g dl^{-1}) in nature, accounting for over 70% of their total body O_2 store (Ponganis, 2011). This elevated blood O_2 storage capacity contributes to their ability to perform repetitive dives of 20 to 25-min duration to depths > 500 m with only 2-3 min surface intervals during foraging trips lasting up to 8 months in duration (Robinson et al., 2012). Additionally, these animals are well-known for their extended and repeated breath-holds (up to 25 min) during sleep apnea events on land, which are also usually followed by brief eupneic periods (Blackwell and Le Boeuf, 1993). Breath-holds of these seals are accompanied by the dive-response (bradycardia, and peripheral ischemia (Andrews et al., 1997; Ponganis et al., 2006)) and routine hypoxemia with arterial hemoglobin saturations reaching as low as 10% (Stockard et al., 2007; Meir et al., 2009). The brief periods of eupnea following breath holds include hyperventilation, tachycardia and vasodilation, which results in reperfusion of tissues with oxygenated blood, thereby increasing the potential for exposure of tissues to reactive oxygen species (ROS) and damage from oxidative stress. Additionally, a typical elephant seal spends 9-10 months per year at sea with over 90% of its time underwater, and when on land will spend a large portion of time in sleep apnea (Blackwell and Le Boeuf, 1993; Robinson et al., 2012). These life history behaviors drastically limit the amount of time the animals spend in eupnea.

Considering endogenous CO is expelled via respiration, the intermittent breathing patterns (sleep apnea and/or diving) of many air-breathing marine divers introduces a potential limitation for removal of CO. Therefore, increased endogenous CO production and delayed removal of CO could elevate COHb content, leading to a decrease in blood

O_2 stores and therefore potentially limiting the duration for aerobic metabolism during a dive. On the other hand, elevated endogenous CO may provide protection against potential reperfusion injury associated with these natural breath-holding behaviors.

To address the potential for elevated endogenous CO production in a species with exceptionally large O_2 stores, we measured levels of COHb in the blood of northern elephant seals and compared these values against hemoglobin concentrations. Further, due to a natural ontogenetic increase in both hemoglobin and myoglobin (Thorson and Le Boeuf, 1994; Tift et al., 2013), we investigated differences in COHb values associated with age. We hypothesized that 1) the amount of COHb would correlate with the total hemoglobin concentration (tHb), and 2) due to larger blood volumes in adults vs. younger seals, the amount of COHb would be highest in adults.

Results

Spectrophotometric absorption peaks of deoxyhemoglobin (HHb), oxyhemoglobin (O_2Hb) and COHb from elephant seals, cattle, and sheep were measured to validate the use of the co-oximeter for evaluation of the hemoglobin properties in elephant seals. All elephant seal hemoglobin varieties had absorption peaks that were identical to the cattle, sheep and multiple other mammalian species, including humans, from Zijlstra et al., 2000 (Fig. 1-1).

Similar to results found in other studies (Thorson and Le Boeuf, 1994), adult elephant seal tHb was significantly higher than pups, but not juveniles ($F_{2,21} = 6.5$, $p = 0.0.066$). Mean tHb in adults was 24.0 ± 1.0 g dl^{-1}, while juveniles and pups had tHb values of 21.7 ± 0.8 g dl^{-1} and 19.6 ± 0.8 g dl^{-1}, respectively.

Adults had significantly higher COHb values (8.7 ± 0.3%) than both juveniles (7.6 ± 0.2%) and pups (7.1 ± 0.3%) (Fig. 1-2). Together, age and tHb explained 80% of the variance in COHb values ($F_{3,20} = 30$, $p < 0.0001$) (Fig. 1-3). Over the course of several hours, COHb remained elevated and showed no significant variation from the mean COHb value ($F_{1,801} = 0.81$, $p = 0.37$) (Fig. 1-4).

Discussion

The values of COHb found in northern elephant seals from this study are higher than values in humans that smoke ≥ 40 cigarettes per day (Law et al., 1997) and comparable to the highest recorded endogenous values (9.7% COHb) found in a critically ill human patient with hemolytic anemia (Hampson, 2007) (Fig. 1-2). The maximum COHb value found in an adult elephant seal (10.4% COHb), was also comparable to 12% COHb values measured in one of the first human clinical pharmaceutical investigations using inhaled CO as a therapeutic agent (Motterlini and Otterbein, 2010). These values in healthy humans were well tolerated and showed no adverse effects compared to control levels.

As originally suggested in Weddell seals, we believe that the high endogenous CO values in northern elephant seals from this study can primarily be attributed to the elevated heme stores associated with increased myoglobin content, blood volume, hematocrit and hemoglobin concentrations (Pugh, 1959; Ponganis et al., 1993; Thorson and Le Boeuf, 1994). The molar equivalents of heme from both hemoglobin and myoglobin stores alone are approximately 4 and 16 times greater, respectively, than those in adult humans (Fig. 1-5). The levels of COHb measured in elephant seals suggest

either 1) more rapid turnover of heme stores (i.e. shorter half-life of heme-proteins or erythrocytes), as is found in human patients with hemolytic anemia (Coburn et al., 1966; Hampson, 2007), or 2) a greater magnitude of heme degradation than has been measured in other mammals, or 3) a combination of both. A greater magnitude of heme degradation could be associated simply with the elevated heme concentrations and/or with an increase in the activity or concentration of heme-oxygenase (HO), the enzyme responsible for heme degradation and endogenous CO production.

Currently, the life-span of heme-proteins and erythrocytes in marine mammals is unknown, but an increased heme turnover rate could elevate the production rate of endogenous CO. Additionally, the natural and repetitive breath-holds both at sea (diving) and on land (sleep apnea) limit the time elephant seals spend in eupnea, and therefore, decrease or at least delay the exhalation of endogenously produced CO. Between these two breath-hold behaviors, a typical elephant seal will spend a majority of its life in an apneic state (Andrews et al., 2000) and thus, this natural breath-holding behavior may also contribute to the build-up of endogenous CO.

Unlike Weddell seals (Pugh, 1959), elephant seals do not have an elevation in the hemoglobin breakdown product bilirubin (D.E. Crocker, Unpublished), to accompany the high blood CO levels seen in these animals. This is especially interesting in elephant seal pups where neonatal hyperbilirubinemia might be expected due to the common occurrence in human infants which have much lower heme stores (Dennery et al., 2001). Neonatal hyperbilirubinemia has also been found in some neonate marine mammals (Dierauf et al., 1984), yet there are relatively low levels of bilirubin found in all age classes of elephant seals (Costa and Ortiz, 1982; Champagne et al., 2013; Crocker,

Unpublished). One possibility is that the clearance rate or recycling of bilirubin and biliverdin relative to absolute total body heme content and heme turnover is adequate in these animals to maintain lower levels of those metabolites.

Effect of elevated CO on O_2 stores and O_2 delivery

The concepts of increased O_2 storage, a low diving metabolic rate, and aerobic diving underlie current interpretations of diving physiology, dive performance, and foraging ecology in apex marine predators (Kooyman, 1989; Butler and Jones, 1997; Ponganis et al., 2003). Total body O_2 stores (lung, blood, muscle) are used to calculate the aerobic dive limit (cADL), which is a prediction of the maximum duration of a dive or breath-hold before a post-dive rise in plasma lactate occurs (Kooyman and Kooyman, 1995; Butler and Jones, 1997; Kooyman and Ponganis, 1998; Costa et al., 2001). For this calculation, which is performed by dividing total body O_2 stores by the rate of breath-hold O_2 consumption, all measured hemoglobin is included in the blood O_2 store (which can make up 70% of the total O_2 store in some species). However, this study shows that over 10% of total hemoglobin content can be bound to CO in the premier pinniped diver, effectively reducing its total body O_2 stores by approximately 7%.

When these CO-related reductions in O_2 stores are taken into consideration in the calculation of a cADL of adult elephant seals (~30 min; Hassrick et al. 2010), there is reduction of approximately 2 min. This reduction might help explain why the majority of dives in female elephant seals are actually 22-23 min (Robinson et al., 2012) instead of the predicted cADL of 30 minutes. The elevated COHb values seen in elephant seals

from this study should heighten awareness of the potential for endogenous CO to reduce the magnitude of total body O_2 stores calculated in other species.

Potential therapeutic benefits of elevated CO in marine mammals

The routine hypoxemia, hypercarbia, reduction in peripheral perfusion, and activation of the sympathetic nervous system during both diving and sleep apnea in elephant seals potentially increase the risks of systemic and pulmonary hypertension, ischemia-reperfusion injury, ROS formation, and subsequent tissue damage (Kooyman, 1989; Butler and Jones, 1997; Kooyman and Ponganis, 1998; Zenteno-Savın et al., 2002). Adaptations that potentially decrease such risks in marine mammals include hypoxic pulmonary vasodilatation and elevations in both antioxidant concentrations and antioxidant enzyme activities (Zenteno-Savın et al., 2002; Olson et al., 2010; Tift et al., 2011; Vazquez-Medina et al., 2011). We propose that the levels of heme degradation, and associated bi-products such as endogenous CO found in elephant seals, may also contribute to the protection against these conditions.

Ischemia-reperfusion injury is commonly seen during a variety of clinical scenarios, including heart attacks, strokes and organ transplantation (Carden and Granger, 2000). Deleterious effects associated with ischemia-reperfusion injury typically include apoptosis, intense inflammation and thrombogenesis. However, exposure to low levels of CO significantly decreases the risk of these injuries during ischemia-reperfusion events, and is currently being applied in clinical investigations (Ozaki et al., 2012). Therefore, the elevated levels of endogenous CO found in elephant seals could provide protection against potential ischemia-reperfusion injury, associated with the marked changes in

peripheral perfusion associated with breath-holds (Ponganis et al., 2008). Additionally, low concentrations of CO are known to promote vasodilation and decrease hypertension in several species. For example, in the llama (*Lama glama,* Linnaeus) at high altitude, elevated pulmonary CO production is associated with decreased pulmonary hypertension (Herrera et al., 2008). And lastly, although smoking during pregnancy is associated with numerous adverse outcomes and low birth weights (Cnattingius and Lambe, 2002), there has been decreased risk of hypertension and preeclampsia associated with smoking during pregnancy (Wikström et al., 2010). Thus, elevated CO may also contribute to vascular regulation for coping with hypoxemia and tissue vasoconstriction during breath-holding in seals (Ponganis et al., 2008; Meir et al., 2009).

While the mechanism is still unknown, placental blood flow is maintained during extreme peripheral vasoconstriction associated with the dive response in a phocid seal (Liggins et al., 1980). Recently demonstrated in the pregnant mouse (Venditti et al., 2013), the elevated CO exposure in seals may help optimize uterine blood flow and placental growth during the at-sea pregnancies of continuously diving female elephant seals. However, complicating this suggestion is the negative relation between smoking (and potentially CO exposure) and endothelial nitric oxide synthase (eNOS) activity, which could reduce vasodilatory capacity in the fetus (Andersen et al., 2009).

Exposure to other heme-protein breakdown products (bilirubin and biliverdin) are also associated with a wide array of potential therapeutic effects in the avoidance of re-perfusion injury and oxidative stress. These include anti-inflammatory, anti-apoptosis and anti-oxidative responses (Wu and Wang, 2005; Ryter et al., 2007; Motterlini and Otterbein, 2010). Thus, although CO decreases O_2 storage, it has the potential to offer

several other beneficial effects in diving animals and other animals exposed to inflammation, apoptosis and oxidative stress.

We conclude with the suggestion that the elevated endogenous CO and COHb concentrations found in deep diving phocid seals represent an excellent opportunity to study and understand the fundamentals and extremes of this unique physiological system (Krogh, 1929). With the growing knowledge on the therapeutic potential of carbon monoxide, deep-diving seals may become a valuable model to investigate the potential cytoprotective effects, mechanisms of action and evolutionary adaptation from long-term exposure to elevated concentrations of this endogenously produced gasotransmitter.

Materials and Methods

Elephant seal COHb absorption spectrum

To validate the use of a Bayer Rapidlab 845 blood gas analyzer with co-oximeter (Siemens Medical Diagnostics, Bayer, Tarrytown, NY, USA) for the determination of elephant seal hemoglobin values, we confirmed that the hemoglobin absorption spectra of elephant seals was similar to those of humans and other terrestrial animals. Hemoglobin from the blood of healthy juvenile northern elephant seals (n = 2, see blood collection technique below), sheep (n = 1) and cattle (n = 1) (sheep and cattle blood obtained from Hemostat Laboratories, Dixon, CA, USA) were spectrophotometrically compared to hemoglobin absorption peak values for human, cattle and sheep from (Zijlstra et al., 2000). Isolated hemoglobin absorption peaks for oxygenated hemoglobin (O_2Hb), deoxygenated hemoglobin (HHb) and COHb were measured and compared using methods similar to that described in Kreutzer et al., 1993 and Zijlstra et al., 2000.

Briefly, blood was collected into chilled vacutainers containing 158 U.S.P units of sodium heparin (BD, Franklin Lakes, NJ, USA). Plasma was discarded and erythrocytes were washed three times with 0.9% NaCl solution. Erythrocytes were then lysed with 1 volume of distilled H_2O and 0.4 volumes of Toluene and left to sit at 4°C for up to 16 hours. This resulted in a top layer of toluene, a middle layer of erythrocyte stromata and a bottom layer of hemoglobin lysate. The bottom layer was collected and centrifuged at 8000g for 20 minutes, and the erythrolysate was then filtered through sterile gauze and diluted with deionized water (Milli-Q, Millipore, Billerica, MA, USA). The diluted erythrolysate was then placed inside a tonometer where respective gases were introduced. The different hemoglobin forms (HHb, O_2Hb and COHb) were prepared by introducing 100% nitrogen (N_2), O_2, and CO, respectively, into the tonometer for up to 2 hours at a rate of 30-40 ml min^{-1}. For O_2Hb and COHb, polystyrene spectrophotometer cuvettes (light-path = 1.0 cm) covered with Parafilm® M were filled with 2.5 ml of erythrolysate collected from the tonometer using an airtight blood-gas syringe, thereby minimizing exposure to the ambient environment. For HHb, an addition of 3 mg of sodium hydrosulfite ($Na_2S_2O_4$), per 2 mL of buffered erythrolysate was included for tonometry to prevent O_2 or CO from binding. Once the lysate was transferred, cuvettes were then immediately capped, sealed with Parafilm® M and immediately analyzed.

Absorbance measurements were made at room temperature (20-25 C) with a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) in the spectral range of 450-700 nm.

Study site and subjects

We sampled 24 northern elephant seals throughout the 2009 winter breeding season (December – April) from Año Nuevo State Reserve, San Mateo County, CA, USA. Every year, a subset of pups at the reserve are marked with flipper-tags following weaning (Jumbo Rototag; Dalton, England), which enables reference to the animals' ages. Subjects were divided into three age classes; pups (< 3 mo. old; n = 9), juveniles (3 mo. - 3 years old; n = 9) and adults (> 3 years old; n = 6). Adult animals were all female, and were sampled from Jan-Feb at the beginning of their breeding season. Pups were sampled in Feb-March and juveniles were sampled April-May.

Animals were chemically immobilized with an intramuscular injection (1 mg kg^{-1}) of Telazol (tiletamine/zolazepam HCl) and subsequent intravenous ketamine injections (0.5 mg kg^{-1}) as necessary to maintain immobilization for the intravenous catheterization of the extradural vein (all drugs from Fort Dodge Labs, Ft Dodge, IA, USA). Venous blood samples (~3ml) were collected into lithium heparinized blood tubes (BD Vacutainer®, Fisher Scientific, Franklin Lakes, NJ, USA) and chilled over ice until analysis (< 3 hours). Standard blood gas analysis was performed using a Bayer Rapidlab 845 blood gas analyzer with co-oximeter (Siemens Medical Diagnostics, Bayer, Tarrytown, NY, USA) to obtain fractions of COHb and tHb concentrations.

To determine the variability of COHb over time and between voluntary periods of apnea and eupnea, we opportunistically sampled the 6 juvenile elephant seals for up to 12 hours. Once sedated, the animals were placed in a holding cage and the extradural vein was percutaneously catheterized with a long-term polyurethane catheter (MILA; 16 ga. X 25 cm) which was affixed to the fur using Loctite glue (Henkel Corporation, Westlake, OH, USA). An extension tube (76 cm) with a three-way stopcock system was attached to

the catheter to allow blood collection during periods of voluntary sleep apnea and eupnea, with minimal disturbance. Over the course of 8-12 hours, we collected a total of 806 blood samples ranging from 36 to 134 samples per individual.

Statistical Analysis

Data were analyzed using the statistical program JMP 11.0. We examined the effects of age class (adult, juvenile and pup) and tHb concentration on the percentage of COHb in blood samples using a multiple regression analysis. Changes in COHb over the course of several hours were evaluated using a repeated measures linear mixed model, with individual ID as the random subject effect. All mean values are reported as mean ± s.e.m. Significance was determined at $p < 0.05$.

Abbreviations

cADL = Calculated aerobic dive limit

CO = Carbon monoxide

COHb = Carboxyhemoglobin

HHb = Deoxyhemoglobin

O_2Hb = Oxyhemoglobin

O_2 = Oxygen

ROS = Reactive oxygen species

tHb = Total hemoglobin concentration

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Chapter 1, in full, is a reprint of the material as it appears in the Journal of Experimental Biology. Tift, Michael S.; Ponganis, Paul J.; Crocker, Daniel E. The dissertation author was the primary investigator and author of this material.

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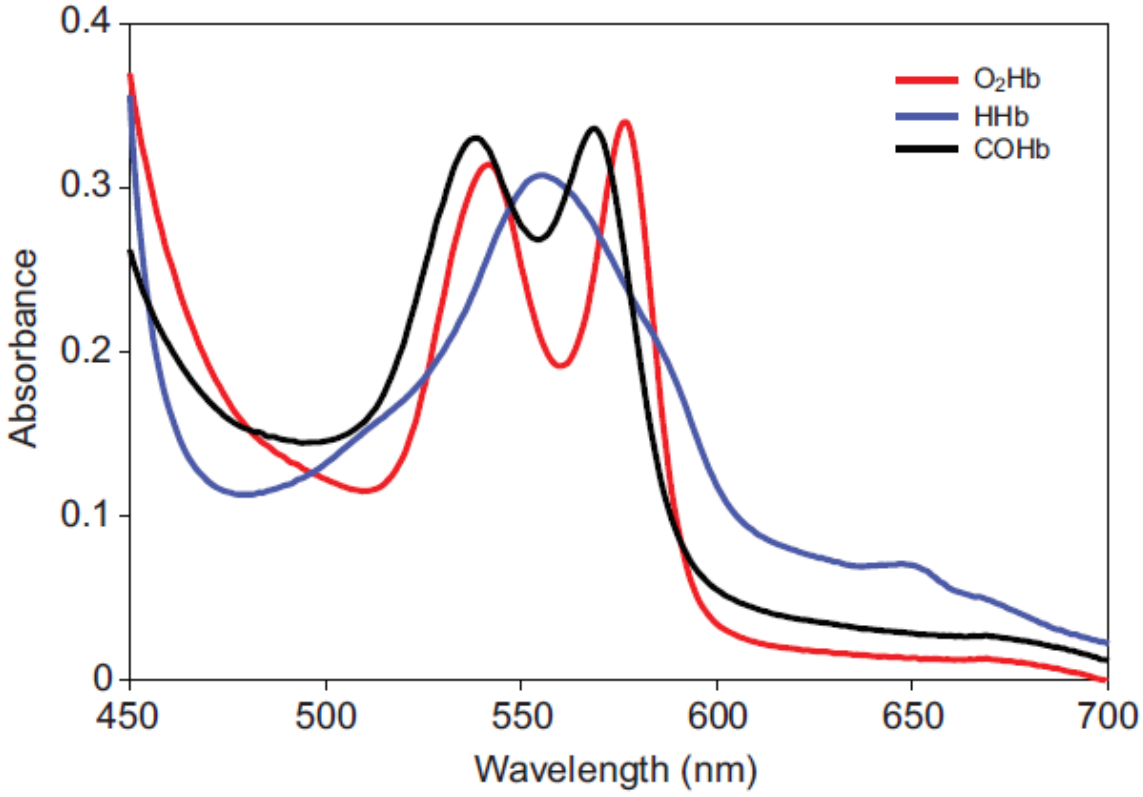

Figure 1-1. Absorption spectra for oxyhemoglobin (O_2Hb), deoxyhemoglobin (HHb) and carboxyhemoglobin (COHb) in northern elephant seals. Peaks for O_2Hb (542 and 577 nm), HHb (555 nm) and COHb (539 and 569 nm) match those of other mammalian species (human, cow, sheep) (from Zijlstra et al., 2000).

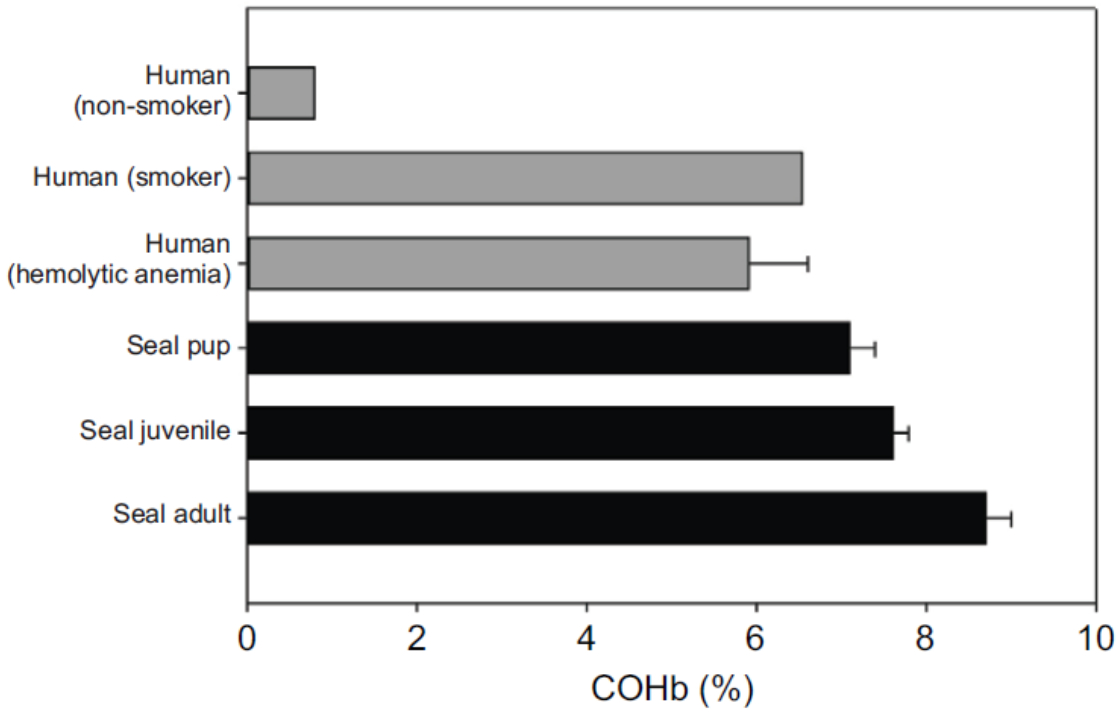

Figure 1-2: Mean carboxyhemoglobin (COHb) values in the blood of human smokers, non-smokers, human patients with hemolytic anemia, and three age classes of northern elephant seals. Human values of COHb for smoking and non-smoking are from Law et al., 1997. Human COHb values from hemolytic patients are from Hampson, 2007. Values are represented as mean + s.e.m.

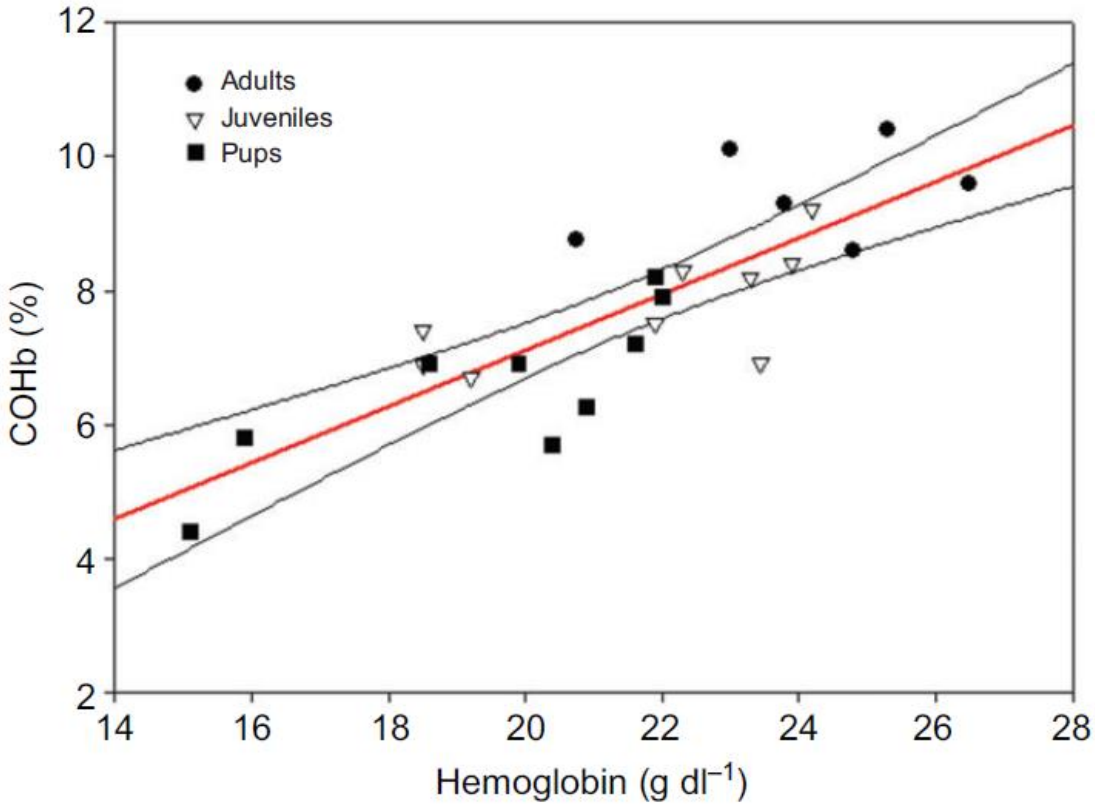

Figure 1-3: Relationship between the total concentration of hemoglobin and percent carboxyhemoglobin (COHb) in the blood from three different age classes of northern elephant seals. The regression line is in red (COHb = -1.31 + 0.42*tHb), and the 95% confidence intervals are in black ($F_{3,20} = 30.1$, $p < 0.0001$, $r^2 = 0.80$).

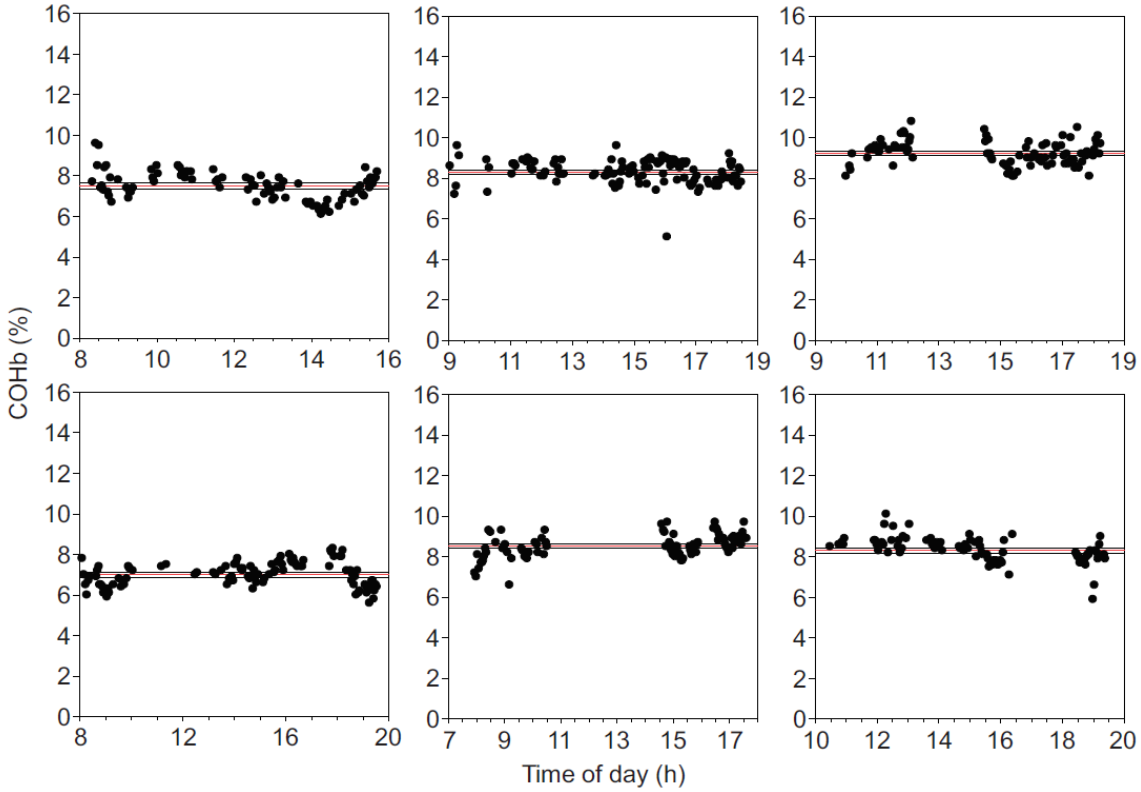

Figure 1-4: Time course of carboxyhemoglobin (COHb) values from six juvenile elephant seals. The red lines represent the best-fit linear regression for the data and the black lines represent 95% confidence intervals.

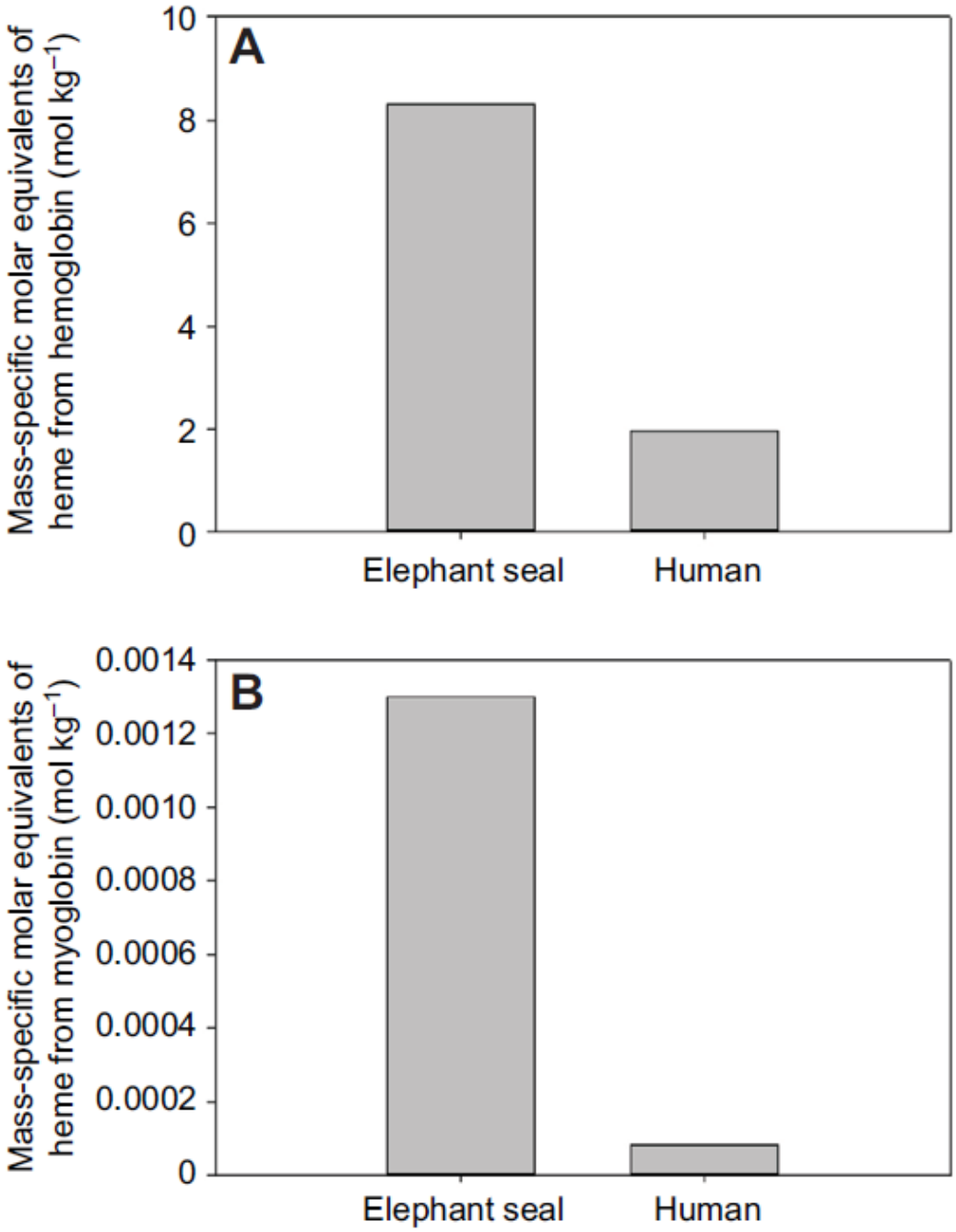

Figure 1-5: Mass-specific molar equivalents of heme calculated from (A) blood (hemoglobin) and (B) muscle (myoglobin) oxygen stores in northern elephant seals and humans. Molar heme equivalents were calculated using a hemoglobin molecular weight of 65,000 g mol^{-1} and myoglobin molecular weight of 17,000 g mol^{-1}. Data for species-specific blood volumes, muscle mass, hemoglobin and myoglobin concentrations are from previous studies (Kendrew et al., 1958; Hill et al., 1962; Åkeson et al., 1968; Oscai et al., 1968; Simpson et al., 1970; Bryden, 1972; Möller and Sylven, 1981; Hassrick et al., 2010; Ponganis et al., 2011).

CHAPTER 2:

The Dose Makes the Poison: Unraveling The Carbon Monoxide (CO) Paradox in Marine Mammals

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Abstract

The mechanism that marine mammals utilize to avoid hypoxic injuries while diving are not well understood. While carbon monoxide (CO) can be toxic, natural turnover of heme-proteins produces small amounts of endogenous CO and moderate amounts of CO can elicit cytoprotective effects. High concentrations of heme-proteins in marine mammals increase their potential for elevated CO production. Here we show elephant seals produce CO at levels similar to those seen in chronic cigarette smokers and also at levels considered cytoprotective in laboratory studies. These CO concentrations enhance hypoxic tolerance by increasing hemoglobin-oxygen affinity and blood oxygen content during severe hypoxemia. Our results support hypotheses of convergent evolution of increased hemoglobin-oxygen affinity as an adaptation to chronic hypoxia, with the first report of endogenous CO to achieve this. We suggest elephant seals represent excellent models for understanding the adaptive and potential cytoprotective benefits from moderate CO exposure.

Introduction

The toxic effect of carbon monoxide (CO), first described by Haldane in the early 1900s[1], is primarily a result of the 240-fold greater affinity that hemoglobin has for CO compared to oxygen. The subsequent formation of carboxyhemoglobin (COHb) occurs when CO binds to hemoglobin, which decreases the quantity of available hemoglobin to transport oxygen to tissues. Elevated COHb also increases hemoglobin's oxygen affinity due to a left shift in the oxygen-hemoglobin dissociation curve[2]. The greater the magnitude of the left shift, the less likely oxygen is to dissociate from hemoglobin during

hypoxia which can result in extreme tissue hypoxia and death[3]. However, exposure to low levels of CO results in a minor left shift in the oxygen-hemoglobin dissociation curve that has been suggested to improve survival during hypoxia[2,4]. For example, under conditions of severe hypoxia, increased hemoglobin-oxygen affinity (i.e. left-shifted curve) results in preservation of higher blood oxygen content and a greater amount of oxygen unloaded to tissues[5,6].

Recent discoveries have shown that exposure to moderate CO concentrations (via inhalation or through carbon monoxide releasing molecules-CORMs) can have cytoprotective properties (e.g. anti-inflammation, anti-apoptosis, anti-proliferation)[7]. Consequently, CO has been investigated as a therapeutic drug for specific pathologies (e.g. inflammatory or hypoxia-related diseases)[8,9]. CO is also constantly produced at low levels in the body[10]. Heme oxygenase enzymes catabolize heme from heme-proteins (e.g. hemoglobin and myoglobin) which leads to the production of equimolar concentrations of ferrous iron, CO, and biliverdin; the latter is rapidly converted to bilirubin by biliverdin reductase[11] (Fig. 1). This natural endogenous CO production results in 0.1-1% COHb in healthy humans and levels as high as 9.7% COHb in hemolytic anemia patients with increased heme-turnover[12]. In chronic cigarette smokers, COHb can be 10-15%[13]. In CO poisoning, death is associated with COHb levels of 30-60% or greater [14,15].

Marine mammals offer a unique opportunity to examine endogenous CO production. First, many species of marine mammals have elevated blood volumes and increased heme-protein concentrations (i.e. hemoglobin and myoglobin)[16] that not only increase oxygen storage but also enhance the potential for greater endogenous production of CO, biliverdin and bilirubin. Second, some marine mammals (e.g. elephant seals

(*Mirounga angustirostris*)) routinely perform remarkable breath-holds during dives (> 30 min duration to > 500 m depth)[17] and sleep apnea events (up to 25 min)[18], that result in repeated exposure to extreme hypoxemia (arterial partial pressure of oxygen (P_aO_2) as low as 12 mmHg, 1.6 kPa)[19]. Yet, marine mammals avoid pathologies that often result from repeated exposure to extreme hypoxia (e.g. hypoxic pulmonary hypertension, hypoxic brain and tissue damage, oxidative stress)[20,21]. The mechanisms these species utilize to avoid hypoxic injuries are still not well understood.

It has been proposed that increased hemoglobin-oxygen affinity is beneficial to deliver more oxygen to tissues under conditions of severe hypoxia[5,22-24]. However, amongst the deepest and longest duration breath-hold divers (e.g. emperor penguins, beaked whales, and elephant seals), only cetaceans and penguins exhibit increased hemoglobin-oxygen affinity[19,25,26]. This pattern is intriguing given that a majority of all mammals and birds adapted to chronic hypoxia, from living in burrows or at high-altitude, have increased hemoglobin-oxygen affinities[5]. For instance, many birds native to high-altitude have hemoglobin with amino acid substitutions that result in higher oxygen affinity[27]. Similarly, terrestrial mammals adapted to tolerate hypoxia achieve increased hemoglobin-oxygen affinity by 1) hyperventilating to reduce carbon dioxide (CO_2) and raise the pH of blood or, 2) reducing the ratio of organic phosphates to hemoglobin concentration in red blood cells [28,29]. It has been hypothesized that some marine mammals, such as deep diving pinnipeds, could utilize elevated endogenous CO to increase their hemoglobin-oxygen affinity[30].

In this paper, we describe the levels of endogenous CO in four species of marine mammals (beluga whales, bottlenose dolphins, killer whales and elephant seals) with a

wide range in heme-protein stores. We show that elephant seals are unique in that they produce and maintain CO at values similar to chronic cigarette smokers and also at levels considered cytoprotective in laboratory studies [7,9]. These levels of CO increase hemoglobin-oxygen affinity in the elephant seal which results in significantly higher blood oxygen contents at the end of dives, when hypoxemia is most extreme. This suggests convergent evolution of increased hemoglobin-oxygen affinity in species that are adapted to tolerate chronic hypoxia, with the first report of endogenous CO as a means to achieve this.

Carbon monoxide levels in different species

We quantified levels of CO in blood and breath samples from four marine mammal species that represent a 3.6-fold range in mass-specific heme content from their respective hemoglobin and myoglobin stores (Table 1, Fig. 2A). CO is removed from the body through the lungs[13], which makes exhaled CO values useful to determine the production rate of CO in the body[31].

The elephant seals had significantly higher CO in blood, with levels similar to those of human chronic cigarette smokers (Fig. 2B). These blood CO levels in the elephant seal (3.7 ± 0.4 ml CO 100 ml blood^{-1}) were up to 20 times higher than those seen in healthy humans that do not smoke and approximately six times higher than the concentrations seen in beluga whales, the marine mammal from this study with the second highest CO levels (Fig. 2B). The mean calculated COHb was 2.4 ± 1.4% in beluga whales, 1.7 ± 1.2% in bottlenose dolphins, 0.8 ± 1.0% in killer whales, and 11.1 ±

1.1% in elephant seals. These elephant seal COHb values agreed well with previously reported levels determined with a co-oximeter[30].

We calculated CO production rates ($\mu l\ min^{-1}\ kg^{-1}$) using the maximum exhaled CO values and minute volumes from the literature (see Methods), which showed a 16.8-fold difference between elephant seals and humans. The mean exhaled CO values had a distribution comparable to the mean levels of CO in the blood from the four species (Fig. 2C). Similar to the relationship seen in human smokers and non-smokers[32], there is a linear relationship between levels of CO in the blood and breath of individuals, which can allow for predictions of blood CO levels from a breath sample (Fig. 2D). The small range of CO concentrations in the blood and breath of cetaceans from this study (Fig. 2D) likely reflects the sampling of similar age classes with comparable hemoglobin and myoglobin stores between individuals[33]. In contrast, the large range of breath and blood CO values seen in elephant seals from this study (Fig. 2D) has been shown previously[30], and is hypothesized to be a result of the large range of heme-stores seen between different age classes of elephant seals[34]. For example, sub-adult elephant seals have significantly lower hemoglobin and myoglobin stores than adults[34], and the two lowest values of CO in breath and blood among elephant seals from this study were from sub-adult animals (Fig. 2D). Among the cetaceans from this study, beluga whales have the highest hemoglobin concentrations and contained the highest levels of CO in both blood and breath samples (Fig. 2C). This confirms heme-protein levels do correlate well with endogenous CO concentrations in marine mammals.

We hypothesize that the large difference in CO values between species in this study is a reflection of an increased red blood cell and hemoglobin turnover rate as well

as higher heme oxygenase activity in the elephant seal. This is based on the evidence of increased CO levels and production in patients with hemolytic and sickle cell anemia who experience high turnover rates of red blood cells and hemoglobin[12,35]. However, to our knowledge, the heme-protein turnover rate has yet to be reported in any marine mammal.

Heme breakdown products (Biliverdin and Bilirubin)

For every mole of CO produced from heme catabolism, there is an equimolar amount of biliverdin and bilirubin produced[11]. Therefore, increased bilirubin is often seen in the blood of patients with increased heme-protein turnover[36]. Typically, biliverdin is rapidly converted to bilirubin via biliverdin reductase. Bilirubin occurs in the body as conjugated (bound to glucuronic acid) or unconjugated bilirubin. Unconjugated bilirubin is generally converted to conjugated bilirubin in the liver, where it then can act as a potent antioxidant in the body, or be excreted into bile where it 1) exits the body through the small intestine or, 2) is reduced by bacteria in the gut to other bilins such as stercobilin or urobilin. Similar to excessive CO, unconjugated bilirubin can have toxic effects at high concentrations[37]. Although increased heme turnover is suggested by high concentrations of CO and iron in the blood of elephant seals[38], bilirubin and biliverdin levels in the blood of marine mammals have been reported as low[20]. This suggests the possibility that marine mammals efficiently excrete large quantities of heme breakdown products in excrement.

We investigated the removal of the heme breakdown products, biliverdin and bilirubin, in the feces from beluga whales (n = 3), bottlenose dolphins (n = 6), killer

whales (n = 10), and elephant seals (n = 4). Similar to the distribution of CO in the species from this study, our results show that elephant seals excrete significantly higher quantities of conjugated and unconjugated bilirubin through the intestine (Fig. 3). All cetaceans had low levels of unconjugated bilirubin, and undetectable amounts of conjugated bilirubin in feces (Fig. 3). Small quantities of biliverdin were seen in the feces of all species from this study (Fig. 3). These results agree with the hypothesis of elevated heme turnover in the elephant seals, with removal of large quantities of heme breakdown products through the intestine. This suggests that levels of bilirubin are low in the blood of elephant seals due to efficient removal of the waste products through excrement. Furthermore, high concentrations of both conjugated and unconjugated fecal bilirubin suggests a unique gut microbiome in elephant seals. In the cetaceans, the presence of biliverdin and the absence of bilirubin in the feces suggests that biliverdin is the primary bile pigment. This route of excretion also occurs in rabbits and in many avian species [39].

Effect of CO on Hemoglobin-Oxygen Affinity

The oxygen affinity of hemoglobin is illustrated in the oxygen-hemoglobin dissociation curve, which plots hemoglobin saturation (S_{O2}, percentage of hemoglobin bound to oxygen) as a function of the partial pressure of oxygen in blood (P_{O2}). In order to compare hemoglobin-oxygen affinity between individuals or species, the P_{50} value is examined. The P_{50} represents the P_{O2} at 50% S_{O2}, and can change with factors such as temperature and the pH of blood, and the concentration of organic phosphates (e.g. 2,3, DPG). Previous reports on the elephant seal oxygen-hemoglobin dissociation curve showed a P_{50} of 30.5 mmHg at a pH of 7.4, and 34.1 mmHg at a pH of 7.3. These P_{50}

values are comparable to the hemoglobin-oxygen affinity values that have been measured in other pinnipeds, humans and many other terrestrial mammals[40]. However, CO is likely removed from the blood during the mixing technique typically used to measure the oxygen-hemoglobin dissociation curve in the elephant seals and other marine mammals. Therefore, the effects of CO on the oxygen-hemoglobin dissociation curve have not previously been considered in these species. We re-measured the elephant seal oxygen-hemoglobin dissociation curve at four different levels of COHb (0, 3.5, 7, 11% COHb) and found P_{50} values of 28.6, 27.4, 26.0 and 22.6 mmHg, respectively (Figs. 4A & 4B). The effect of CO on the elephant seal oxygen-hemoglobin dissociation curve shows the expected positive relationship between CO and hemoglobin-oxygen affinity.

It has previously been shown that a reduced P_{50} (higher hemoglobin-oxygen affinity) promotes performance and survival in vertebrates exposed to chronic hypoxia[5]. Many mammals and birds that face chronic hypoxia achieve higher hemoglobin-oxygen affinity through a variety of mechanisms. These include amino acid substitutions in the hemoglobin molecule, a reduction in the ratio of organic phosphates to hemoglobin levels in red blood cells[28], or behavior modifications such as hyperventilation to reduce CO_2 and raise the pH of blood[27]. For example, human climbers at 8400 m elevation on Mount Everest responded to hypobaric hypoxia by hyperventilating, which reduced P_aCO_2 to 10-16 mmHg, resulting in arterial pH values of 7.45 – 7.6[41].

Some marine mammals (e.g. Weddell seals (*Leptonychotes weddellii*)) hyperventilate at the surface prior to a dive, which moderately lowers end-tidal CO_2 at the start of the dive (~ 30 mmHg)[42]. Despite this, end-tidal CO_2 and P_aCO_2 are only slightly elevated (> 50 mmHg) and arterial pH does not drop below 7.3 during long dives (> 17

min) of Weddell seals [43]. Similarly, the pH of arterial and venous blood does not typically drop below 7.3 and the P_aCO_2 does not rise above 60 mmHg at the end of prolonged sleep apneas (> 15 min) of elephant seals[44,45]. These results show the excellent buffering capacity of the blood of these animals. Therefore, it is unlikely that drastic changes in pH occur during dives of these animals, and it is also unlikely that hyperventilation prior to the dive would result in a significant reduction in hemoglobin-oxygen affinity.

Although CO was relatively low in the three cetacean species from this study, it is notable that all three species have low P_{50} values (increased hemoglobin-oxygen affinity) previously measured with mixing techniques (beluga whale P_{50} = 24.5, bottlenose dolphin P_{50} = 24.6, killer whale P_{50} = 25.2)[46,47]. These low values suggest that these cetaceans could have amino acid substitutions in their hemoglobin molecules. Although the complete amino acid structures of marine mammal hemoglobins have not yet been reported, the ratio of 2,3-DPG to hemoglobin levels fall within the range of healthy humans which suggests it is unlikely that alterations in these organic phosphates would result in a significant change in P_{50} values[47]. Similarly, the temperature of blood in diving marine mammals stays near 37°C while diving[48,49], which makes it unlikely for temperature shifts to significantly affect hemoglobin-oxygen affinity during dives of these animals.

The theoretical optimal P_{50}^* for an animal can be calculated using the equation below[5].

$$P_{50}{}^* = (P_aO_2 * P_vO_2)^{0.5}$$

Where P_aO_2 and P_vO_2 represent the minimum partial pressure of oxygen the animal regularly experiences in arterial and venous blood, respectively. In the case of the

elephant seal, the average minimum P_aO_2 and P_vO_2 during dives is 33.5 and 19.0 mmHg, respectively[19]. This results in a P_{50}^* of 25.2 mmHg, which is strikingly similar to the P_{50} value of the elephant seal oxygen-hemoglobin dissociation curve with 7-8% COHb. Values of COHb in this range are commonly seen in adult elephant seals[30]. It is worth noting that this P_{50}^* in elephant seals is also similar to previously measured P_{50} values in the three cetaceans from this study[46,47]. Another marine mammal that supports this conclusion is the manatee which has a P_{50} value of 16 mmHg[50]. This strengthens the hypothesis that increased hemoglobin-oxygen affinity is selected for in mammalian breath-hold divers, and species adapted to tolerate chronic hypoxia[5].

It has been suggested that deep-diving pinnipeds would benefit from low-oxygen affinity hemoglobin because this would promote diffusion of oxygen into the tissues[40]. Instead, we find that moderate amounts of CO in elephant seals shifts the oxygen-hemoglobin dissociation curve to the left, an illustration of convergent evolution among other hypoxia tolerant species, including cetaceans, to achieve increased hemoglobin-oxygen affinity (Fig. 4A). This is the first evidence of increased hemoglobin-oxygen affinity due to endogenous CO levels in a hypoxia-tolerant species.

Effects of CO on oxygen transport in free-diving elephant seals

In a previous study, continuous measurements of P_aO_2 and P_VO_2 in freely diving elephant seals demonstrated extreme hypoxemic tolerance (Figs. 5A & 5D)[32]. These measurements allowed the calculation of corresponding S_{O2} values based on oxygen-hemoglobin dissociation curves obtained with the classic mixing technique. Here we present the effects of different levels of COHb on the availability of arterial and venous blood oxygen stores in a free-diving elephant seal (Fig. 5).

In the majority of all dives, increased CO in the blood results in higher hemoglobin saturation and oxygen content in arterial and venous blood at the end of dives, when hypoxemia was most extreme (Figs. 5 B, C, E & F and Table 2). However, increased hemoglobin-oxygen affinity due to CO has the potential to reduce oxygen delivery to tissues. Yet, even with COHb values as high as 11%, venous oxygen is almost completely depleted by the end of the dives (Fig. 5D). The arteriovenous oxygen content difference at the end of dives was near 5 ml O_2 dl^{-1} (Figs. 5E & 5F), typical of that in many mammals[5]. These findings indicate that blood oxygen extraction by tissues was efficient during hypoxemia and even in the presence of increased hemoglobin-oxygen affinity induced by CO in the elephant seal.

A decrease in the pH of blood from 7.4 to 7.3 at the end of dives would shift the oxygen-hemoglobin dissociation curve to the right and decrease hemoglobin-oxygen affinity, which could potentially diminish the gain in blood oxygen content due to increased CO in elephant seals. In the absence of CO in elephant seals, a reduction in pH from 7.4 to 7.3 would decrease S_{O2} by 3.5% and 5.8% saturation at common end-of-dive P_aO_2 values of 15 and 20mmHg, respectively[47]. Even with this decrease in S_{O2} from a reduced pH, there is a greater S_aO_2 and arterial oxygen content at the end of the dive when COHb values are above 7% (Table 2). Although investigation of pH effects on the oxygen-hemoglobin dissociation curve in the presence of CO is still needed, the above changes in S_{O2} due to pH alone are small and suggest that CO still results in a net gain in S_{O2} and oxygen content at the end of dives.

Conclusions

Here we examine the levels of endogenous CO in four species of marine mammals, and describe the effects of significantly increased CO production in the elephant seal on hemoglobin-oxygen affinity and blood oxygen transport. We show increased endogenous CO results in greater hemoglobin-oxygen affinity and higher oxygen availability at the end of dives, when hypoxemia is most severe. These results support the hypothesis of convergent evolution of increased hemoglobin-oxygen affinity in species adapted to tolerate chronic hypoxia, with the first report of endogenous CO as a means to achieve this. The additional potential benefits from increased CO production in the elephant seal make the species an excellent model to understand the mechanisms for potential cytoprotection against hypoxia or ischemia-reperfusion events they face during breath-holds. The avoidance of hyperbilirubinemia and increased concentrations of fecal bilirubin in elephant seals, and fecal biliverdin in all cetaceans, suggests that these species efficiently secrete heme degradation products into bile and that they may also possess unique microbiota. While cetaceans from this study did not have elevated levels of CO, previous studies report that many cetaceans have increased hemoglobin-oxygen affinity[46,47] which suggests they may have amino acid substitutions in their hemoglobin to achieve this. We suspect other deep-diving species with large heme-protein stores may also benefit from increased endogenous CO production (e.g. southern elephant seals and Weddell seals[51]). Future studies should consider endogenous CO to interpret the available oxygen stores and cytoprotective effects in animals that are adapted to tolerate hypoxia.

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Author Contributions

M.S.T. and P.J.P. acquired funds for the project. M.S.T. and D.E.C. collected breath and blood samples from elephant seals. M.S.T. collected breath and blood samples from cetaceans. M.S.T. and T.J.L. extracted CO from blood samples. M.S.T. and P.A.J.

analyzed bilirubin, biliverdin and hemin in samples. P.C. performed the hemoglobin-oxygen dissociation curves. J.U.M. collected the elephant seal P_{O2} and depth data. M.S.T. performed statistics on the data. M.S.T. and P.J.P. wrote manuscript. All authors discussed results and edited the manuscript.

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Table 2-1. Blood volume and levels of hemoglobin and myoglobin in humans and four species of marine mammals. Human values are from Davy and Seals, 1994[52], and Möller and Sylvėn, 1981[53]. Marine mammal values are from Ponganis, 2015[54].

Species	Blood volume (ml kg^{-1})	Hemoglobin (g dl^{-1})	Myoglobin (g 100 g^{-1})
Human	70	14	0.5
Bottlenose dolphin	71	14	3.2
Killer whale	90	18	3.1
Beluga whale	128	21	3.4
Elephant seal	216	25	7.8

Table 2-2. Mean and median difference in S_aO_2 (%) and arterial oxygen content (O_2ct; ml O_2 dl blood^{-1}) at the end of 25 deep dives (> 100m) and 25 shallow dives (≤ 100m) from a free-ranging northern elephant seal for different levels of COHb. Mean values are reported as mean ± standard error. Median values in parentheses. Original P_{O2} and depth data for these calculation are from Meir et al., 2009[19].

	Δ S_aO_2 (11 vs 0% COHb)	Δ S_aO_2 (7 vs 0% COHb)	Δ S_aO_2 (3.5 vs 0% COHb)	ΔO_2ct (11 vs 0% COHb)	ΔO_2ct (7 vs 0% COHb)	ΔO_2ct (3.5 vs 0% COHb)
Deep (> 100m)	15.9 ± 0.1 (16.2)	7.7 ± 0.07 (7.8)	3.3 ± 0.04 (3.4)	4.0 ± 0.02 (4.0)	1.9 ± 0.009 (1.9)	0.8 ± 0.006 (0.9)
Shallow (≤ 100m)	13.4 ± 0.6 (14.5)	6.0 ± 0.4 (6.7)	2.9 ± 0.2 (3.2)	2.3 ± 0.3 (2.7)	0.8 ± 0.2 (1.1)	0.4 ± 0.08 (0.5)

Table 2-3. Heme stores from four species of marine mammals and humans. We used a similar mass for hemoglobin (64000 g mol^{-1}) and myoglobin (g mol^{-1}).

Species	Hemoglobin (g dl^{-1})	Blood volume (L kg^{-1})	Myoglobin (g $100g^{-1}$)	Muscle (%)	Muscle mass (g)	Mass (kg)
Elephant seal	25	0.216	7.8	0.28	112000	400
Beluga whale	21	0.128	3.4	0.36	360000	1000
Killer whale	18	0.09	3.1	0.36	1386000	3850
Bottlenose dolphin	14	0.071	3.2	0.36	72000	200
Human	14	0.07	0.5	0.38	26600	70

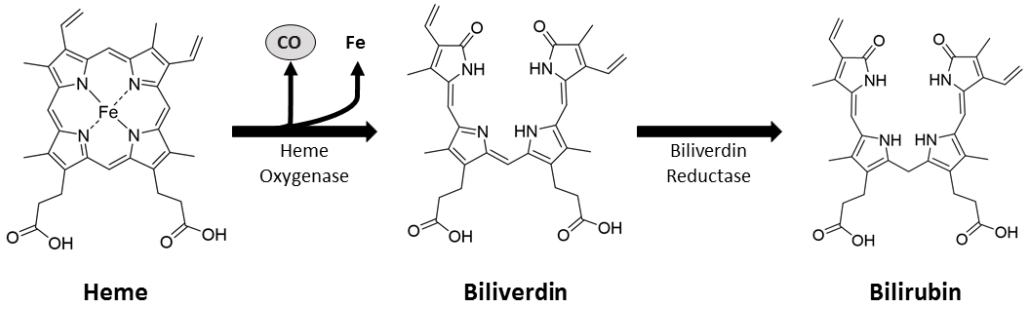

Figure 2-1. Heme oxygenase/carbon monoxide pathway. Heme, from heme-proteins, is catabolized by heme oxygenase enzymes to produce equimolar concentrations of ferrous iron, CO and biliverdin. Biliverdin is then rapidly converted to bilirubin by biliverdin reductase.

A & B

C & D

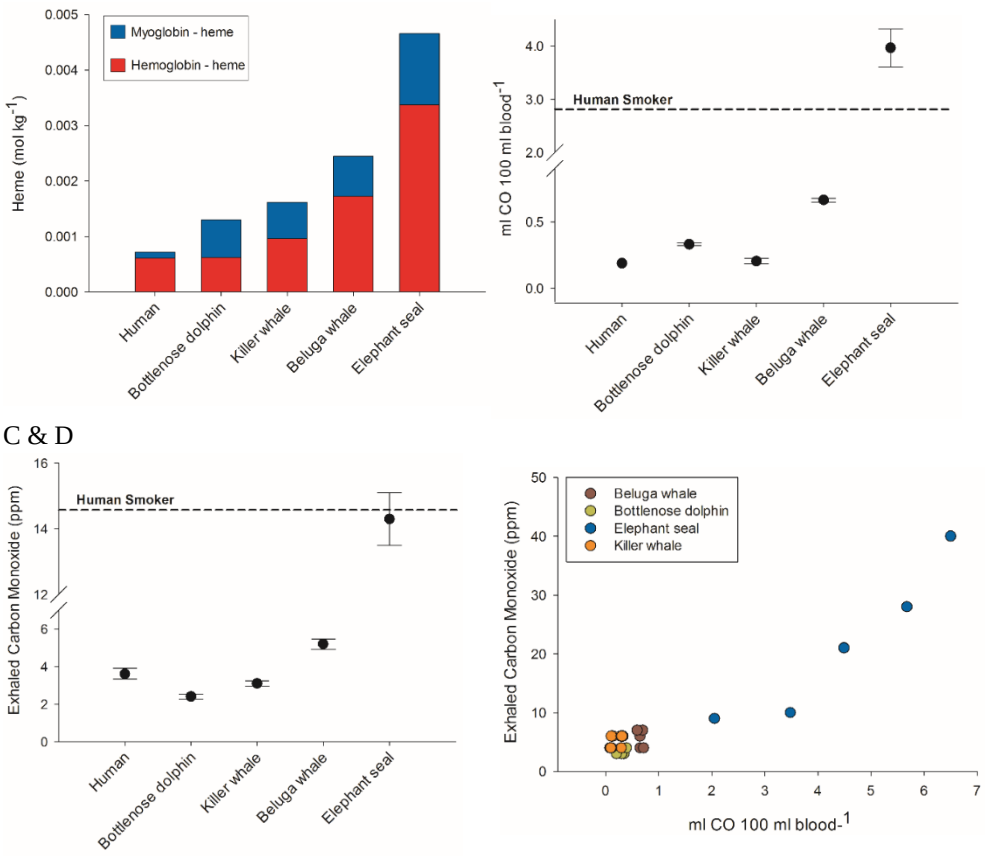

Figure 2-2. Mass-specific molar equivalents of heme and levels of carbon monoxide in the blood and breath from four species of marine mammals and humans. Values and references for calculation of marine mammal and human heme stores can be found in Table 2-3. Human values are from non-smokers and the dashed line represents values from chronic cigarette smokers. Blood CO values from cigarette smokers are from Wald et al., 1981[13], using a hemoglobin value of 14 g dl^{-1} and COHb value of 12%. Breath CO values from cigarette smokers are from Deveci et al., 2003[55] A) Mass-specific molar equivalents of heme (mol kg^{-1}) in four species of marine mammals and humans based on hemoglobin (red) and myoglobin (blue) stores. B) CO content extracted from the blood of beluga whales (n = 5), bottlenose dolphins (n = 6), elephant seals (n = 7), and killer whales (n = 9) in comparison to human levels. Elephant seals had significantly higher blood CO than all other marine mammals ($F_{3,24}$ = 21.4, $p < 0.0001$). Values from marine mammals are reported as mean ± standard error. C) Exhaled CO concentrations from beluga whales (n = 5), bottlenose dolphins (n = 6), elephant seals (n = 5), and killer whales (n = 8) in comparison to human values. To ensure the quality of breath samples collected, end-tidal CO_2 was also measured and a minimum value of 40 mmHg, or 5.3 kPa, was accepted[56]. Similar to blood CO values, elephant seals had significantly higher values than the other marine mammal species ($F_{3,21}$= 17.3, $p < 0.0001$). All breath values are reported as mean ± standard error. D) Relation of mean levels of CO extracted from blood and maximum CO in breath samples from beluga whales (n = 5), bottlenose dolphins (n = 6), elephant seals (n = 5), and killer whales (n = 8). The low range of values in cetaceans likely reflects their reduced heme-protein stores and magnitude of turnover of heme, in relation to elephant seals. In elephant seals, the two lower values are from juvenile seals with lower hemoglobin stores, and the three higher values are from adults. There is a positive relation between mean values of CO in the blood and maximum CO in the breath of elephant seals (Breath CO = -9.5 + 7.0*CO; r^2 = 0.91), where CO is the ml of CO extracted from 100 ml of blood.

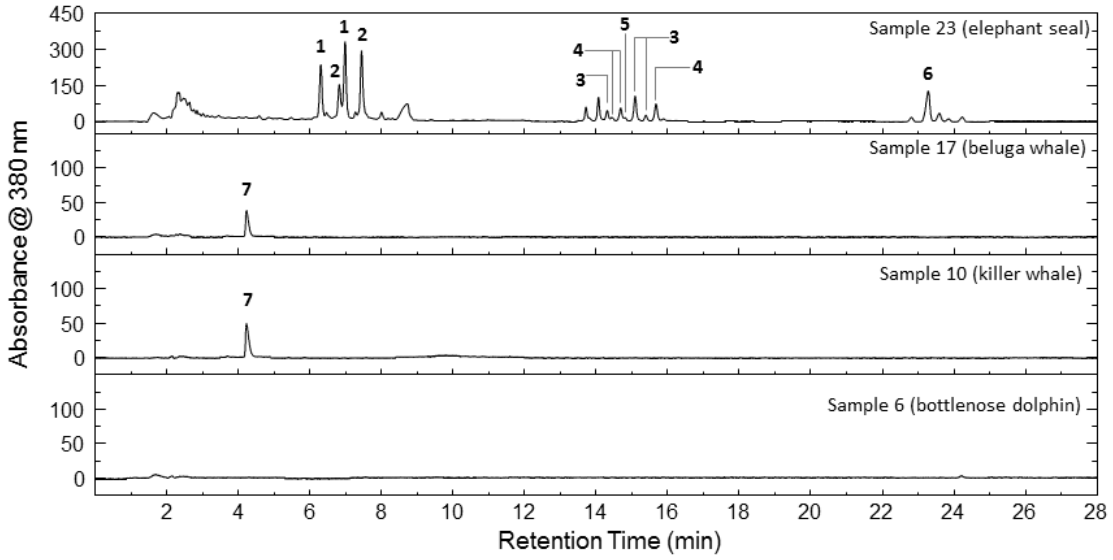

Figure 2-3. Fecal metabolite analysis of methanol extracts from four species of marine mammals. A) Metabolite analyses for the four species of marine mammals from this study. B) Metabolite analyses for 3 adult elephant seals. Compound ID, 1) Bilirubin-glucose conjugate; 2) Bilirubin-glucuronic acid conjugate; 3) Dihydrobilirubin; 4) Mesobilirubin; 5) Dihydromesobilirubin; 6) Unconjugated bilirubin; 7) Biliverdin. UV chromatograms measured at 380 nm are indicated for each animal.

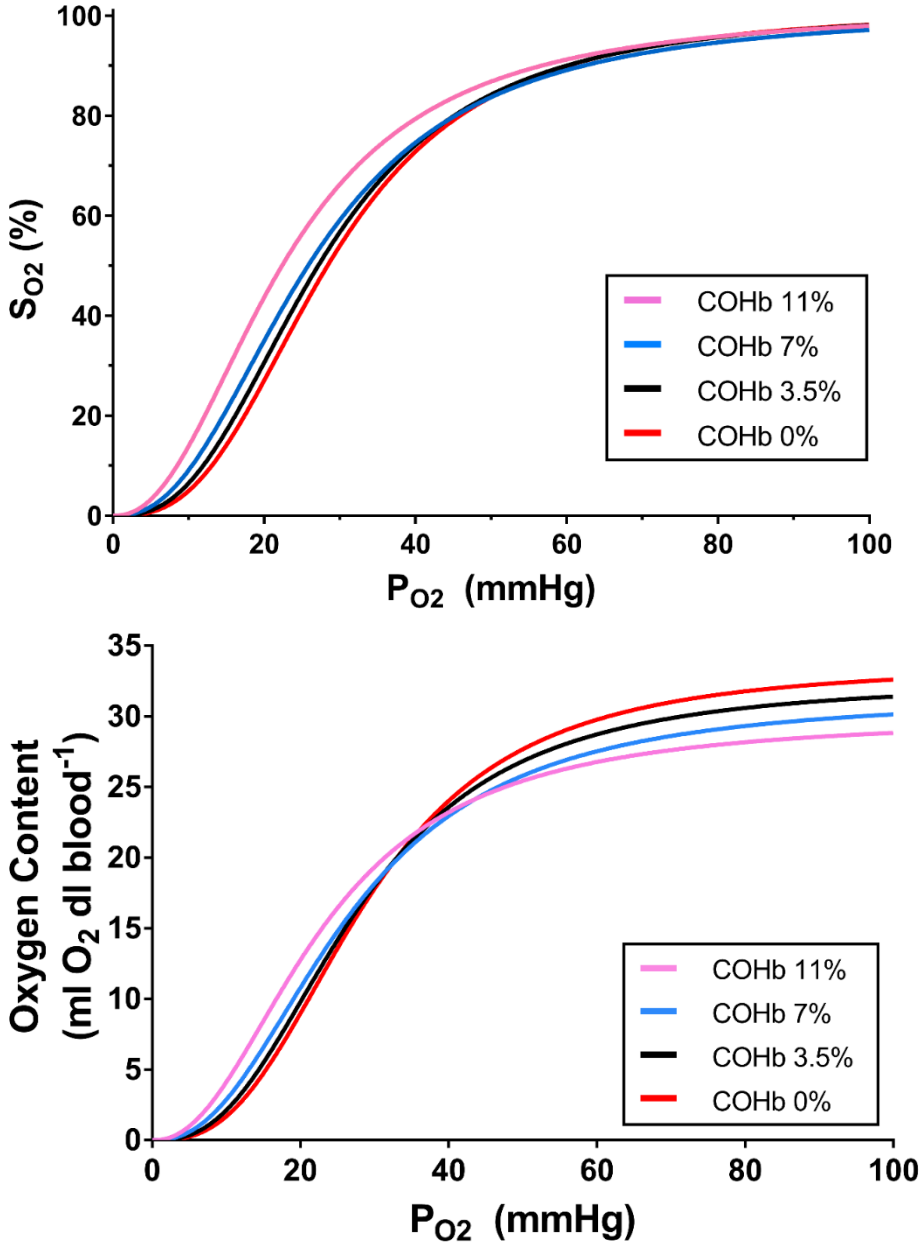

Figure 2-4. Effect of four levels of COHb on the oxygen-hemoglobin (O_2-Hb) dissociation curve and oxygen-equilibrium curve from adult elephant seals (n = 3) A) Oxygen-hemoglobin (O_2 – Hb) dissociation curves for four different levels of COHb in adult elephant seal blood. The hill slope and P_{50} value for each level are; COHb 0% (2.82, 28.6 mmHg), COHb 3.5% (2.65, 27.4 mmHg), COHb 7% (2.42, 26.0), COHb 11% (2.25, 22.6 mmHg). Each curve plots the percentage saturation of available hemoglobin (not bound to CO) versus P_{O2}. B) The relationship between P_{O2} and oxygen content of blood in adult elephant seals. Oxygen contents are calculated using the saturation curves for each respective level of COHb. Hemoglobin concentrations of 25, 24.125, 23.25, and 22.25 g dl^{-1} were used to calculate oxygen contents for 0, 3.5, 7 and 11% COHb, respectively. 1 kPa = 7.50 mmHg

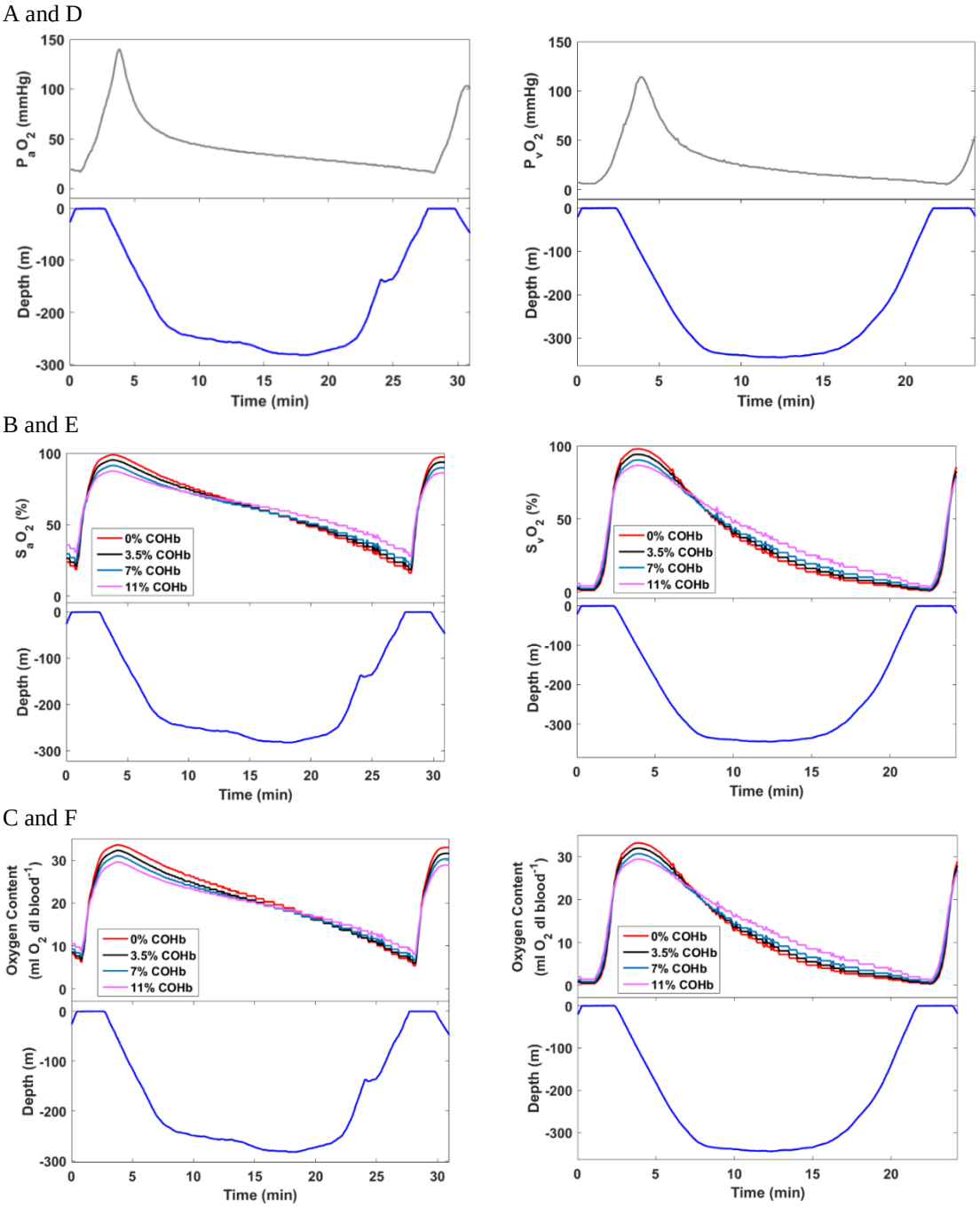

Figure 2-5. Arterial and venous partial pressure of oxygen (P_{O2}), percentage of hemoglobin saturation (S_{O2}), and blood oxygen content profiles from free-diving elephant seals (Meir et al., 2009)[19], at four levels of carboxyhemoglobin (COHb). Original P_aO_2 (A), P_vO_2 (D) and depth data (1 kPa = 7.50 mmHg). Elephant seals surface for ~1-2 minutes, where they quickly re-oxygenate before and after dives. Calculated S_aO_2 (B) and S_vO_2 (E) at four levels of COHb. Calculated arterial (C) and venous (F) oxygen content (ml O_2 dl blood^{-1}) at four levels of COHb. The oxygen content equation is: $O_2ct = 1.34 * Hb * (S_{O2}/100) + 0.003 * P_{O2}$. Where Hb is the hemoglobin concentration, S_{O2} is the arterial hemoglobin saturation, 0.003 is the dissolved oxygen constant for plasma, and P_{O2} is the arterial partial pressure of oxygen. Oxygen content values assume hemoglobin concentrations of 25, 24.125, 23.25, and 22.25 g dl^{-1} for 0, 3.5, 7 and 11% COHb, respectively.

Materials and Methods

Blood Collection

Cetaceans voluntarily presented their tail fluke, where veins could be percutaneously catheterized to collect blood samples by SeaWorld veterinarians. Elephant seals were chemically immobilized with an intramuscular injection (1 mg kg^{-1}) of Telazol (tiletamine/zolazepam HCl) where the extradural vein could then be percutaneously catheterized to collect blood samples. After sample collection was finished, elephant seals were monitored until they regained full range of movement. Blood was collected directly into a chilled, heparinized vacutainer (BD Vacutainer®, Fisher Scientific, Franklin Lakes, NJ, USA), that had the vacuum removed upon collection of the sample by quickly puncturing the top of the tube with a needle. Blood was chilled over ice and analyzed immediately upon returning to the laboratory (0.5 - 12 hours).

Blood carbon monoxide measurements

We followed a modified method for analyzing levels of CO in blood via headspace gas analysis[57]. All samples were measured in duplicate. Blood (1-3 ml) was drawn from the vacutainer into a blood gas syringe (Smiths Medical, Dublin, OH, USA) and was mixed with 5 ml of 1.8 M sulfuric acid in a sealed 25 ml Erlenmeyer flask (volume calibrated to ± 0.1ml) that had been previously flushed with > 5 vol N_2 gas (scrubbed free of CO by Sofnocat®423). The blood and acid were then stirred at 40°C for 40 min in the sealed Erlenmeyer flask. A 1500 ml flask (volume calibrated to ± 0.1ml) was then flushed with ≥ 10 vol of CO-free N_2 gas. Using a gas-tight syringe, 5 ml of headspace was removed from the Erlenmeyer flask and injected into a line where CO-

free N_2 gas flushed the sample into the bottom of the 1500 ml flask. The sample 1500 ml flask was then sealed and allowed to sit for ≥ 24 hrs to ensure adequate mixing. The 1500 ml flask was then connected to a trace-gas analyzer system and the mole fraction of CO analyzed on a Picarro Model 2401 CO analyzer (Sunnyvale, CA, USA). The daily flask analyses were bracketed with reference gases traceable to The National Institute of Standards and Technology gases (NIST, Gaithersburg, Maryland, USA). Individual flask analysis precision ± 11 ppb (n=90, 1 sec data). COHb was calculated using the equation below.

$$COHb(\%) = (ml\ CO\ 100ml\ blood^{-1} * 100) * (1.34 * Hb)$$

Where 1.34 is the hemoglobin carrying capacity (ml O_2 g^{-1} hemoglobin), and Hb is the average hemoglobin concentration (g dl^{-1} blood) for the different species (Table 1).

Biliverdin and bilirubin measurements in feces

Methanol Extracts of Feces: Fecal samples were voluntarily collected from cetaceans using a catheter and syringe by SeaWorld trainers and veterinarians. Fecal samples from elephant seals were collected with sterile tongue depressors from anesthetized animals, or from sleeping animals. Fecal samples were freeze dried and the dried powder pulverized with a glass tissue homogenizer before being sifted through a stainless steel screen (60-mesh: opening = 230 micron) to remove large insoluble debris. Dry powder (5 mg) for each sample was placed in a 1.5 mL microcentrifuge tube and 1 mL of mass spectrometry grade methanol was added. The methanol suspension was placed in a water bath sonicator for 20 minutes, followed by 20 minutes of centrifugation

at 16.1 relative centrifugal force. The supernatant was removed by pipette and methanol extraction was repeated in triplicate.

Analysis of Fecal Bilins by UV-Vis and LCMS: Fecal methanol extracts were combined, filtered and 10 µL of each sample analyzed by liquid chromatography (LC) mass spectrometry (MS) on an Agilent 6530 Accurate-Mass Q-TOF Mass Specrometer equipped with a dual electrospray ionization source and an Agilent 1260 LC system with diode array detector. Polar bilins (e.g. urobilin and stercobilin) were separated on a Phenomenex Kinetex 2.6µ XB-C18 100 A, 150 x 4.6 mm column with "Method A", an acetonitrile (MeCN)-water gradient (0-3 min isocratic 10% MeCN, 3-23 minutes 10-100% MeCN, + 0.1% formic acid). Biliverdin, non-polar bilins (e.g. mesobilirubin, dihydrobilirubin, urobilinogen etc.) as well as conjugated and unconjugated bilirubin were separated with same Phenomenex column using "Method B" (0-3 min isocratic 50% MeCN; 3-12 min 50-90% MeCN; 12-26 min 90-100% MeCN, + 0.1% formic acid). Biliverdin and bilirubin were positively identified based upon retention time of authentic standards (see below) and mass spectrometry. Related bilirubin breakdown products were positively identified based upon UV-Vis absorption, mass spectrometry and tandem mass spectrometry (collision induced dissociation at 20 eV) and their structures deduced from parent ion exact mass and expected fragment ions (see Table S1) based upon the previously reported MS fragmentation behavior for such molecules[58]

Standard Curve and Quantification: Stock solutions of biliverdin (Sigma 30891, 140 µg/mL) and bilirubin (Spectrum BI110, 130 µg/mL) were prepared in 10 mL of methanol buffered with 50 µL of 14.8 N ammonium hydroxide (for solubility) and serial dilutions of these stocks were used to generate a standard curve for biliverdin and

bilirubin quantification. UV-Vis absorbencies and peak areas were measured at 380 nm and 430 for biliverdin and bilirubin, respectively. Both glucose and glucuronic acid conjugates of bilirubin could be identified in fecal methanol extracts of elephant seals. These were quantified with the same standard curved for bilirubin as sugar conjugation is not expected to affect the bilirubin chromophore.

Hemoglobin dissociation curves

Hemoglobin dissociation curves for blood were obtained by deoxygenation of pre-equilibrated diluted blood in Hemox buffer at 37°C, using a Hemox Analyzer (TCS Scientific, New Hope, PA). Briefly, 50uL of blood were mixed into 5 mL of Hemox buffer and equilibrated with compressed gas (21% oxygen/5% carbon dioxide balanced nitrogen) remove any existing carbon monoxide, and ii) define the 100% Hb saturation in the Hemox Analyzer. Dissociation curves for blood without carbon monoxide was obtained by deoxygenating the diluted blood with gas mixture (95% nitrogen/5% carbon dioxide). To increase the carbon monoxide content, blood was re-oxygenated with compressed (21% oxygen/5% carbon dioxide balanced nitrogen) in a seal vial, and then small amounts of pure carbon monoxide gas were injected into the sealed vial until COHb reached desired levels. COHb levels were confirmed spectrophotometrically using a Lambda 20 UV/VIS spectrometer (Perkin Elmer, Foster City, CA). Dissociation curves for blood at set levels of carbon monoxide was also obtained via deoxygenating the carbon monoxide equilibrated blood with gas mixture (95% nitrogen/5% carbon gas). Equations, P_{50} values and hill slopes for the oxygen-hemoglobin dissociation curves at the four different levels of COHb are reported below.

0% COHb

$$SO2 = 100 * \frac{PO2^{2.82}}{(28.6^{2.82} + PO2^{2.82})}$$

Hill slope	2.82
p50	28.6

3.5% COHb

$$SO2 = 100 * \frac{PO2^{2.65}}{(27.4^{2.65} + PO2^{2.65})}$$

Hill slope	2.65
p50	27.4

7% COHb

$$SO2 = 100 * \frac{PO2^{2.42}}{(26.0^{2.42} + PO2^{2.42})}$$

Hill slope	2.42
p50	26.0

11% COHb

$$SO2 = 100 * \frac{PO2^{2.25}}{(22.6^{2.25} + PO2^{2.25})}$$

Hill slope	2.25
p50	22.6

End-tidal CO and CO_2 measurements

Breath CO measurements (ppm) are useful to determine the removal rate and production rate of CO[59]. Cetaceans were trained to exhale fully into a device which captures end-tidal samples. The gas sample could then be pumped out of the breath-collection device and into the appropriate analyzers. End-tidal carbon monoxide (ETCO) values were collected from each individual and CO was sampled on a portable carbon monoxide breathalyzer (± 5%) (ToxCO: Covita, Santa Barbara, CA, USA). The breathalyzer was calibrated with 50 ppm CO every day samples were collected. In animals where tidal volume was too large, or breath samples contained too much moisture to collect the sample directly into the breathalyzer, we trapped end-tidal gasses in a flow-through chamber with a series of one-way valves, where we could then subsample the gases. To confirm end-tidal samples were being collected from cetaceans, we used a cutoff value of 40 mmHg CO_2 for end-tidal sample collection on the killer whale, beluga whale and bottlenose dolphins[56]. End-tidal CO_2 values were measured on a CO_2 analyzer (Model #17515, VacuMed, Ventura, CA, USA). This analyzer was calibrated with room air and 10% CO_2 every day before sampling. Elephant seals reliably exhale fully while under anesthesia which removed the need to simultaneously sample CO_2. The consistently high end-tidal CO values in individuals also confirmed the likelihood of end-tidal sampling. Mass-specific CO production rates were calculated for the elephant seal by multiplying the maximum ETCO (40 ppm) and previously measured minute volume (219 L min^{-1}). Human mass-specific CO production rates are based on healthy humans (mass = 70 kg) from Scharte et al., 2000[31].

Statistics

The difference in blood and breath CO levels between species was investigated using a linear mixed-effect model with individual as the random effect. Residuals of models were assessed for normality. A Tukey's post-hoc analysis revealed the species which had significantly different values from each other. Significance was determined at $\alpha = 0.05$.

CHAPTER 3:

Flipper stroke rate and venous oxygen levels in free-ranging California sea lions

Michael S Tift, Luis A. Hückstädt, Birgitte I. McDonald, Philip H. Thorson, Paul J. Ponganis

Abstract

The depletion rate of the blood oxygen store, development of hypoxemia, and dive capacity are dependent on the distribution and rate of blood oxygen delivery to tissues while diving. Although blood oxygen extraction by working muscle would increase the blood oxygen depletion rate in a swimming animal, there is little information on the relationship between muscle workload and blood oxygen depletion during dives. Therefore, we examined flipper stroke rate, a proxy of muscle workload, and posterior vena cava oxygen profiles in four adult female California sea lions (*Zalophus californianus*) during foraging trips at sea. Flipper stroke rate analysis revealed that sea lions minimized muscle metabolism with a stroke-glide strategy when diving, and exhibited prolonged glides during the descent of deeper dives (> 100 m). During the descent phase of these deep dives, 55 ± 21% of descent was spent gliding with the longest glides lasting over 160 s and covering a vertical distance of 340 m. Animals also consistently glided to the surface from 15-25 m depth during these deeper dives. Venous hemoglobin saturation (S_vO_2) profiles were highly variable throughout dives, with values occasionally increasing during shallow dives. The relationship between S_vO_2 and flipper stroke rate was weak during deeper dives, while this relationship was stronger during shallow dives. We conclude that 1) the depletion of oxygen in the posterior vena cava in deep diving sea lions is not dependent on stroke effort, and 2) stroke-glide patterns during dives contribute to a reduction of muscle metabolic rate.

Introduction

Optimal management of oxygen stores underlies the breath-hold capacity of air-breathing divers. The utilization of oxygen stores during dives is dependent on the regulation of heart rate, the magnitude/distribution of peripheral blood flow, tissue oxygen uptake, and muscle workload. The severe bradycardia and peripheral vasoconstriction observed in the classic dive response conserves oxygen in blood by directing it towards hypoxia-sensitive tissues, such as the brain and heart (Irving et al., 1941; Scholander et al., 1942). However, blood oxygen supplementation to working muscle can occur during moderate bradycardias that are common during routine dives and breath-holds of diving animals (Guyton et al., 1995; Jobsis et al., 2001; Ponganis et al., 2008; Williams et al., 2011). In addition, the positive relationship between heart rate and flipper stroke rate in some diving mammals has led to the hypothesis that exercise and muscle workload modulate the dive response and increase heart rate during short duration dives (Davis and Williams, 2012). In the presence of muscle blood flow, blood oxygen extraction by working muscles during a dive should result in a decline in venous hemoglobin saturation (S_vO_2). Such declines in venous oxygen content are proportional to muscle workload in terrestrial mammals, despite increases in muscle blood flow and oxygen delivery during exercise (Taylor et al., 1987).

The California sea lion (*Zalophus californianus*) is an ideal model to investigate the effect of muscle workload (flipper stroke rate) on the depletion of blood oxygen stores during dives. These animals are known for being active swimmers and can dive as deep as 540 m and as long as 10 min, during which they demonstrate a wide range of heart rate responses, and blood oxygen depletion patterns (Kuhn and Costa, 2014;

McDonald and Ponganis, 2012; McDonald and Ponganis, 2013; McDonald and Ponganis, 2014). For this study, we equipped free-ranging, adult female California sea lions with bio-logging devices that simultaneously recorded depth, tri-axial acceleration, and venous partial pressure of oxygen (P_vO_2). From these data, we documented stroke rate profiles, evaluated the effect of muscle workload on changes in S_vO_2, and determined if blood oxygen extraction by muscle contributed significantly to S_vO_2 depletion in dives of different depths and durations.

Materials and Methods

Instrumentation

This study was conducted on San Nicolas Island, California in August 2013. Four animals were captured with hoop nets and anesthetized using a portable vaporizer-breathing circuit set up with an initial mixture of 5% isoflurane and 100% oxygen. Once anesthetized, a custom built P_{O2} datalogger (UUB-2PT; UFI, Morro Bay, CA, USA) with custom housing (Meer Instruments, Palomar Mountain, CA, USA), time-depth recorder with 3-axis accelerometer (TDR10; Wildlife Computers, Redmond, WA, USA) and VHF radio transmitter (mm160B; Advanced Telemetry Systems, Isanti, MN, USA) were affixed to the dorsal, midline pelage of the animal using epoxy (Loctite, Henkel Corp., Westlake, OH, USA). Using a Doppler ultrasound (SonoSite Inc., Bothell, WA, USA), the caudal gluteal vein was identified and percutaneously catheterized with a peel-away catheter (5 Fr, Cook Medical, Bloomington, IN, USA). A P_{O2} electrode (Licox C1.1, Integra Life Sciences, Plainsboro, NJ, USA) was then inserted in the catheter and threaded up into the posterior vena cava. These procedures have been explained in more

detail in previous publications (McDonald and Ponganis, 2013; Meir et al., 2009; Ponganis et al., 1997; Ponganis et al., 1991; Ponganis et al., 2007; Stockard et al., 2007). Depth and P_{O2} were recorded at 1Hz, and three axes of acceleration were recorded at 16Hz.

After instrumentation, the animals were placed in a large canine kennel to allow them to safely recover from anesthesia (~30-60 min) and weighed (±0.2 kg, MSI-7200 Dyna-link; Measurement Systems International, Seattle, WA, USA). Once the animals were fully alert, they were released back onto the same beach where they were originally captured. We recaptured the sea lions after 1-3 trips to sea, removed the instruments under manual restraint, and released them (~5-10 min procedure). All procedures were approved by the University of California, San Diego Animal Subjects Committee (no. S11303) and National Marine Fisheries Services (no. 14676).

Data processing and statistics

Dive data were analyzed using a custom written MATLAB program (IKNOS; Y. Tremblay). Briefly, this dive analysis program calculated a zero-offset correction at the surface and identifies dives using a specified minimum depth (10 m) and duration (20 s). This depth threshold was selected as dives under 10 m were too short to evaluate changes in physiological parameters. The following dive phases were identified in the data: descent (surface to 80% maximum depth), bottom (depths within 80% maximum depth), and ascent (80% maximum depth to surface). Most California sea lion dives are shallower than 100 m, yet many adult female California sea lions from San Nicolas Island are known to be deep divers (Kuhn and Costa, 2014; McHuron et al., 2016).

Therefore, to compare shallow and deep dives, dives to maximum depths > 100 m were classified as deep, and dives to maximum depths ≤ 100 m were classified as shallow.

Foreflipper stroke rate was calculated using a custom written algorithm in MATLAB. The low frequency static acceleration data was filtered out using a 0.2 Hz high-pass Butterworth filter. The resulting dynamic acceleration was then analyzed using Power Spectral Density analysis was performed to identify the dominant frequency of a stroke for each individual animal (approximately 0.8 – 1.2 strokes s^{-1}). A peak detection algorithm, similar to those in other studies (Jeanniard-du-Dot et al., 2016; Sato et al., 2011), was used to identify flipper strokes. A single flipper stroke was identified there was a prominent acceleration peak (≥ 0.4 – 0.5 m s^{-2}) in the X-axis ('forward surge') or Z-axis ('heave surge') (Fig. 1). A preliminary experiment with a captive sub-adult male California sea lion was conducted at the National Marine Mammal Foundation. This animal was equipped with the same 3-axis accelerometer (TDR10) and performed submerged swimming behaviors across several lengths of a pen, where we could visually observe and video-record the number of strokes. Peaks of acceleration in the two axes mentioned above (X and Z) matched the observed strokes, confirming that the strokes of California sea lions can be detected using these two acceleration axes. Stroke rate was calculated as an average stroke rate along a moving window of 10 s throughout the diving record.

The P_{O2} electrodes were calibrated in the laboratory before deployment (Ponganis et al., 2007; Stockard et al., 2007). Values of S_vO_2 were calculated using the hemoglobin-dissociation curve from McDonald and Ponganis, 2013. Because the minimum calculated aerobic dive limit (cADL) for this species is just over three minutes (Weise and Costa,

2007), P_{O2} values collected before three minutes into the dive were converted to S_VO_2 using the equation for a pH of 7.4 ($\log[S_{O2}/(100-S_{O2})] = 2.473 \times \log(P_{O2}) - 3.632$) and all P_{O2} values collected beyond three minutes into the dive were converted to S_VO_2 using the equation for a pH of 7.3 ($\log[S_{O2}/(100-S_{O2})] = 2.363 \times \log(P_{O2}) - 3.576$). Previous data suggests that California sea lions, and other deep diving pinnipeds, typically keep core body temperature relatively constant at 37°C while diving (McDonald and Ponganis, 2013; Meir and Ponganis, 2010), therefore P_{O2} electrodes were only calibrated at this temperature and temperature effects on the S_vO_2 values were not considered. Mean ΔS_vO_2 ($\Delta\%\ s^{-1}$) was calculated for the same 10-s moving window as flipper stroke rates to facilitate comparison. The mean ΔS_vO_2 was also calculated for the three dive phases (descent, bottom, ascent) and the total dive duration.

To investigate whether flipper stroke rate activity effects ΔS_vO_2 for dives of different durations, we used linear mixed effect models with flipper stroke rate, dive duration, and an interaction term (flipper stroke rate * dive duration) as fixed effects and individual as a random effect. This model was evaluated for shallow and deep dives.

Because periods of gliding (i.e. no flipper strokes recorded) were consistently noticed during the descent and ascent phases of deeper dives, the duration and depth at which these glide phases occurred were identified for dives greater than 100 m. The starting and ending depth of the glide and glide durations were compared against mass, maximum depth and ΔS_vO_2 for those phases of the dives using a linear mixed effect model, with individual as the random effect. Residuals and AICc values from all models were assessed to determine the quality of the model predictions.

Results

Venous P_{O2}, depth and acceleration data were simultaneously collected from four adult, female California sea lions. Flipper stroke rate analysis revealed a stroke-glide pattern with periods of prolonged gliding during the descent of deep dives (Fig. 3-1A). This resulted in data collection from a total of 3,306 dives. Similar to previous reports on California sea lions from San Nicolas Island (McDonald and Ponganis, 2013; McDonald and Ponganis, 2014; McHuron et al., 2016), all animals reached depths over 200m, while the majority of dives were shallow (< 50m). Distribution of maximum dive depths was bi-phasic, with very few dives occurring at depths of 100-150 m. This pattern influenced how we defined shallow (≤ 100m) and deep (> 100m) dives. Dive statistics are reported in Table 3-1.

Posterior vena caval S_vO_2 profiles were variable during shallow dives, with a wide range of values throughout dives and inconsistent patterns in ΔS_vO_2 (Fig. 3-2B). On deeper dives, there was a steady drop in S_vO_2 throughout the dive until later in ascent, when values often increased prior to surfacing (Figs. 3-2A, 3-3 and 3-4B). There was no significant relationship between flipper stroke rate and ΔS_vO_2 during deep dives (Table 3-2). However, there was a significant relationship between the interaction term of dive duration and flipper stroke rate with ΔS_vO_2 during shallow dives (Table 3-2).

On deeper dives, animals would regularly glide down to depth (Fig. 3-1A, 3-2A, 3-3, and 3-4A). This resulted in low stroke rates during the descent of deep dives (Fig. 3-5). The depth at which gliding started on the descent phase of deeper dives did not vary with maximum depth of the dives ($p = 0.8$) (Table 3-3). As expected, the duration of the descent glide increased significantly with maximum depth of the dive ($F_{1,159} = 59.3$, $p <$

0.001). The depth at which the descent glide started did not significantly predict patterns of ΔS_vO_2 during the descent period ($p = 0.8$).

At the end of the ascent from deeper dives, animals would also utilize a gliding strategy to reach the surface (Figs. 3-1A and 3-3). The depth at which gliding started on the ascent phase did not vary with maximum depth of the dive ($p = 0.6$). The duration of the ascent glide did not vary significantly with maximum depth ($p = 0.5$). The depth at which gliding started on the ascent phase and the duration of the ascent glide phase were not related to ΔS_vO_2 during the ascent phase ($p = 0.3$, $p = 0.2$, respectively) (Table 3-3).

Discussion

Flipper stroke patterns

We documented flipper stroke rate patterns in free-ranging, adult, female California sea lions for the first time. Similar to techniques using 1 axis of acceleration to identify flipper strokes in free-diving animals (Maresh et al., 2014; Sato et al., 2003), we used both the surge (X) and heave (Z) acceleration axes to reliably identify flipper strokes in all dive phases (Fig. 3-1). Our results show that the average flipper stroke rates during different dive phases from this study were consistent with data from other free-ranging otariids (~0.4 – 0.6 Hz: Fig. 3-5) (Insley et al., 2008; Jeanniard-du-Dot et al., 2016). Similar to free-ranging phocids (Davis et al., 2001; Williams et al., 2000), California sea lions exhibited periods of prolonged gliding on the descent phase of deep dives, with maximum glides reaching over 160 seconds and covering 340 m vertical distance (Figs. 3-1A, 3-2A, and 3-3). California sea lions are streamlined, with high fineness ratios resulting in low drag, making them efficient at gliding through the water (Feldkamp,

1987). Gliding during descent will conserve blood and muscle oxygen stores for subsequent activities such as prey capture and stroke effort during ascent.

During deep dives, sea lions glided an average of 55% of both the depth and duration of the descent phase with a maximum of 88% of the descent depth and 92% of the descent duration. Prolonged gliding on the descent phase was consistently associated with dives that reached the range of the estimated depth of lung collapse in this species (~200 m) (McDonald and Ponganis, 2012)(Fig. 3-5). On dives deeper than 100 m, the average depth at which gliding started on the descent was relatively shallow (60 ± 20 m) (Table 3-3). This is not surprising as buoyancy will decrease as lung volume decreases (approximately 50% of surface lung volume at 10m depth, and 14% at 60m according to Boyle's law). Interestingly, elephant seals also began prolonged glides during deep dives at about 60m depth (Davis et al., 2001).

The average flipper stroke rate was 0.42 ± 0.002 Hz during the ascent phase of deeper dives (Fig. 3-5). However, ascent flipper stroke rates usually were higher at the beginning of the ascent (~1 Hz) and tapered off to a glide by the time the animals reached 15-25m (Figs. 3-1A and 3-3). The depth at which the glide started varied between individuals, and this was not influenced by sea lion mass or dive depth or duration. This gliding pattern on the ascent phase has also been documented in diving birds, and was suggested to be influenced by respiratory air volume (Sato et al., 2011; Watanuki et al., 2006). Ascent glides in sea lions are likely secondary to increased buoyancy near the surface due to lung re-expansion during ascent.

In contrast to the consistent stroke rate patterns seen in deep dives (Figs. 3-2A and 3-5), our data showed that there was a large variation in flipper stroke rates on dives with

maximum depths shallower than 100 m (Figs. 3-2B and 3-5). Similar to deep dives, the highest stroke rates during shallow dives occurred at the beginning of the dive, while the lowest stroke rates were often at the end of the dive (Fig. 3-3 and 3-4C). This pattern is consistent with expected buoyancy changes with depth. Overall, the mean flipper stroke rate for the entire dive duration was similar between both shallow dives and deep dives (Fig. 3-5). However, the range and variability of flipper stroke rates for the entire dive duration was larger during shallow dives (Fig. 3-5).

Relationship of blood oxygen use to flipper stroke rate in shallow dives

Profiles of S_vO_2 were highly variable during shallow dives, with S_vO_2 even increasing for the duration of the dive (Figs. 3-2B and 3-4D). In addition, some shallow dives began with low S_vO_2; the sea lions did not completely re-saturate venous blood prior to starting these dives (Fig. 3-2B). In some cases, ΔS_vO_2 appeared to be affected by stroke rate patterns as would be expected during exercise and consistent with recent suggestions that heart rate (and presumably some muscle blood flow) is modulated by exercise (Davis and Williams, 2012; Williams et al., 2015). During shallow dives, California sea lions exhibit higher heart rates than those seen during deep dives (McDonald and Ponganis, 2014). These higher heart rates are likely associated with increased blood flow to muscle, and could account for the relationship between flipper stroke rates and venous blood oxygen depletion during shallow dives. However, the weak correlation between the two parameters, and in some instances, increases in S_vO_2 throughout the dives, despite high stroke rates, argues that this does not always occur.

Full interpretation of S_vO_2 during shallow dives is also limited by the lack of arterial hemoglobin saturation data during shallow dives of California sea lions. The relationship between S_vO_2 profiles and the interaction of dive duration and stroke rate of shallow dives may also be partially secondary to greater arterial hemoglobin desaturation during longer shallow dives. In other words, a decrease in S_vO_2 may reflect a decrease in arterial oxygen content delivery rather than an increase in blood oxygen extraction by tissue. Ideally simultaneous measurements of arterial/venous blood oxygen content, heart rate, flipper stroke patterns and/or muscle myoglobin saturation would resolve this question.

From our current data, it appears that for shallow, short duration dives, regulation of posterior vena caval S_vO_2 is not critical or highly controlled, and that the peripheral vascular response is quite variable. Such plasticity in blood flow distribution has also been observed in diving emperor penguins (Williams et al., 2011).

Relationship of blood oxygen use to flipper stroke rate in deep dives

Assessment of locomotory effort on S_vO_2 profiles during deep dives is aided by our prior study which demonstrated that arterial hemoglobin saturation is maintained during deep dives (McDonald and Ponganis, 2012). Consequently, changes in S_vO_2 during deep dives are more likely due to changes in perfusion and tissue oxygen extraction rather than a decrease in arterial oxygen content delivery.

Posterior vena caval S_vO_2 profiles in the sea lions were similar to those of a previous study from our laboratory (McDonald and Ponganis, 2013). In our current study, we demonstrate that flipper stroke rates had little impact on ΔS_vO_2 during most deep

dives of sea lions (Table 3-2). We also saw that S_vO_2 often increased during the ascent, despite the animal's active stroking to reach the surface (Figs. 3-2A, 3-3, and 3-4B). Furthermore, the greatest decreases in S_vO_2 occurred during the low stroke rates and prolonged glides of the descents of deep dives (Figs. 3-3 and 3-5). In contrast, some of the largest increases in S_vO_2 occurred while the animals were actively stroking during ascent from deep dives (Figs. 3-3 and 3-5). As in our prior studies, we postulate that the re-oxygenation of venous blood prior to surfacing is indicative of maintenance of pulmonary gas exchange at shallow depths, increased peripheral perfusion, and possible use of arterio-venous shunts during the ascent (McDonald and Ponganis, 2012; McDonald and Ponganis, 2013; McDonald and Ponganis, 2014).

Based on these findings, we conclude that ΔS_vO_2 in the posterior vena cava is not significantly affected by flipper stroke rate during deep dives. Rather, as we have previously hypothesized (McDonald and Ponganis, 2013), it is more likely that S_vO_2 is determined by the degree of bradycardia, the magnitude of reduction in peripheral blood flow, and the associated changes in tissue transit time during dives. Prolonged tissue transit times during severe bradycardia should result in greater blood oxygen extraction and lower hemoglobin saturations in any blood slowly draining into the posterior vena cava. In addition, given that arterial hemoglobin saturation is well-maintained throughout deep dives (McDonald and Ponganis, 2012), desaturation of venous blood is unlikely secondary to a decrease in arterial blood oxygen content.

The maintenance of high arterial hemoglobin saturation, despite the posterior vena caval S_vO_2 decreasing to extremely low values during deep dives, suggests the presence of a central venous oxygen pool which is slowly depleted during the severe

bradycardias seen in deep dives. Otherwise, in the presence of lung collapse and lack of gas exchange, arterial hemoglobin saturation should reflect the low venous values observed in the posterior vena cava. Such a central blood oxygen store would be analogous to the hepatic sinus oxygen store described in elephant seals (Elsner et al., 1964). The existence of such a central venous oxygen store remains to be demonstrated in otariids with either anterior vena cava or pulmonary artery hemoglobin saturation measurements.

Conclusion

California sea lions use a gliding strategy during the descent phase of most dives beyond 100m. On these deeper dives, animals also glide to the surface from depths of 15-25m, probably due to the increased buoyancy associated with lung re-expansion during ascent. The lack of a strong relationship between posterior vena caval S_vO_2 and flipper stroke rate during deep and some shallow dives demonstrates that posterior vena caval hemoglobin desaturation is not dependent on muscle work load. This independence of blood oxygen depletion from locomotory effort implies that 1) muscle blood flow is restricted during deep dives, and not consistently regulated during shallow dives, and 2) the posterior vena caval oxygen profile is more related to heart rate and the magnitude of tissue perfusion/oxygen extraction.

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Competing Interests

The authors report no competing interests.

Author Contributions

P.J.P obtained grant. M.S.T, P.J.P and B.I.M conceived the experiments. M.S.T, P.J.P, L.A.H and P.H.T collected the data. M.S.T and L.A.H performed the analyses. M.S.T and P.J.P wrote the manuscript; B.I.M and L.A.H edited the manuscript.

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Table 3-1: Dive characteristics from four adult female California sea lions. Values on top for dive duration and dive depth represent mean ± standard deviation, while values in parentheses represent the range of values collected.

Sea lion	Mass (kg)	No. dives	Dive duration (s)	Dive depth (m)
21314	91.8	1046	181.7 ± 141.2 (13-504)	127.2 ± 128.2 (10-424)
21316	85.8	530	119.9 ± 76.6 (17-470)	57.5 ± 102.5 (10-385)
210034	93.2	1295	132.6 ± 86.1 (15-376)	77.5 ± 71.6 (10-289.5)
210036	71.6	435	165.7 ± 68.0 (15-466)	97.5 ± 53.8 (10-377.5)
Summary			150.4 ± 106.6 (15-504)	92.6 ± 94.9 (10-424)

Table 3-2: Results from a linear mixed effect model with dive duration, flipper stroke rate and the interaction between dive duration and flipper stroke rate as fixed effects against ΔS_VO_2. Individual was the random effect. Values of flipper stroke rate and ΔS_VO_2 were averaged every 10 seconds into the dive record.

Depth Phase	**Fixed Effects**	**Mean ΔS_vO_2**	**SEM**	**DF**	**t-value**	**p-value**
Shallow	Intercept	-0.6843	0.324	9659	-2.1	0.0349
	Dive Duration	-0.0002	0.001	9659	-0.1	0.8839
	Stroke Rate	1.9518	0.290	9659	6.7	<0.001
	Dive Duration*Stroke Rate	-0.0065	0.003	9659	-2.6	0.0094
Deep	Intercept	-0.6183	0.296	12468	-2.1	0.0370
	Dive Duration	-0.0008	0.001	12468	-1.7	0.0891
	Stroke Rate	0.4321	0.366	12468	1.2	0.2372
	Dive Duration*Stroke Rate	-0.0010	0.001	12468	-1.0	0.3360

Table 3-3: Total glide durations and depths of the start and end of the descent and ascent gliding periods for four California sea lions on dives deeper than 100m. Top values are reported as mean ± SEM, and the bottom values in parentheses represents the range.

Descent glide start (m)	**Descent glide end (m)**	**Descent glide duration (s)**	**Ascent glide start (m)**	**Ascent glide end (m)**	**Ascent glide duration (s)**
60.5 ± 1.6 (13.5 – 147)	199.8 ± 5.8 (55 – 388)	70.5 ± 3.0 (6.5 – 161.0)	20.3 ± 0.4 (4.5 – 33)	3.4 ± 0.2 (0 – 12.5)	9.6 ± 0.2 (2.8 – 16.2)

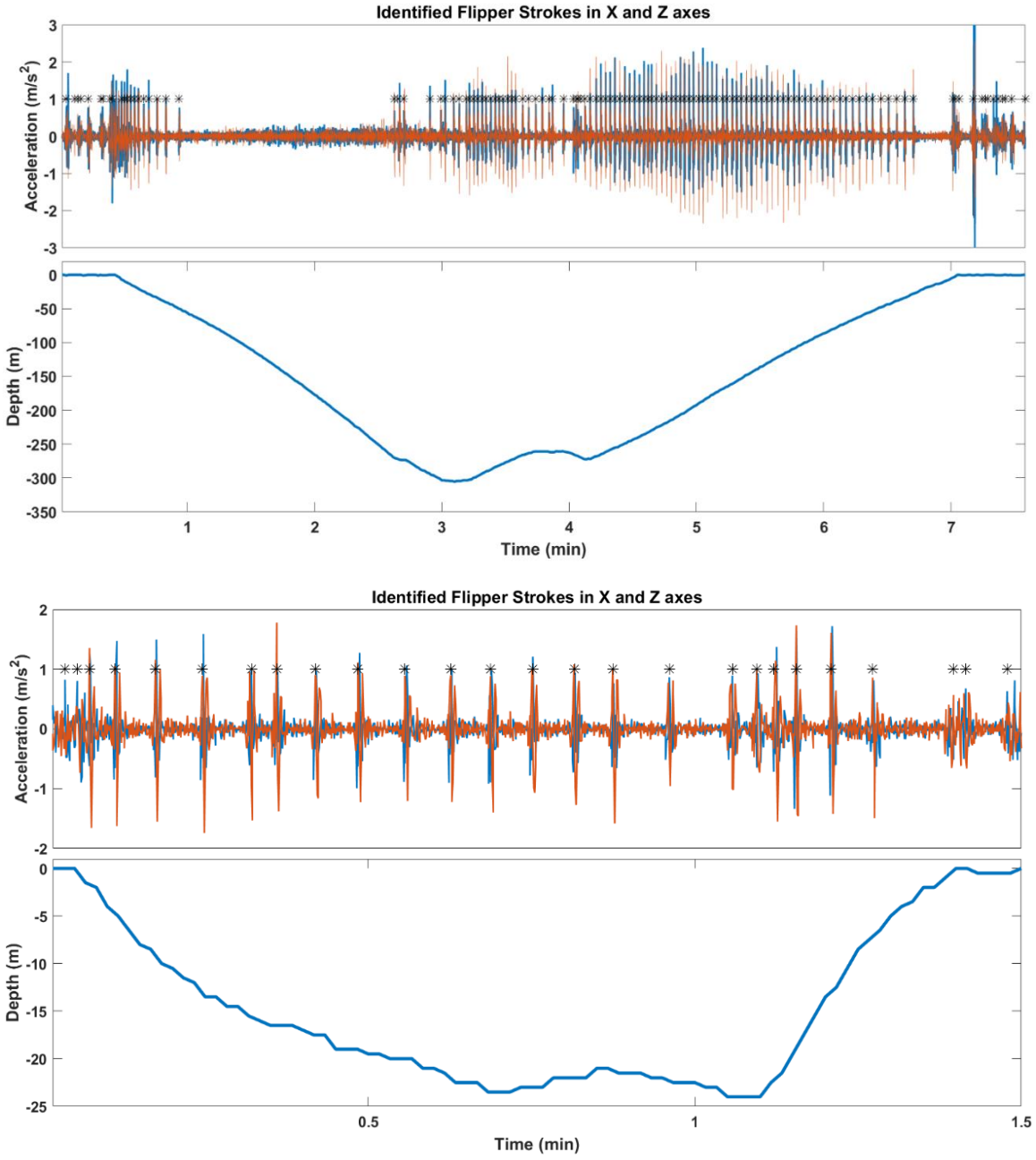

Figure 3-1: Flipper stroke detection in a deep (A) and shallow (B) dive from an adult -female California sea lion using two axes (x: blue, and z: orange). Identified strokes are noted with asterisks. During the deep dive, gliding periods occur during the descent and ascent portions of the dive.

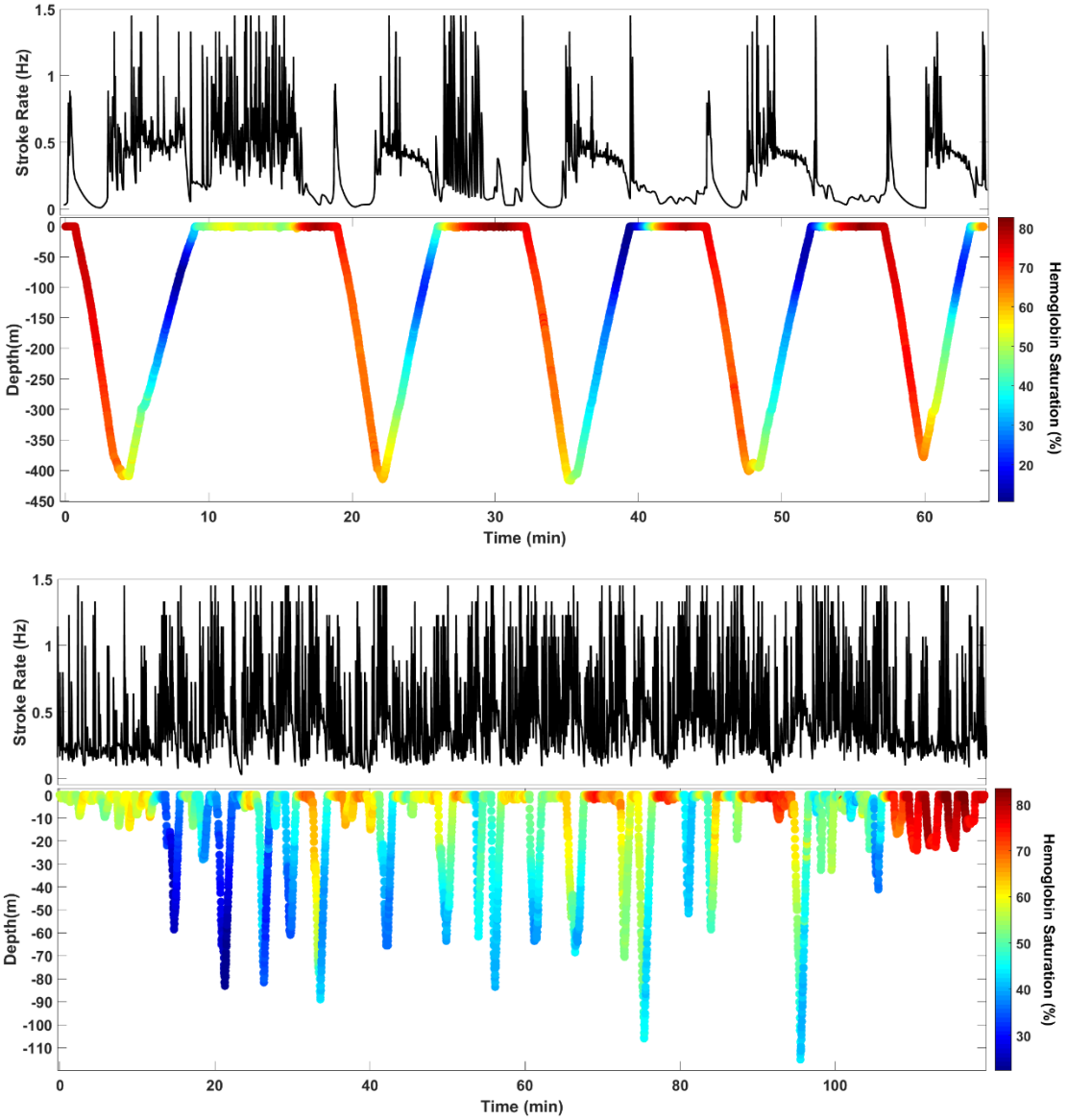

Figure 3-2: Flipper stroke rate and depth profiles color-coded with posterior vena caval hemoglobin saturation (S_VO_2) for deep (A) and shallow (B) dives. Note the wide variations in both flipper stroke rates and S_VO_2 during shallow dives, while deep dives exhibit repetitive cyclic patterns.

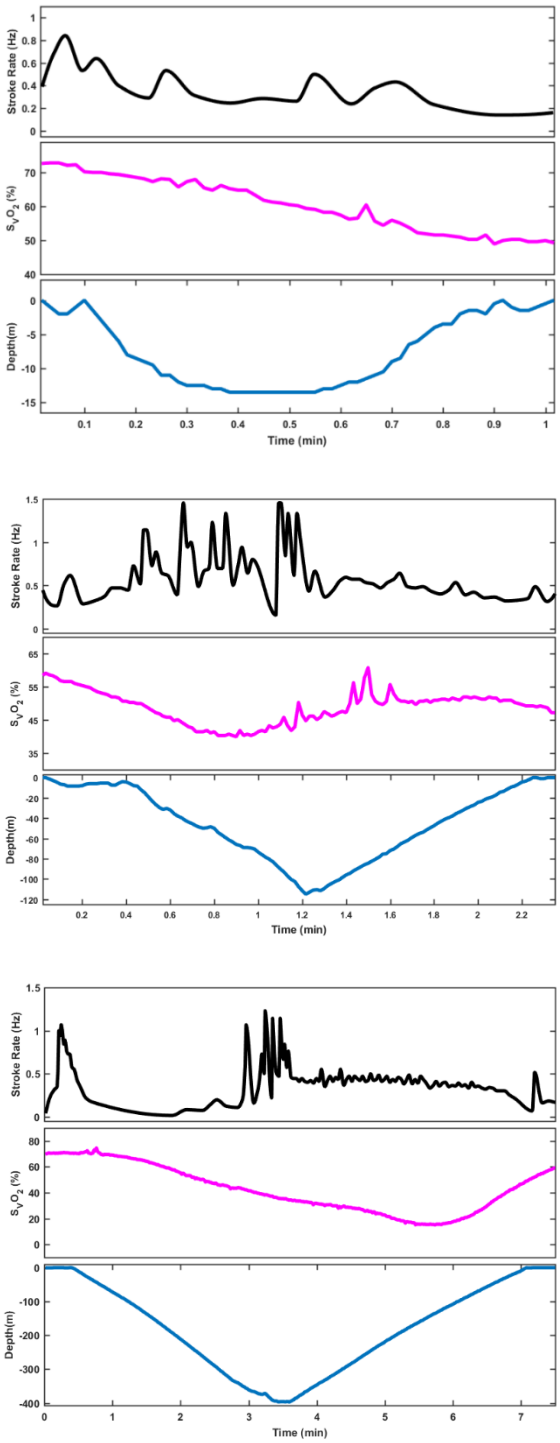

Figure 3-3: Flipper stroke rate (black), posterior vena caval hemoglobin saturation (pink) and depth profiles (blue) during dives to three different depths (13, 117, and 395 m).

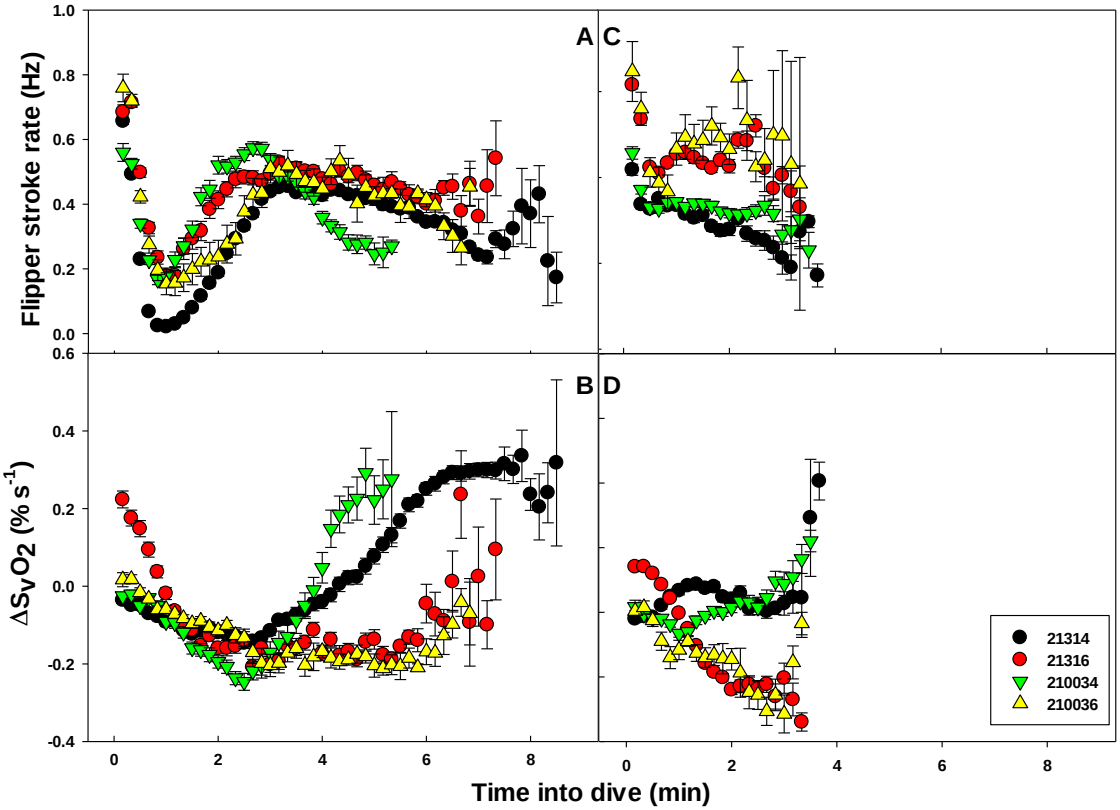

Figure 3-4: Mean ± SEM values of flipper stroke rate (A and C) and ΔS_VO_2 (B and D) every 10 s into dives. A and B represent deep dives (> 100m), while C and D represent shallow dives (< 100m). Data are represented for the four individual sea lions from the study. Negative values of ΔS_VO_2 denote oxygen depletion and positive values denote a gain of oxygen.

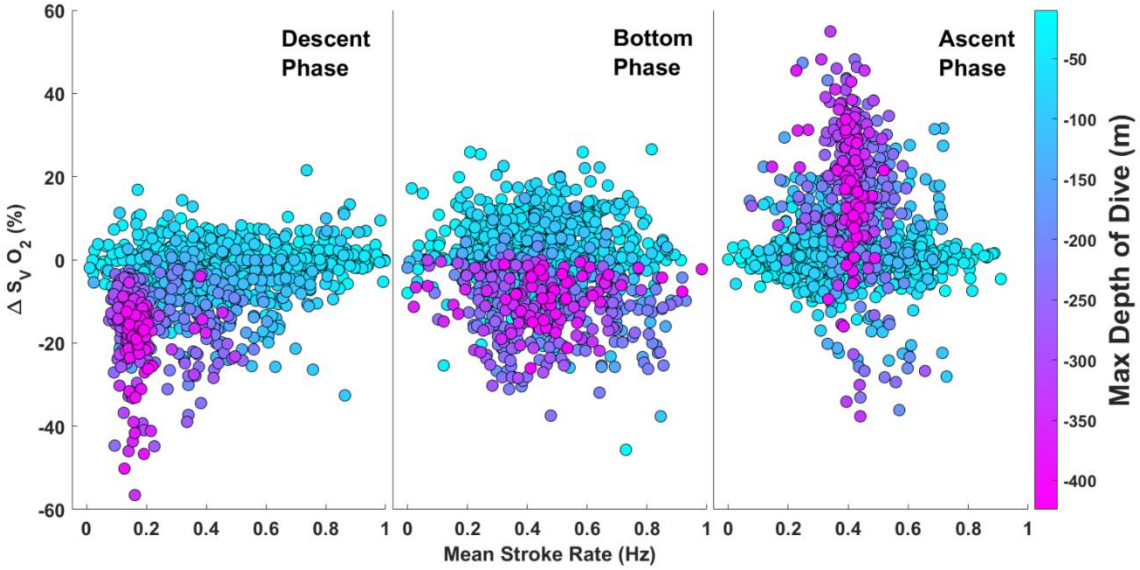

Figure 3-5: Mean flipper stroke rate and mean ΔS_VO_2 for the three different dive phases. Each point represents the mean value for a given phase during one dive. Points are color coded based on the maximum depth of that individual dive. Negative values of ΔS_VO_2 denote oxygen depletion and positive values denote a gain of oxygen.