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Investigation of the potential for vascular bubble formation in a repetitively diving dolphin

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SUMMARY

The production of venous gas emboli (VGE) resulting from altered dive behavior is postulated as contributing to the stranding of beaked whales exposed to mid-frequency active sonar. To test whether nitrogen gas uptake during repetitive breath-hold diving is sufficient for asymptomatic VGE formation in odontocetes, a bottlenose dolphin (*Tursiops truncatus* Montagu) was trained to perform 10–12 serial dives with 60 s surface intervals to depths of 30, 50, 70 or 100 m. The dolphin remained at the bottom depth for 90 s on each dive. Doppler and/or two-dimensional imaging ultrasound did not detect VGE in the portal and brachiocephalic veins following a dive series. Van Slyke analyses of serial, post-dive blood samples drawn from the fluke yielded blood nitrogen partial pressure (P_{N_2}) values that were negligibly different from control samples. Mean heart rate (HR; ±1 s.d.) recorded during diving was 50±3 beats min^{-1} and was not significantly different between the 50, 70 and 100 m dive sessions. The absence of VGE and elevated blood P_{N_2} during post-dive periods do not support the hypothesis that N_2 supersaturation during repetitive dives contributes to VGE formation in the dolphin. The diving HR pattern and the presumed rapid N_2 washout during the surface-interval tachycardia probably minimized N_2 accumulation in the blood during dive sessions.

Key words: dolphin, ultrasound, bubble emboli, diving.

INTRODUCTION

Mid-frequency active (MFA) sonar has been implicated in a number of beaked whale (Family Ziphiidae) stranding events associated in time and space with naval exercises involving sonar (Simmonds and Lopez-Jurado, 1991; Frantzis, 1998; Evans and England, 2001; Jepson et al., 2003). Several hypotheses have been proposed as a cause–effect relationship between MFA sonar use and these stranding events (Cox et al., 2006; Rommel et al., 2006). One of these is that beaked whales alter their dive behavior in response to MFA sonar exposure in such a manner that behavioral or physiological mechanisms employed for protecting against the formation of nitrogen gas (N_2) bubbles are overridden (Fernández et al., 2005; Cox et al., 2006). According to this proposal, bubble evolution occurs as a result of alterations in dive behavior (e.g. extremely rapid surfacing or remaining at the surface and possibly vigorously swimming).

Although various models have indirectly addressed the potential for the evolution of N_2 bubbles by predicting dive-dependent N_2 saturation states in marine mammals (Houser et al., 2001; Fahlman et al., 2006; Fahlman et al., 2009; Zimmer and Tyack, 2007), the greatest support for the hypothesis of N_2 bubble formation as a causative stranding mechanism comes from the post-mortem analysis of stranded animals. Gross and histological analyses of beaked whales stranded in association with Naval activities near the Canary Islands in 2002 demonstrated the presence of distributed gas and fat emboli (Fernández et al., 2005), in addition to other gross traumas (e.g. subarachnoidal hemorrhage) observed in other mass strandings of beaked whales either putatively or known to be associated with the presence of MFA sonar (Evans, 2002; Ketten, 2005). The observed presence of intravascular bubbles and tissue separation representative of bubble formation within tissues is consistent with the presentation of decompression insults in laboratory animals and humans (Gersh et al., 1944; Shim et al., 1967; Kitano and Hayashi, 1981; Francis and Mitchell, 2003). The presence of fat emboli is consistent with initial formation of N_2 bubbles in fat bodies, such as bone marrow, and/or a possible activation of complement in the immune system that accompanies intravascular bubble formation (Kitano and Hayashi, 1981; Francis and Mitchell, 2003). The relationship between the magnitude of the fat embolic response and the degree of the decompression insult remains uncertain and there is evidence that fat emboli can form without any of the debilitating effects associated with decompression sickness [DCS (Shim et al., 1967)]. Collectively, these findings have been used to suggest that N_2 bubble formation occurred in beaked whales that stranded coincident with MFA sonar activities and it has been hypothesized that the magnitude and distribution of bubble and fat emboli signify an explosive decompression event that could have ultimately led to the stranding events (Fernández et al., 2005).

The formation of N_2 bubbles, whether it is formed autochthonously or by activation of bubble nuclei, is related to the degree of N_2 supersaturation within the tissue compartments of the body. In human divers, considerable effort has been placed into modeling and predicting the conditions under which bubble formation occurs. Within marine mammals, very little is known about N_2 kinetics during voluntary breath-hold dives. The few studies of N_2 kinetics during forced and natural dives of marine mammals have predominantly used pinnipeds (i.e. seals and sea lions) as the model species (Kooyman et al., 1970; Kooyman et al., 1972; Kooyman and Sinnett, 1982; Falke et al., 1985), but only one study of N_2 kinetics has been performed in a diving cetacean, the bottlenose dolphin (Ridgway and Howard, 1979). Results of that work suggested that intramuscular N_2 tension was 224–276% of

that supported by ambient pressure at the water surface following the completion of a series of dives to depths of 100 m. The results of early work on decompression in laboratory animals suggest that bubble formation would not be expected in animals until saturation states exceeded approximately 300% ambient saturation levels (Gersh et al., 1944). However, in humans, it has been shown that a 5 min ascent from a saturation dive as shallow as 4 m can produce venous bubbles, i.e. at a much lower degree of supersaturation (Eckenhoff et al., 1990). The degree of gas supersaturation required for venous bubble production in any naturally diving marine mammal is unknown.

The deleterious accumulation of N_2 in marine mammals has been generally considered to be mitigated by pressure-induced alveolar collapse and subsequent lack of gas exchange with the lung at depth (Scholander, 1940; Ridgway et al., 1969; Kooyman et al., 1970). More recently, it has been hypothesized that, even in the absence of alveolar collapse, changes in cardiac output and tissue perfusion associated with the heart rate profile during a dive may be sufficient to account for blood N_2 levels in marine mammals (Fahlman et al., 2006; Fahlman et al., 2009). Regardless of the mechanism, deleterious N_2 accumulation and bubble formation do not appear to occur under normal circumstances in marine mammals. However, this does not mean that N_2 bubble formation never occurs in a marine mammal, as findings putatively related to dysbaric osteonecrosis have been observed in sperm whale skeletons (Moore and Early, 2004). To date, no investigation for the presence of N_2 bubbles in a repetitively diving marine mammal has been performed. It may be that N_2 bubbles do form in marine mammals, but are safely filtered out of the venous system by the lung (Butler and Hills, 1979; Vik et al., 1990) or, within odontocete cetaceans, *via* the intrathoracic rete mirabile (Nagel et al., 1968; Viamonte et al., 1968; Ridgway and Howard, 1982; Vogl and Fisher, 1982). If there is merit to the proposition that altered dive behavior in beaked whales contributed to the formation of N_2 bubbles and a subsequent DCS-like condition, then N_2 bubbles would be expected to occur at asymptomatic levels under less severe deviations in diving behavior than may have occurred in beaked whale stranding events associated with MFA sonar operations.

The presence of vascular N_2 bubbles is unlikely to be evaluated in beaked whales because trained individuals are unavailable. Bottlenose dolphins (*Tursiops truncatus* Montagu) can be used as surrogates. Dolphins are not beaked whales, but both are odontocete breath-hold divers. Given that the absorption of N_2 is passive and that N_2 is metabolically inert, the accumulation of N_2 is primarily dictated by the depth at which alveolar collapse occurs. The subsequent distribution throughout the body is a function of tissue-specific N_2 solubility and perfusion. Since N_2 uptake from the lung is limited to the depths above which complete alveolar collapse occurs, dolphins can be trained to perform dive profiles that maximize N_2 uptake and produce levels potentially greater than those expected to occur in diving beaked whales performing a comparable number of dives and with similar initial tissue saturations. In this study, we used ultrasound techniques to detect asymptomatic intravascular N_2 bubbles, blood N_2 analyses to examine post-dive blood nitrogen partial pressure (P_{N_2}) levels, and digital electrocardiogram (ECG) records to determine heart rate responses in a bottlenose dolphin trained to perform dives that maximize N_2 accumulation from the dolphin's lung.

MATERIALS AND METHODS

Subject

An adult male bottlenose dolphin of the coastal ecotype was trained to perform open water diving and present himself for ultrasound inspection and blood sampling. Although coastal bottlenose dolphins typically perform dives less than 50 m, in part due to bathymetric constraints of the near-shore environment, they are capable of dives of up to 308 m (Ridgway et al., 1969). For purposes of this study, the dolphin was requested to perform repetitive dives to depths of up to 100 m with constrained surface intervals (see below). The dolphin was 23 years of age at the start of the study and had a mass of 190 kg. The subject was maintained by the United States Navy Marine Mammal Program at the Space and Naval Warfare Systems Center Pacific (SSC Pacific) in San Diego, California. The study followed a protocol approved by the Institutional Animal Care and Use Committee of the Biosciences Division, SSC Pacific.

Dive protocol

The dolphin was trained to beach himself onto a padded transport mat that was pulled into a 6.7 m Boston Whaler. The dolphin was then transported to one of several dive locations within or outside of San Diego Bay with water depths varying between approximately 15 and 130 m. Transport times lasted up to 40 min and extended to distances of up to 16 km from the shore. A second boat (8.2 m Radon) was used to transport dive station equipment to the dive location. Upon arrival at the dive location, the dolphin was returned to the water and performed basic husbandry behaviors while the dive station was lowered to depth.

The dive station consisted of a custom aluminum or polyvinyl chloride frame to which a biteplate was attached. The biteplate consisted of a neoprene covered nylon plate. The attachment was bitten by the dolphin while at depth to ensure that he maintained a constant depth position. A light-emitting-diode Multi-SeaCam 1065 underwater video camera with titanium housing (Deep Sea Power and Light, San Diego, CA, USA) was attached to the top of the dive station in order to visually verify that the dolphin was positioned on the biteplate. The camera was connected to a ToolBox camera control unit (CCU-4000; Deep Sea Power and Light) *via* 130 m of Kevlar-reinforced triax (KTRI) underwater video cable (Falmat, San Marcos, CA, USA). The dive station and camera were deployed by davit (a lowering device) from the Radon.

Just prior to performing the dive session, the dolphin was fitted with a MK-9 time–depth recorder (Wildlife Computers, Redmond, WA, USA) set with a 1 s sampling interval. The recorder was placed in a nylon housing and attached to the dolphin's pectoral flipper with a neoprene strap. The time–depth recorder was used to complement the video system in verification of the requested dive behavior and to monitor the diving pattern of the dolphin for variability. At the end of every dive session the data from the time–depth recorder were downloaded for analysis.

A diving session consisted of a series of dives performed to a specified depth. Each dive consisted of (1) diving to depth, (2) biting and holding onto the biteplate for a period of 90 s, (3) and returning to the surface for a 60 s interval prior to performing another dive. At the surface, the dolphin was cued to begin diving by a hand signal given by a trainer. While on the biteplate, the animal was cued to release and return to the surface by an audible underwater 'buzzer' deployed from the side of the Radon. Each session consisted of 10-12 dives. For a given session, the dive station was lowered to a constant depth of 30, 50, 70 or 100 m. Immediately following completion of the session (i.e. the last dive of the series), the dolphin was requested to either present itself for ultrasound inspection or for serial blood sampling (see below).

The particular diving profile was designed to maximize the amount of N_2 accumulation at various depths up to and exceeding the depth at which lung collapse has been hypothesized to occur. The estimated

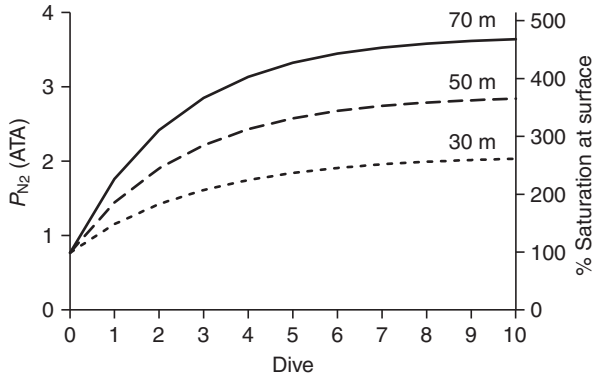

Fig. 1. Estimated intramuscular P_{N_2} at the surface of a series of dives. Estimates were made according to a single compartment model previously used by Ridgway and Howard (Ridgway and Howard, 1979) and Houser and colleagues (Houser et al., 2001). ATA, atmospheres absolute.

depth of lung collapse in the bottlenose dolphin is approximately 70 m (Ridgway and Howard, 1979) and is consistent with changes in buoyancy that suggest a minimal impact of the lung on buoyancy between 60–80 m of depth (Skrovan et al., 1999). Given that there is some uncertainty in the accuracy of this measure, a depth of 100 m was chosen as a dive station depth at which lung collapse was anticipated to have occurred. It should be noted, however, that pulmonary shunt due to compression has been observed to be incomplete (though it is greater than 70%) in harbor seals and sea lions during simulated dives to 100 m in a pressure chamber (Kooyman and Sinnett, 1982). It is unknown how the anatomical structure of the dolphin lung might contribute to gradation in the pulmonary shunt with increasing compression, so it cannot be stated with certainty that alveolar collapse was complete at the 100 m diving depth.

Estimates of intramuscular N_2 saturation were made utilizing a kinetic model derived from the Workman decompression schedules (Ridgway and Howard, 1979), which has previously been applied to predictions of intramuscular N_2 loading in diving cetaceans (Houser et al., 2001). Estimates of N_2 saturation using this model suggest that dolphins performing the prescribed dive profile would have intramuscular N_2 saturation between 265 and 474% of surface pressure saturation levels upon surfacing from the last dive in the series (Fig. 1). This model assumes an instantaneous cessation of lung gas exchange at 70 m. This assumption is most likely incorrect and a gradual increase in pulmonary shunting with increasing depth probably occurs. Indeed, alternative models exist for predicting N_2 saturation in different tissue compartments as a function of dive behavior and physiological responses to diving in marine mammals [e.g. gradual lung collapse, variability in perfusion and cardiac output (Bostrom et al., 2008; Fahlman et al., 2006; Fahlman et al., 2009; Zimmer and Tyack, 2007)]. Each of these models has a different set of assumptions and model parameterizations, which lead to differences in the N_2 saturation values that are predicted. Discussion of each possible permutation of these models is beyond the scope of this study but the reader is referred to them for alternative estimates of dive-related N_2 saturation in the muscle and other tissue compartments. We rely on the simplest of the models because it (1) addresses only the tissue compartment for which N_2 measurements have been made in a diving cetacean and (2) the dive profile conducted prior to the N_2 measurements was similar to that used in this study, with the exception of the extended bottom time.

Absorption of N_2 from the lung must necessarily occur at depths where the lung is functional in gas exchange. The dolphin dive profile used in this study should result in greater N_2 absorption from the lung than for a dolphin that immediately swims beyond the depth of complete alveolar collapse and immediately returns to the surface once ascent has begun. Given the similarities in the dive profiles between those given by Ridgway and Howard (Ridgway and Howard, 1979) and this study, and that bottlenose dolphins were used in both studies, the results of the model employed here can be used qualitatively to conclude that post-dive N_2 saturations in the muscle, and overall absorption of

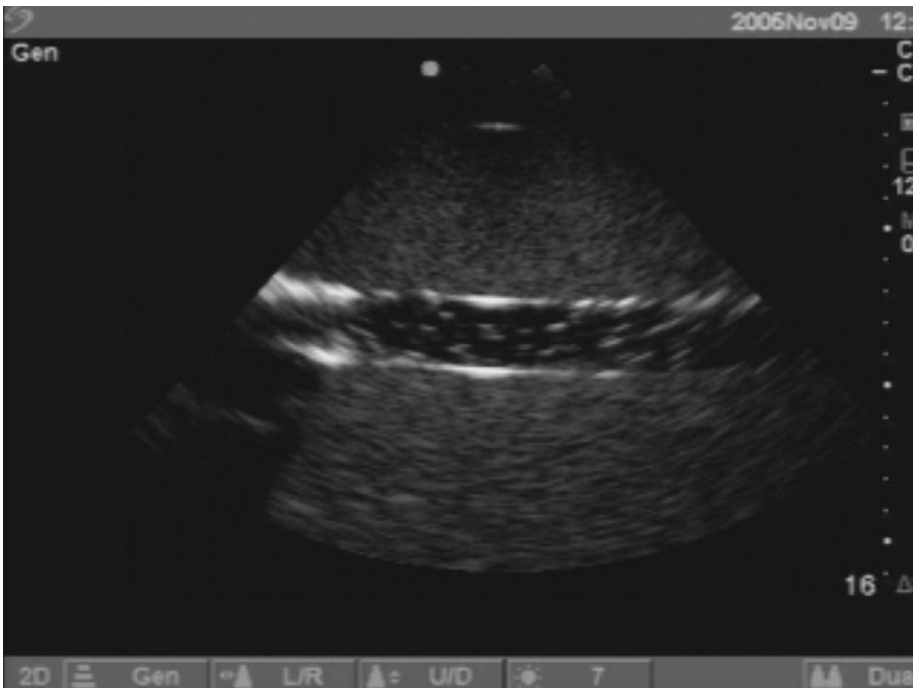

Fig. 2. Ultrasound image of small bubbles flowing through the tissue and vessel phantom. The bright spots within the vessel are bubbles.

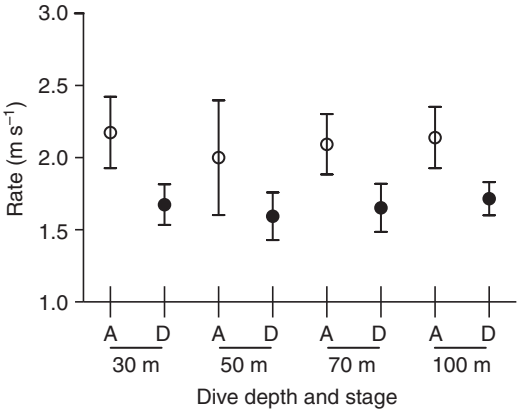

Fig. 3. Ascent (A) and descent (D) rates measured for the different dive series depths. Values are means ±1 s.d. Sample sizes for each dive depth are as follows: 30 m, *N*=20; 50 m, *N*=81; 70 m, *N*=42; 100 m, *N*=45.

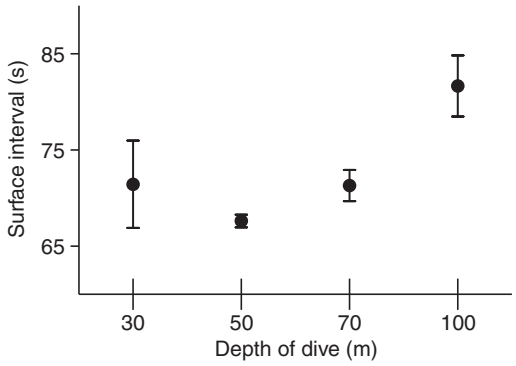

Fig. 4. Surface interval measured for the different dive series depths. Values are means ±1 s.d. Sample sizes for each dive depth are as follows: 30 m, *N*=18; 50 m, *N*=72; 70 m, *N*=38; 100 m, *N*=41.

nitrogen from the lung, was greater than that observed by Ridgway and Howard. Assuming comparable mass-specific lung volumes and depths of lung collapse between species, and accounting for the observed dive behavior of various beaked whale species (Baird et al., 2006; Tyack et al., 2006), the prescribed dive behavior performed by dolphins in this study is also expected to result in a greater accumulation of intramuscular N_2 than that which occurs in beaked whales performing a similar number of dives.

Ultrasound inspection

Upon completion of a dive series, the dolphin either beached itself onto the transport mat within the boat or presented its right lateral or ventral surface to the animal trainer while remaining in the water at the side of the boat. The two presentation methods were used to account for the potential of the animal's vascular dynamics to be altered while beached out of the water. A Titan ultrasound system with a C15 (4–2 MHz) abdominal probe (Sonosite©, Bothell, WA, USA) was used to inspect either the portal vein or the brachiocephalic vein of the dolphin for venous gas emboli (VGE). Vessel inspections began immediately following (within seconds) the dolphin's return to the surface on the last dive of the series and were continued for 5–10 min following surfacing. Occasional spot checks of vessels were made upon returning the animal to its enclosure approximately 1 h after the cessation of the dive session. Inspections were performed with two-dimensional (2-D) imaging and pulsed-wave (PW) Doppler modes. Continuous-wave (CW) Doppler was investigated as a monitoring tool, but PW Doppler was used because it demonstrated a higher fidelity signal while inspecting the deep portal vein (8–15 cm from the surface of the animal), probably because it diminished background noise and used a smaller sample volume obtained *via* range gating (Nishi et al., 2003). Ultrasound images and associated audio (Doppler only) were recorded on a portable digital video recorder for later analysis. Owing to the reflectivity of light off the Titan video screen, real-time observation of ultrasound images was achieved by using video display goggles (i-O Display Systems LLC, Sacramento, CA, USA) under an opaque hood.

Prior to use of the ultrasound system on the bottlenose dolphin, an ultrasound tissue phantom (Blue Phantom™, Advanced Medical Technologies LLC, Kirkland, WA, USA) with a 2 cm artificial blood vessel was used to test the effectiveness of the ultrasound system for bubble detection. The diameter and depth of the vessel were consistent with the diameter and depth of the descending portion of the portal vein in the bottlenose dolphin. Tubing was coupled to the tissue phantom and water circulated through the phantom at rates of 90–528 ml min^{-1}. Bubbles were generated by inserting a micro-bore (10 μm) needle into the flow path prior to the phantom-tube coupling and applying a small amount of air pressure to the needle. Both 2-D imaging and Doppler modes were able to detect small bubbles at an inspection depth of 15 cm (Fig. 2). However, because of flow interference at the phantom-tube coupling (i.e. turbulent flow, cavitation and bubble coalescence), determination of the minimal detectable size of bubbles was not achievable *via* microscopic inspection of bubbles in the tube prior to the coupling. Therefore, images captured from the ultrasound system were output to the image processing package Analyze 6.0, produced by the Biomedical Imaging Resource of the Mayo Clinic (Robb, 1999; Robb and Barillot, 1989; Robb et al., 1989). Based on image intensity differences between a bubble and the surrounding water, a microscopic bubble down to the size of a single pixel could be isolated. Using this information and the resolution of the ultrasound display, which varied depending on the depth of ultrasound inspection, the detectable bubble size was estimated to be approximately 333 μm. However, this estimate assumes that the dimensions of the bubble producing an echo corresponding to a detectable image intensity difference are equivalent to the pixel dimensions. Detectable image intensity differences may occur if the intensity of echoes from bubbles smaller than the pixel dimensions is sufficiently high, suggesting that the actual threshold of detection lies between the theoretical lower limit of detection of 10 μm (Daniels et al., 1979) and 333 μm.

The portal vein drains the spleen, pancreas and gut and flows into the liver, whereas the brachiocephalic vein drains the cranial tissues *via* the jugular, and the pectoral flippers and chest wall *via* the axillary and thoracic veins. Many of the tissues drained by these vessels are probably fast tissues with tissue half-times less than 5 min; however, the thoracic muscle beds and acoustic fats drained by the brachiocephalic probably have intermediate to long half-times [e.g. Ridgway and Howard (Ridgway and Howard, 1979) measured muscle half-times of 5–6 min from the dorsal epaxial muscles of the dolphin following a dive bout]. The portal and brachiocephalic veins were chosen for inspection for two reasons. First, the vessels are relatively large and easy to locate. Many vessels of the bottlenose

dolphin are deep within the animal and are associated with other anatomical structures that obstruct the field of view. The portal vein can be in excess of 2 cm in diameter and is easily accessed from a right lateral perspective, just posterior to the right flipper (Brook et al., 2001). The brachiocephalic, though smaller, is easily imaged from the ventral surface of the dolphin (Ridgway et al., 2006), but requires the animal to be in the water for the examination. Second, the portal veins have been observed as a site of bubbling following decompression in dogs, with subsequent extraction of the bubbles by the liver sinusoids (Butler and Morris, 1995). The observation of macroscopic gas-filled cavities in the liver of stranded cetaceans, with evidence of antemortem presentation, suggests the possibility of a similar manifestation in cetaceans although the etiology may be very different (Jepson et al., 2003). Cardiac monitoring was considered as part of the ultrasound inspection procedure, but transthoracic echocardiography is inhibited by aeration of the lungs in the dolphin. Transesophageal echocardiography (TEE) was considered as an alternative means of cardiac inspection (Butler and Morris, 1995) since TEE has been performed in dolphins under controlled conditions (Sklansky et al., 2005). However, because of the combined requirement of the dolphin to voluntarily accept the esophageal probe and the uncontrolled nature of the test environment (e.g. swells, wave action, relative motion between boat and animal), the potential for trauma to the dolphin was considered too great and the procedure was not pursued.

Blood sampling and N_2 analysis

Upon completion of a dive series to 50, 70 and 100 m, serial voluntary blood samples were taken from the primary branching vein of the fluke *via* a 21 gauge butterfly needle. Blood samples were pulled into 5 ml gas-tight syringes over a 20-min period, usually producing between 12 and 15 samples over the course of the blood sampling. Blood collection began within 90 s of the dolphin surfacing from the final dive of the bout. Blood samples were immediately returned to the laboratory for analysis of N_2 partial pressures (P_{N_2}). The P_{N_2} of the samples was determined with a Van Slyke manometer as has been previously reported (Kooyman et al., 1973; Ponganis et al., 1999). After the 70 and 100 m dives, sub-samples of blood were analyzed for oxygen and carbon dioxide partial pressures (P_{O_2} and P_{CO_2}), pH and lactate concentration (Stockard et al., 2007) with an I-stat portable blood analyzer (Abbot Point of Care, East Windsor, NJ, USA). Control blood samples for P_{N_2} of a dolphin at rest were obtained from the fluke of the test subject while the dolphin was in his holding pen. Control arterial and venous blood gas data of a dolphin 'at rest' were obtained on six occasions from four additional adult dolphins that were beached for routine medical examinations after injection of local 1% xylocaine in the skin of the caudal peduncle. Blood samples from the peduncle artery were collected *via* 14–18 gauge needles or 19–20 gauge catheters inserted percutaneously through the needle. Blood samples were collected by the same team in all cases and control blood samples were processed with the same I-stat portable blood analyzer used during the dive study.

Heart rate determinations

Heart rate was recorded during dive sessions to 50, 70 and 100 m depths with the use of a backpack digital electrocardiogram (ECG) recorder (Meir et al., 2008) attached to a custom-designed body harness and connected to two suction cup ECG electrodes (Ponganis and Kooyman, 1999; Williams et al., 1999a). Electrodes were placed dorsoventrally at the level of the pectoral fins. After application of a high frequency filter, ECG complexes were detected with AcqKnowledge software (BIOPAC Systems, Goleta, CA, USA) and processed with Excel and Origin programs (Stockard et al., 2007).

RESULTS

Dive behavior

A total of 23 dive series were performed by the dolphin (30 m, N=3; 50 m, N=8; 70 m, N=6; 100 m, N=6). Due to stretching of the line to which the dive station was attached, dive depths were slightly deeper than planned; average depths were 35.1±0.5, 53.8±2.2, 78.0±0.8 and 104.4±5.5 m for each of the respective dive sessions. The dolphin consistently demonstrated a stereotypical dive profile characterized by rapid descent to station and rapid ascent to the surface. Mean rates of ascent and descent were not statistically different from one another with respect to dive depth (Fig. 3), but the mean rate of ascent for a given depth was always greater than the mean rate of descent. For 30, 50 and 70 m dive sessions, the surface interval was generally maintained between 65 and 75 s and the dolphin promptly responded to cues to dive. During the 100 m dive sessions, the dolphin began to increase the surface intervals after completing several dives, even though he had been given the cue to dive. This hesitancy resulted in an approximately 10 s increase in the mean surface interval (Fig. 4); however, the mean increase in the surface intervals was typically due to the extension of one or two surface intervals to 90 to 120 s.

Ultrasound inspection

Ultrasound inspections were performed on all but five of the dive sessions; three of the dive sessions were committed to blood sampling, and difficulty with the ultrasound system was encountered during two sessions. No instances of vascular bubbles were found using the 2-D imaging or the PW Doppler for inspection of the portal vein (Fig. 5). No vascular bubbles were observed in the brachiocephalic using 2-D imaging; owing to the relative motion between the boat and the animal floating in the water, PW Doppler inspection of the brachiocephalic vein was not performed. Consistent with measurements in humans (reviewed by Kok et al., 1999), blood flow in the portal vein was sluggish, with mean values of approximately 20 cm s^{-1} and maximal values of approximately 45 cm s^{-1}. No instances of vascular bubbles were observed in any of the spot checks made at the dolphin pen approximately 1 h after the conclusion of the dive session.

Blood gas analysis

The N_2 solubility coefficient (1.90 ml N_2 100 ml^{-1} blood atmosphere^{-1} N_2) was determined in blood tonometered with 100% N_2 at 37°C. The P_{N_2} of the dolphin at rest during tail fluke blood sampling was 0.87 ATA (atmospheres absolute) whereas the P_{N_2} of serial blood samples collected after the 50, 70 and 100 m dive sessions ranged from 0.74 to 1.04 ATA (Fig. 6). The average P_{CO_2} and P_{O_2} of blood samples was 59±4.1 and 48±2.6 mmHg, respectively, from blood samples collected 5 to 21 min after the last dive of a 70 m and 100 m dive session (Table 1). These values were characteristic of partially arterialized venous blood in that P_{O_2} was between that of arterial and venous values and P_{CO_2} was within the range of both arterial and venous values of four additional beached animals at rest, which were collected during routine veterinary examinations (Table 2).

Heart rate profiles

During pre-dive session periods (1 min) and post-dive session periods (8–20 min), heart rates (HRs) oscillated in a sinus arrhythmia pattern between 40 and 140 beats min^{-1}, with mean HR ranging

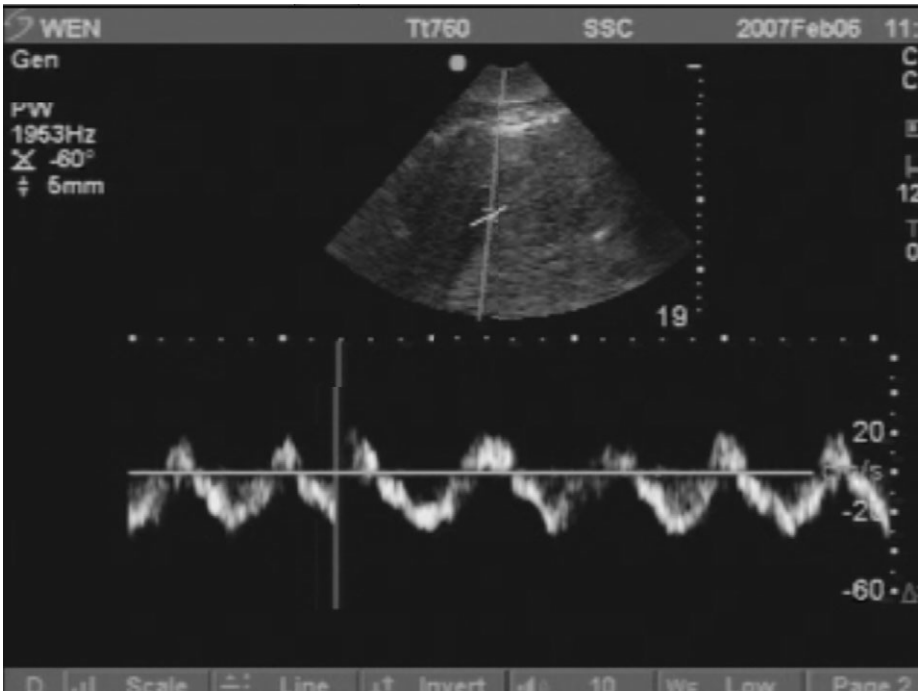

Fig. 5. Pulsed-wave Doppler ultrasound acquired from the descending portal vein following 10 dives to a depth of 70 m. The spectral trace in the lower portion of the image demonstrates the velocity of blood flow through the portal vein. Blood flow velocity is measured in cm s^{-1}.

between 83±24 and 101±33 beats min^{-1}, and 67±25 and 85±25 beats min^{-1}, respectively. The same general pattern and level of HR occurred in post-session periods whether the dolphin was in the water or whether he was beached for ultrasound examinations. During all dives, heart rate consistently declined to <30 beats min^{-1} within 15 s, and remained at 20-40 beats min^{-1} until the ascent period, during which HR increased to 120-130 beats min^{-1}. Mean dive and surface-interval HRs were 47±4 and 121±7 beats min^{-1}, 50±2 and 132±5 beats min^{-1}, 52±3 and 137±6 beats min^{-1} for dive sessions to 50, 70, and 100 m, respectively. Mean HR during diving did not significantly vary with the depth of diving and the pooled mean HR was 50±3 beats min^{-1}. Fig. 7 illustrates the HR profile before, during and after the 100 m dive session. The same HR pattern was observed in all dive sessions in which HR was monitored.

DISCUSSION

Ultrasound investigations

The presence of vascular bubbles, although indicative of decompression stress, does not necessarily correspond to the occurrence of acute decompression sickness (Eckenhoff et al., 1990;

Table 1. Serial post-dive blood gases and lactate contents obtained from the fluke after the last dive of a 70 m and 100 m dive session of the dolphin

Minutes post-dive	pH	P_{CO_2} (mmHg)	P_{O_2} (mmHg)	[Lactate] (mmol l^{-1})
70 m dive session				
6	7.32	59	43	2.55
10	7.33	58	51	2.13
16	7.31	60	44	2.89
21	7.32	59	47	2.55
100 m dive session				
5	7.30	64	46	3.06
9	7.45	54	54	3.20
13	7.34	60	47	3.28
19	7.46	59	43	3.14

Table 2. Arterial and venous blood gases and lactate content of dolphins 'at rest' during routine medical exams while beached

Dolphin ID	Body mass (kg)	pH	P_{CO_2} (mmHg)	P_{O_2} (mmHg)	[Lactate] (mmol l^{-1})	Source	Sedation (Midazolam)
Arterial							
Tt1	216	7.34	53	68	1.51	Catheter	–
$Tt2_{(1)}$	259	7.46	46	84	4.30	Needle	15 mg
Tt3	269	7.23	58	81	0.64	Needle	15 mg
$Tt2_{(2)}$	232	7.43	39	108	1.78	Catheter	–
Tt4	261	7.31	66	68	1.77	Catheter	20 mg
Venous							
$Tt2_{(3)}$	238	7.31	57	41	3.80	Catheter	15 mg

All samples were drawn through the peduncle artery or vein.
Tt1–Tt4 refers to four dolphins from which samples were collected. The subscripts for dolphin Tt2 indicate that this dolphin was sampled three times.

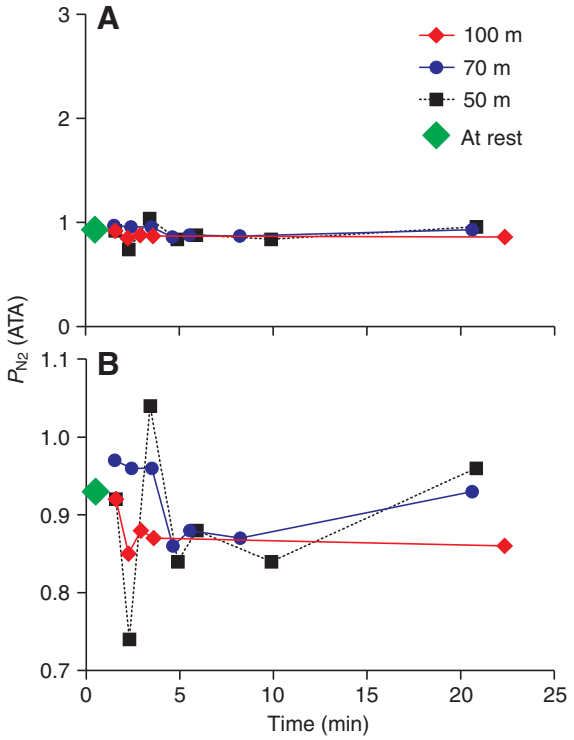

Fig. 6. (A) Post-dive serial blood samples obtained from the fluke of the dolphin after dive sessions to 50, 70 and 100 m. Values are the P_{N_2} of the blood sample plotted against time. ATA, atmospheres absolute. (B) A magnified view of A, to clarify the degree of variation in the P_{N_2} of the samples.

Nishi et al., 2003; Eftedal et al., 2007). Indeed, the human body has been shown to tolerate the presence of VGE without the accompaniment of decompression sickness (i.e. 'silent bubbles') but the presence and magnitude of VGE as a predictor of decompression sickness is equivocal (Conkin et al., 1996; Nishi et al., 2003). If beaked whales that stranded in association with naval use of MFA sonar experienced a severe decompression stress and explosive VGE formation following a behavioral reaction to sonar exposure, then asymptomatic VGE would be expected to occur under less severe levels of decompression stress.

No evidence for vascular N_2 bubble formation was found in the portal or brachiocephalic vein of a dolphin trained to repeatedly dive and remain at depths ranging from 30 to 100 m. This finding does not conclusively rule out the potential for *in vivo* bubble formation in the dolphin for several reasons: (1) only two vessels were inspected and VGE may have occurred elsewhere; (2) animal orientation may not have been ideal as there is evidence in humans that orientation of the subject (e.g. standing *versus* supine) can impact bubble detection (Nishi et al., 2003); and (3) the time course of testing for the occurrence of bubbling may not have been ideal, i.e. onset and peak bubble activity has been observed to vary across studies, species and dive profiles (Lynch et al., 1985; Vik et al., 1993; Butler and Morris, 1995; Conkin et al., 1996). Nonetheless, the results do not support the proposition that repetitive breath-hold diving within the depths at which lung gas exchange occurs is sufficient to produce asymptomatic N_2 bubbles. The dive profiles employed were intentionally created to maximize N_2 uptake across the dive series (i.e. maximize time at depth where alveolar gas exchange occurs relative to time at the surface). The lack of occurrence of asymptomatic bubbles suggests that even under these conditions, which are atypical of many coastal and pelagic dolphin dive patterns in the wild (Wursig and Wursig, 1979; Davis et al., 1996; Baird et al., 2001), the threshold for bubble formation was not exceeded.

One factor potentially present in beaked whale responses to MFA sonar exposure, and which was not addressed in this study, is an increase in the rate of ascent during surfacing. The dolphin's rate of ascent in this study was self-determined and there was no effort to elevate the rate of ascent through conditioning or through creation of a startle response. The rate of ascent to the surface is generally believed to be related to the probability of the formation of the free gas phase (e.g. Carturan et al., 2002) as the rate of change in hydrostatic pressure is directly related to the degree of gas saturation of the various tissue compartments. Thus, fast ascent rates would generally be expected to have higher incidence of VGE than slower rates of ascent. However, modeling efforts suggest that rates of ascent of up to 20.0 m s^{-1} are probably insufficient to decrease

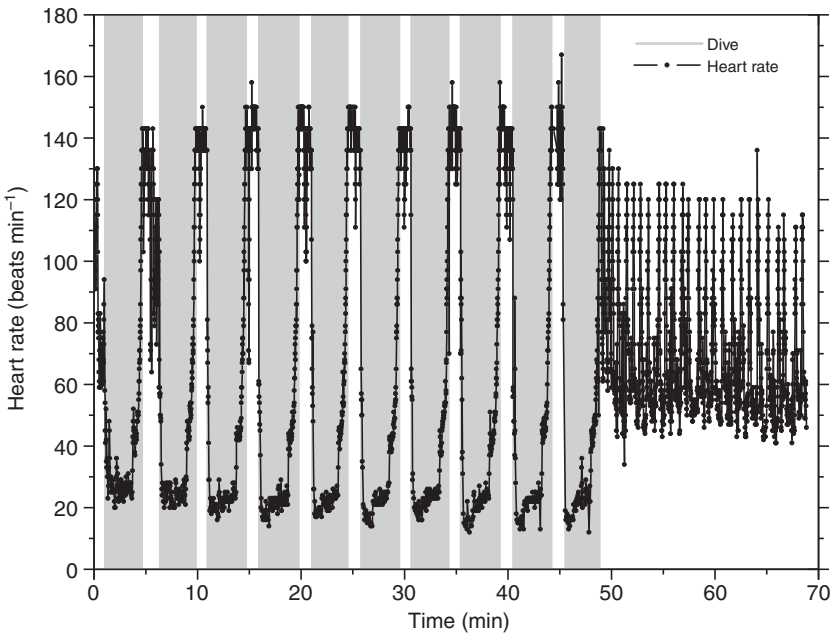

Fig. 7. Heart rate profile during a 100 m dive session, including a 1 min pre-dive period, and a 20 min post-dive period during which the dolphin was beached for an ultrasound study. The sinus arrhythmia pattern during the post-dive period on the mat also occurred during post-dive periods while the animal was still buoyant in the water. Each dive (descent–stationing–ascent) is denoted by a grey box.

the threshold saturation for bubble formation in beaked whales; the half-times of tissues are so slow relative to the rate of ascent under normal conditions (1 to 2 m s^{-1}) that an increase to 20.0 m s^{-1} does not appreciably elevate tissue P_{N_2} (Zimmer and Tyack, 2007). Other modeling results contend this point, depending on the parameters and assumptions applied (Fahlman et al., 2006).

Post-dive blood P_{N_2}

The lack of elevation in post-dive blood P_{N_2} is consistent with the absence of intravascular bubble formation in this dolphin. Several factors may account for the low P_{N_2} and explain why they contrast with the high post-dive intramuscular P_{N_2} reported in dolphins by Ridgway and Howard (Ridgway and Howard, 1979). The P_{N_2} in blood samples obtained from the fluke are potentially affected by (1) the P_{N_2} of arterial blood that is mixed with venous blood in the peri-arterial venous rete, (2) the post-dive washout of N_2 from other tissue compartments, and (3) the likely reduction in peripheral tissue uptake of N_2 during diving. At the surface, the P_{N_2} of arterial blood, even after a deep dive, is generally considered to be at the level of an animal at rest due to rapid equilibration between inspired gas and pulmonary blood (Tikuisis and Gerth, 2003). The P_{O_2} and P_{CO_2} in the post-dive samples reflect a mix of arterial and venous blood; the P_{O_2} was greater than the venous value of a beached dolphin at rest and the P_{CO_2} was greater than the arterial value of a beached dolphin at rest (Tables 1 and 2). The potential mixture of arterial and venous blood because of arteriovenous shunting in the fluke (Scholander and Schevill, 1955) should reduce the P_{N_2} of samples collected from the peri-arterial venous rete *via* dilution of the venous blood with the presumably less saturated arterial blood. In addition, the fluke tissue P_{N_2} should not be elevated due to the bradycardia of diving (Elsner et al., 1966; Williams et al., 1999a; Williams et al., 1999b) and the reduction of peripheral blood flow during the dive. Indeed, blubber P_{N_2} has been found to be only minimally elevated in the post-dive period in dolphins (Ridgway and Howard, 1979). Collectively, these factors suggest and agree with the observation of minimal post-dive washout of N_2 from the fluke.

The results are also consistent with observations of arterial and venous P_{N_2} during forced and natural dives in phocid seals. Venous and arterial P_{N_2} measured in harbor and elephant seals return to surface ambient levels within minutes of 'surfacing' from forced dives (Kooyman et al., 1972). Similarly, arterial P_{N_2} in naturally diving Weddell seals was observed to return to surface ambient values within 4 min of surfacing from a natural dive (Falke et al., 1985). Although no measures of P_{N_2} were obtained from peripheral blood vessels in these studies, one might predict that the continued distribution of N_2 to peripheral tissues, pulmonary elimination of N_2 from the blood, and a mix of arterial and venous blood would lead to peripheral blood P_{N_2} in seals similar to that observed here. However, for an adequate comparison between species, more information is required on the N_2 kinetics of the central venous and arterial pools in the diving dolphin.

A better assessment of N_2 absorption during the dive and N_2 washout after the dive requires arterial blood sampling during the dive and central venous sampling after the dive. These approaches, however, were not considered currently feasible by veterinary and training staff.

Heart rate responses

The beat-to-beat HR profiles during the dive sessions of this dolphin were characterized by an abrupt bradycardia (HR <30 beats min^{-1}) within 15 s of the start of the dive, maintenance of a low HR (20–40 beats min^{-1}) during most of the descent and bottom time at the biteplate, and an ascent tachycardia which began as the dolphin left the biteplate and which increased to 120–130 beats min^{-1} by the time the dolphin reached the surface. An interdive tachycardia and a post-session HR which demonstrated a sinus arrhythmia pattern were also observed. The similarity of these beat-to-beat HR patterns to averaged HR records in other diving dolphins (Williams et al., 1999a; Williams et al., 1999b), and to the analogue ECG record of a dolphin stationing at an underwater target in a pool (Elsner et al., 1966), suggest that this general profile is characteristic of bottlenose dolphins diving to depth. The HR profile of the diving dolphin has significant implications for N_2 absorption and distribution and for basic assumptions in mathematical models of N_2 kinetics in these diving animals (Fahlman et al., 2006; Fahlman et al., 2009; Houser et al., 2001; Ridgway and Howard, 1979).

A severe diving bradycardia should limit net N_2 absorption and restrict the volume of distribution for N_2 (Fahlman et al., 2006; Ponganis et al., 1999) until the ascent, at which time the volume of distribution should increase with the onset of the ascent tachycardia and increased peripheral perfusion (see Falke et al., 1985). The transfer of N_2 back into the lung should begin once the alveoli re-expand and gas exchange resumes during the latter part of the ascent (i.e. above the threshold depth of lung collapse). In addition, N_2 washout from the tissues and clearance in the lung should be near maximal at the high surface interval HRs. Collectively, these factors should minimize the accumulation of blood and tissue N_2 during the dive session and can potentially account for the absence of post-dive asymptomatic vascular bubbles and lack of elevated post-dive blood P_{N_2} in samples collected from the fluke.

The asymmetry in the HR between descent and ascent suggests assumptions of equivalent N_2 wash-in and wash-out rates in dolphin N_2 kinetics should be revisited (Ridgway and Howard, 1979; Houser et al., 2001). Post-session surface HR in the current study was high and consistent with rapid gas exchange supporting Ridgway and Howard's (Ridgway and Howard, 1979) estimation of tissue N_2 half time based on muscle P_{N_2} recorded after the end of a dive series. However, in the current study, the post-session HR was considerably lower than that measured during the interdive surface intervals. This observation calls into question whether the tissue half-time estimated following the dive series is applicable to the interdive surface interval. If the tissue half-times are different between the post-dive and interdive periods because of a change in perfusion, the calculation of the 70-m depth threshold for lung collapse in dolphins based upon tissue half-times determined at the conclusion of a dive series may be incorrect, as has been previously suggested (Fahlman et al., 2006; Ridgway and Howard, 1979).

It should be noted that if the HR profiles observed in the current study are characteristic of dolphins diving to depth, then any elevation in post-dive muscle P_{N_2} is probably secondary to N_2 delivery to muscle during the ascent tachycardia and return of peripheral perfusion. Since HR is <30 beats min^{-1} within 15 s of the start of the dive, it is unlikely that muscle blood flow persists during most of the descent and bottom time. As previously suggested in seals (Fedak and Thompson, 1993), it is proposed that muscle blood flow would resume during the ascent tachycardia and that N_2 delivery would parallel a return of the peripheral perfusion.

Relevance of findings to the vascular bubble hypothesis

The post-mortem findings in beaked whales (e.g. gas and fat emboli) that stranded coincidently with MFA sonar activities are provocatively similar to previous observations of severe decompression insults in

man and some terrestrial mammals and require an explanation (Haymaker and Davidson, 1950; Cockett et al., 1965; Shim et al., 1967; Kitano and Hayashi, 1981). The findings of this study do not support the hypothesis that repetitive shallow-water diving leads to decompression risk in beaked whales (Zimmer and Tyack, 2007). However, to any significant degree, the dive profile used in this study would only supersaturate relatively short half-time tissues [e.g. muscle tissue with half-times in the order of 5.2–6.6 min (Ridgway and Howard, 1979)]. Based on human models, long half-time tissues (e.g. fat, bone marrow) may have time constants from tens of minutes to in excess of 400 min (Lundin, 1960; Schreimer, 1967; Sićko et al., 2003; Workman, 1965; Wienke, 2003). Significant supersaturation of these tissues would take considerably longer dive bouts and/or longer individual dives than performed in this study. Beaked whales typically make foraging dives between 800 and 1400 m and have recorded dives in excess of 1800 m (Baird et al., 2006; Tyack et al., 2006). Dive durations in these species can approach an hour. As has been suggested from modeling efforts (Hooker et al., 2009), it seems feasible that the natural dive behavior of these animals across a lifetime could lead to saturation of long half-time tissues such that the tissues are supersaturated during surfacing periods.

Tissue compartments with short half-times ('fast' tissues) predominantly control tissue desaturation for short duration dives whereas tissue compartments with long half-time constants ('slow' tissues) have a greater influence on tissue desaturation states following deep or prolonged dives (Tikuisis and Gerth, 2003). The interaction of these compartments during depressurization dictates, in part, the probability of VGE formation; indeed, it has been suggested that bubble formation is predominantly related to the relationship between short half-time compartments and the longer half-time compartments which supply them N_2 (Eckenhoff and Olstad, 1991). A number of physiological adaptations exist that probably manage P_{N_2} so that deleterious VGE formation is prevented (e.g. selective ischemia, diving bradycardia, alveolar collapse), and other mechanisms have been proposed (Hooker et al., 2009). However, as recent work by Moore et al. (Moore et al., 2009) has demonstrated, tissue gas supersaturation is sufficient to cause bubbles if a marine mammal dies at depth and is quickly brought to the surface (i.e. all physiological and behavioral mitigations are obviated). Taking these factors into account, the question with respect to beaked whales and marine mammals as a whole then becomes a matter of the circumstances under which the N_2 dynamics between slow and fast tissues would be favorable to VGE formation.

One possibility that has not yet been investigated is that bubble and fat emboli found in beaked whales stranded in relation to MFA sonar activity were a result of the stranding, not a cause of the stranding. Necropsy results from both the Bahamas and Canary Island beaked whale mass stranding events associated with MFA sonar activity indicated that stranded whales died from cardiovascular collapse (Evans and England, 2001; Fernández et al., 2005). Cardiovascular collapse is the failure of the circulatory system, resulting in regions of reduced perfusion and blood pooling. Within regions of reduced perfusion, supersaturated tissues will continue to off-gas; however, with compromised vascular flow to the lung for elimination of blood N_2, autochthonous bubble formation would increase. This would be particularly true for long half-time tissues (e.g. fat bodies) since poorly perfused tissues with slow gas wash-out rates are considered the most vulnerable to autochthonous bubble formation (Francis and Mitchell, 2003). Any agonal action on the part of the beached whale may also contribute to VGE formation *via* tribonucleation (Hayward, 1967), a process that may explain the increased susceptibility to DCS in men and animals exercising following decompression (Kumar and Powell, 1994; Pollard et al., 1995).

Fernández et al. (Fernández et al., 2005) compared fat emboli found in the lungs of beaked whales stranded in the Canary Islands in 2002, and putatively related to MFA sonar activities, to 86 lung tissue samples from other cetaceans stranded along the coast of the Canary Islands. Of these control samples, only six had fat emboli and two of these could be explained as resulting from traumatic injury resulting from ship strike. Similarly, the incidence of bubble formation in stranded marine mammals is low (Jepson et al., 2005; Moore et al., 2009). If gas supersaturation is a possible explanation for the observance of gas and fat emboli in beaked whales stranded in association with MFA sonar exercises, one explanation for why it is not observed in other stranded specimens may be the rapidity with which the stranding occurs. An instantaneous decompression of a diving marine mammal, emulated by bringing marine mammals that have drowned in gillnets to the surface, shows a relatively high rate of vascular and tissue bubble formation [15 of 23 cases (Moore et al., 2009)]. By contrast, moribund cetaceans probably spend a period of time at sea with reduced depth and repetitiveness of diving prior to stranding. During this time, the degassing of tissues to near-surface saturation values would occur. Beaked whales stranding in association with MFA sonar activity rapidly stranded. Inadequate desaturation of long half-time tissues prior to stranding could result in significant oversaturation of these tissues once stranded. Under these conditions, the time stranded would be critically related to the formation of gas and fat emboli, i.e. the progression toward cardiovascular collapse would increase the potential for emboli formation. Unfortunately, relatively little information on the times that beaked whales stranded in association with MFA sonar activity exists. Of the whales that died in the Bahamas (2000) and Canary Islands (2002) stranding events, the actual time of stranding and the time elapsed between stranding and death is unknown (Evans and England, 2001; Fernández et al., 2005).

Summary

Ultrasound inspections of blood vessels following bouts of repetitive, prolonged dives up to 100 m depth, suggest the acute accumulation of N_2 within short half-time tissue compartments is insufficient to generate asymptomatic intravascular bubbles in bottlenose dolphins. Post-dive P_{N_2} data suggest efficient pulmonary clearance of blood N_2 at the surface and negligible peripheral accumulation of N_2 while diving. HR data in the dolphin are consistent with a low cardiac output, restricted volume of distribution, and relatively low N_2 uptake until the ascent portion of the dive. With the increase in heart rate during ascent, cardiac output and the volume of distribution should increase and, once gas exchange resumes, efficient N_2 transfer back into the lung should begin.

LIST OF SYMBOLS AND ABBREVIATIONS

ATA	atmospheres absolute
CW	continuous wave
DCS	decompression sickness
ECG	electrocardiogram
HR	heart rate
MFA	mid-frequency active
P_{CO_2}	partial pressure of carbon dioxide
P_{N_2}	partial pressure of nitrogen
P_{O_2}	partial pressure of oxygen
PW	pulsed-wave
TEE	transesophageal echocardiography
VGE	venous gas emboli

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